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DEVELOPMENT OF QUALIFICATION OF FRESH AND FROZEN STALLION SEMEN, INVESTIGATION OF FACTORS AFFECTING SPERM QUALITY USING A NEW EVALUATION METHOD

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Szüleim és nővérem emlékére

Dedicated to the memory of my parents and sister

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“Life shield” from Tayos gold library (Ecuador)
/collection of Padre Carlos Crespi,
presumed age of the artifact is 8000-10000 years/

„A csodák nem a természeti törvényekkel állnak ellentmondásban, csupán azzal, amit a természetről tudunk” - Szent Ágoston-

"*Miracles are not contrary to nature, but only contrary to what we know about nature*."
- Saint Augustine -

1. GENERAL INTRODUCTION

The horse has been in close contact with humans for thousands of years. This species - presented in the largest populations of mammals in Eurasia 25 thousand years ago-, was an important part of human life in prehistoric times, which is attested by the 20-30-thousand-year-old Palaeolithic art cave paintings from Lascaux Caves, Pech-Merle Cave, etc. Horses had also survived the glacial period. The date and place of horse domestication has long been subject to research. However, several evidence supports the hypothesis that horses were domesticated in the Eurasian Steppes (Dereivka centered in Ukraine) approximately 4000-3500 BCE, recent discoveries on Botai culture in northern Kazakhstan suggest that location of the earliest domestication of the horse was in this region during the Copper Age, dating to about 3500 BCE. Pathological characteristics indicate that some horses were bridled, perhaps ridden and Botai culture used horses as a source of meat and milk (Outram et al. 2009). DNA studies seem to indicate that domestication probably occurred in multiple locations simultaneously (Jansen et al. 2002). Horse domestication was a great breakthrough, bringing horsepower to communications, transportation, farming and warfare. In the Scythian Empire, the horse was an important animal, enabling the Eurasian steppe population to cover long distances. It was also an essential companion in the afterlife as the archaeological findings from the Unesco World Heritage Frozen Scythian Tombs of the Altai Mountains shows. Phylogenetical analysis showed that these

ancient horse's mitochondrial DNA sequences were distributed throughout the tree defined by modern horses' sequences and are closely related to them (Keyser-Tracqui et al. 2005). The horse later, for centuries had represented a decisive role in human cultures. The selection of excellent sires and dams had strategic importance to the expansion, conquest, and in battles.

According to the legend the horse was the first animal upon which artificial insemination (AI) was practised successfully. In 1322 an Arab chieftain sent a spy to the neighbouring rival's camp. This man then scooped some of the semen from a recently mated mare's vagina, diluted it with camel milk and rushed with this „diluted semen" back to base. The legend does not relate how the diluted semen was inseminated into the boss's mare but it does avow that a healthy and exceptionally beautiful foal was born the following year (Bowen 1969, Pickett et al. 2000, Allen 2005). The first successful real insemination was performed by Spallanzani (1784) in a dog, which gave birth to three puppies 62 days later (Foote 2002). In 1897, Heape and other scientists in several countries reported successful use of AI in dogs, rabbits and horses. Pioneering efforts to establish AI as a practical procedure were begun in Russia in 1899 by Ivanov who studied AI in many domestic farm animals, dogs, foxes, rabbits, and poultry. Some of this research, especially in horses, is included in his paper from 1922. The Japanese scientist Ishikawa studied with Ivanov and after he had returned to Japan began a similar program in horses in 1912 (Nishikawa 1962). In Sweden, Lagerlöf became known for his research on infertility problems in bulls. Other Scandinavians, such as Blom (1950), followed, publishing a steady stream of excellent papers on abnormal sperm morphology (Foote 2002). Intensive growth of AI occurred in the 1940s in the United States with close collaboration between dairy cow farmers and researchers. In Hungary Kaldrovics around 1890 then Threisz from 1902 successfully utilized equine AI in the Kisbér and Mezőhegyes Studs (Bölcsházy and Mészáros 1962). Dezső Hámori after the World War II. introduced generally artificial insemination in order to stop the spread of infectious Trypanosomiasis. One year after the other artificial insemination stations had been established throughout the country. In 1950 seventy four Artificial Insemination Stations were working and altogether 42.000 mares were inseminated with sperm of 240 stallions. In average 175 mares were booked for one stallion. Several stallions had more than 300 mares for breeding (Mészáros 1951). In 1953 the number of AI stations and mares inseminated was culminated (63.000 horses in 120 breeding stations) and one-third of the broodmares were bred with AI. The reorganization of agriculture from the mid-1950s caused that importance of horse breeding gradually declined, artificial insemination was also getting underplayed. In 1960 only 9000 mares were inseminated, to the year of 1965

the application of AI had been almost completely gone. In the years of 1970s, Magdolna Alhegyi started cryopreservation of stallion semen in the National Artificial Insemination Station because of a dramatically decline in the number of horses, only this way was feasible to ensure preservation of genetic diversity of bloodlines of rare breeds.

In the last decades the use of artificial insemination (AI) in equine reproduction has been increasing again worldwide in the horse industry, offering many advantages over natural service. Some of the reasons for this include the choice of a great number of stallions, safety for both the mare and the stallion, reduced risk of infectious disease transmission, and transport inconvenience. Although pregnancy rates have been shown to be equal or even higher after AI with fresh or chilled semen compared to natural service, lower fertility occurs when mares are inseminated with frozen semen (Jasko et al. 1992, Samper 2001, Janett et al. 2003). Some experimental and field studies however indicate that acceptable fertility with frozen semen is possible with good quality semen, selection of mares and stallions and good mare management, and may not differ significantly from the fertility of commercial cooled semen (Metcalf 1995, Barbacini et al. 1999, Loomis 2001). Based on my clinical experiences I agree with these findings.

Smith and Polge (1950) first froze equine spermatozoa in 1950 and the first equine pregnancy using frozen semen was reported in Barker and Gandier (1957). Recently the total AI with cryopreserved or cooled equine semen is 0.5 million AI/year all over the countries. From the total number in 0.1 million AI/year has been used frozen semen. Around 350.000 foals are born each year after successful artificial insemination (Central European Management Intelligence /CEMI/, frozen + fresh data from 2006). Frozen semen offers breeders additional benefits not available with cooled semen: Stallions need not be available for every second day collections, allowing for involvement in performance events during the breeding season; Illness, injury or death of a stallion does not prevent insemination of mares with his semen; Frozen semen can be shipped in advance and maintained on the farm until the optimum time for insemination; It allows for international distribution; Less semen is wasted while all the semen collected for freezing is processed and stored resulting in an average of 10–12 insemination doses per ejaculate However some disadvantages of utilization of frozen semen limiting their general usage: Frozen semen results in lower fertility than with cooled semen for many stallions; More technical expertise is required for processing frozen semen than cooled semen; Increased costs associated with management of mares due to more frequent examinations (Loomis 2001).

The equine artificial insemination was getting a new impetus from the end of 90's also in Hungary. In 2003 forty two equine AI stations were working in the country with 314 stallions and around 5000 mares were inseminated which was half of the total number of broodmares involved in the breeding. After the governmental financial support system was stopped many of them have closed. In 2010 twenty three AI breeding stations were working with 96 stallions (data of Central Agricultural Office /Nemzeti Élelmiszerlánc-biztonsági Hivatal/ and National Horse Information System /OLIR/, personal communication of Dr. Ferenc Flink and Csilla Végi). The ratio of AI in all breeding services decreased however the utilization of frozen semen and imported cooled semen increased in the recent years. The "conservation of genetic resources" programme and supports for in vitro and/or ex situ preservation of protected native and endangered farm animal breeds starting in 2012 hopefully will bring again upsurge in Hungarian horse breeding.

In the recent years modern breeding technologies like hysteroscopic low-dose insemination, fluorescence-activated sex sorting of stallion spermatozoa, embryo transfer, embryo freezing and bisection, transvaginal ultrasound-guided oocyte collection (OPU), intracytoplasmic sperm injection for fertilization (ICSI), gamete intrafallopian transfer (GIFT) and nuclear transfer (cloning), have all been applied to equids with encouraging success (Allen 2005).

In the horse breeding selection of sire is based on performance, genetic potential and exterior. However excellent sport or race performance and noble appearance are not related to great fertility of the stallion. Unlike bulls, stallions have been not selected by the artificial insemination (AI) industry for many years and generations based on semen production, sperm quality and freezability. This explains that there is a wide variation in semen characteristics among individuals and in remarkable rate the semen quality is not sufficient. In some breeds (Kavak et al. 2004) or rather in some genetic lines increased proportion of morphologically abnormal spermatozoa was observed which is considered inheritable due to inbreeding, e.g. in the case of Kuhaylan Zaid Arabian line (Mészáros et al. 1951, Bölcsházy and Mészáros 1962). In more countries like in France or Netherlands semen evaluation is performed in the prospecting breeding stallions. In the Netherlands it has been recommended that minimum values of semen quality of 3-year-old stallions for registration in the studbook is a mean total number of motile, morphologically normal spermatozoa of 2×10^9 and a mean of 50% for motility and 50% of morphologically normal spermatozoa (Parlevliet et al. 1994) No such official recommendation exists in Hungary for the young stallions, although there is a Hungarian Standard for breeding stallion semen (7034/1999) which proposes at least

40 x 10^6 spermatozoa in 1 ml gel-free ejaculate, 20 ml gel-free volume, 40 % motility and ≤ 30% sperm with any morphologic aberrations, if less than half of these abnormal cells have primary defect. Moreover one insemination dose of diluted extended stallion semen is recommended to have at least 3-5 x 10^8 total number of live spermatozoa and to have ≥ 40 % progressive motile sperm at the time of insemination. Both of healthy reproductive status of the broodmare and good quality semen of the breeding stallion are playing key-role in the successful fertilization and producing offspring. AI, cooled- and frozen storage of the semen give opportunity to allocate more doses from one ejaculate of the breeding stallion and to inseminate several mares even in more-hundred kilometres distance from the stud farm where the sperm was collected. This is unrealizable if the stallion is used for naturel service. Considering this tendency in the world it is need to lay greater emphasis on examination of stallion sperm quality even before receiving the breeding permit. Conventional semen evaluation is very subjective mainly based on sperm concentration and movement of the spermatozoa. In spite of its limited applicability, motility and progressive motility are the most commonly used parameters in the evaluation of stallion semen, in both laboratories and studfarms, because it is easily accessible and quick to perform (Katila et al. 2001a). Various techniques and protocols are available for evaluation of the spermatozoon. However besides routine examination to combine several tests or use combined analyses of more features are needed for quality and fertility evaluation of equine semen (Katila 2001a, Colenbrander et al. 2003). It should be taken into consideration that if parameters of the fresh semen are behind the desired level, after chilled-transportation or cryopreservation these would be declined. Using multi-parametric semen analysis methods subfertile and infertile stallions would be identified and reason for decreased pregnancy results may be revealed (Colenbrander et al. 2003).

It is important that a reliable sperm quality control will be available in both of introduction of a new technology or annual or daily routine examination of the stallions in the practice. Kovács-Foote staining procedure (Kovács and Foote 1992) has been tested in many species since its first debut and may be suitable for complex quality control of the semen. In the equine species for the correct evaluation it was necessary to improve and modify the assessment technology, to achieve the ideal settings in every steps of the staining. Comparing the treatments in two sperm manipulation process and investigation of subfertile and fertile stallions, I defined new classification which could make the comparisons more sophisticated and which is capable of more complex evaluation.

2. LITERATURE OVERVIEW

2.1 Breeding soundness examination of the stallion

Annual assessments for actively breeding stallions prior to the breeding season can help with management decisions and provide a baseline for comparison if a problem should arise. Complex breeding soundness examination (BSE) of the stallion is an attempt to provide an estimate of a stallion's potential fertility and is composed of the following: History (past breeding records, illness, medications, pedigree); General physical examination (body condition, inheritable conditions, external genitalia, testicular evaluations, and measurements); Evaluation of libido and mating behavior; Quantity and quality evaluation of semen; Ancillary procedures (bacterial cultures, cytology, special stains, hormone analysis) (Juhász et al. 2000, Steiner 2002). The Society for Theriogenology has developed Guidelines for Classification of Breeding Stallions (Kenney et al. 1983) based on breeding 40 mares by natural service or 120 mares by artificial insemination with a result of at least 75 % of the mares become pregnant. Stallions are classified as satisfactory, questionable, or unsatisfactory breeding prospects. To be classified as satisfactory, the following criteria must be met: stallion demonstrates good libido and mating ability, normal penis without inflammatory lesions, free of venereal or transmissible diseases or potentially heritable defects; negative EIA (Coggins) test; two scrotal testes and epididymes which have normal size, consistency, and shape; total scrotal width greater than 8.0 centimeters; a stallion produces a minimum of one billion morphologically normal, progressively motile spermatozoa (corrected for season, e.g. 2 billions in May) in each of 2 ejaculates collected 1 hour apart. Under these criteria the stallion is classified as a questionable or unsatisfactory. Several stallions are not used for 40/120 mares and may be able to settle a smaller book of mares with adequate reproductive management. A recent report indicates that the mare book of Thoroughbred stallions in North America has increased considerably in the last 20 years, and that in 2005, 11% of stallions had a book greater than the traditional book of 40 mares. More importantly, over 50% of Thoroughbred mares were bred by stallions with books greater than 40 mares, and 35% of the mares were bred by stallions with books greater than 80 mares. Interestingly, foaling rates increased as the book increased, indicating that selected fertile stallions are able to couple with the larger books (Turner and McDonnell 2007, Brito 2007).

2.2 Stallion semen

In the process of BSE two semen samples are collected from the stallion one hour apart. The two ejaculates are considered representative if volume of the ejaculates are similar and the second contains approximately one-half the number of spermatozoa as the first with comparable or increased sperm motility. Immediately after collection, the sample is evaluated for colour, clarity, and volume. The gel portion of the ejaculate should be removed by filtration. Next, the motility of the sperm should be assessed and sperm concentration determined using a densometer, hemocytometer, or spectrophotometer. Sperm concentration multiplied by gel-free semen volume will give the total number of sperm. Live/dead ratio of spermatozoa and sperm morphology is also addressed as well as longevity of spermatozoa (Steiner 2002). Composition of semen shows species specific and individual differences within species moreover changes occasionally in different ejaculates. Individual differences are related to spermatogenesis and secretion of accessory sex glands. Sperm production of the stallion depends on age, testicular volume, sperm reserve capacity of the epididymis and is also influenced by extrinsic factors as nutrition, temperature, breeding season, frequency of ejaculation, medication (Haraszti and Zöldág 1993, Pickett 1993a). Volume of equine semen is in avarege 70 ml (30-300 ml), sperm concentration is 30-800 x 10^6 sperm/ml. The light-absorbance of semen is primarily influenced by the spermatozoa, therefore opacity of the ejaculate is proportional to sperm concentration. The pH of the semen is determined by accessory sexual glands which is pH. 6.6-7.6 (Pécsi 2007). The semen is composed by two main parts: cellular elements (5-10% dispersed portion) and seminal plasma (90-95%). Spermatozoa compose the main part of cellular elements and other cells like nucleated epithelial cells of reproductive tracts, immature spermatozoa, lymphocytes, leucocytes are also found in this fraction. In addition protein, lipid, pigment particles and prostatic amyloids are also analysed from the semen. Seminal plasma is the product of accessory sex glands. Different types of bacteria may be also presented in the sperm related to bacterial flora of the praeputium (Haraszti and Zöldág 1993). Values for the most frequently used parameters of fresh equine semen are presented in Table 1.

Evaluation of daily sperm output (DSO) is a criterion used to assess the breeding potential of a stallion. In order to accurately predict the number of mares a stallion can breed or the number of AI doses a stallion can yield during a given time period it is also necessary to know the number of morphologically normal spermatozoa in ejaculates. (Stich et al. 2002, Einarsson et al. 2009). Collecting and evaluating total

spermatozoa number (TSN) per ejaculate found that DSO determinations can begin as early as on the 5th day for stallions with smaller testes, and on the 6–7th day for stallions with larger testes (Kavak et al. 2003b).

.

Table 1. Quantitative and qualitative parameters of fresh equine semen (Juhász et al. 2000, Juhász and Nagy 2003)

Parameter	Mean ± SD	Number of Stallions	Reference
Gel free volume (ml)	65 ± 26	398	Parlevliet et al. 1994
	45 ± 30	417	Pickett 1993b
	33.7 ± 2.13	165	Dowsett and Knott 1996
	51.6 ± 31.5	8	Long et al. 1993
	45.3 ± 30.9	47	Dowsett and Pattie 1982
	63.1	245*	Juhász and Nagy 2003
Concentration (10^6/ml)	206.1 ± 168.5	398	Parlevliet et al. 1994
	335 ± 232	417	Pickett 1993b
	164.13 ± 39.35	165	Dowsett and Knott 1996
	223 ± 148	8	Long et al. 1993
	178 ± 168	47	Dowsett and Pattie 1982
	192.5 ± 169.6	245*	Juhász 2003
Total sperm number (10^9)	11.29 ± 7.13	398	Parlevliet et al. 1994
	11.9 ± 9	417	Pickett 1993b
	6.34 ± 1.93	165	Dowsett and Knott 1996
	9.1 ± 4.7	8	Long et al. 1993
	7.21 ± 6.87	47	Dowsett and Pattie 1982
	8.44 ± 4.61	245*	Juhász and Nagy 2003
Total motility (%)	53 ± 15	417	Pickett 1993b
	76.43	165	Dowsett and Knott 1996
	72.1 ± 16	47	Dowsett and Pattie 1982
	70.3 ± 17.4	64	Jakso et al. 1991
	70.3 ± 17.4	245*	Juhász and Nagy 2003
Progressive motility (%)	68 ± 9	398	Parlevliet et al. 1994
	53.1 ± 16.2	8	Long et al. 1993
	52.7 ± 23.8	64	Jakso et al. 1991
	66.9 ± 10.4	245*	Juhász and Nagy 2003
Live spermatozoa (%)	65 ± 16	398	Parlevliet et al. 1994
	82.56	165	Dowsett and Knott 1996
	78.8	47	Dowsett and Pattie 1982

*data based on 245 ejaculates of 8 different stallions

Collecting one ejaculate per day for 10 consecutive days found that TSN decreased successively from day 1 to 8 in full-size horses, and from day 1 to 4 in small-size

horses, thereafter remaining approximately at the same number. The DSO, calculated as the mean of TSN of days 8–10 was 6.4 x 10^9 (Kavak et al. 2003b, Einarsson et al. 2009).

2.3 Spermatozoa production, spermatogenesis, sperm maturation

Spermatogenesis is a process of division and differentiation of cells from diploid germinal cells termed spermatogonia, to haploid, mature spermatozoa in seminiferous tubules of the testis. Spermatozoa are released every 12.2 days in horses, however, the duration of spermatogenesis is around 57 days (Johnson et al. 1997). The 57-day-period may be divided into three phases, including: spermatocytogenesis, where stem cell spermatogonia divide by mitosis to produce other stem cells in order to continue the lineage throughout the adult life of the male, they also divide cyclically to produce committed spermatogonia and primary spermatocytes (19.4 days); meiosis, the period of replication (primary spermatocytes) and then reduction of genetic material into haploid spermatids (19.4 days); and spermiogenesis or spermiomorphogenezis which is a Sertoli cell aided differentiation of spermatids (18.6 days). Spermiation is the release of spermatids as spermatozoa into the lumen of seminiferous tubule. Transit of sperm through the efferent ductules and the epididymis is associated with significant maturation changes such as gaining the capacity for progressive motility; final condensation of the nucleus and further modification of the form of the acrosome; alteration of plasma membrane surface (release, modification and adsorption of proteins and lipids); migration of cytoplasmic droplet from a proximal to distal midpiece position. The most important functional changes in sperm occur in the efferent ductules, caput and corpus of epididymis. Sperm taken from the distal part of corpus and cauda epididymis already have the potential to fertilize (Bart and Oko 1989, Gadella et al. 2001). Approximately 9 days are required for transportation of spermatozoa through the excurrent duct system. Therefore, a new population of spermatozoa is ejaculated every 64–66 days (Amann 1993, Card 2005). The cauda epididymis has properties that allow the sperm to be stored for several weeks. Seminal fluids secreted by accessory sex glands (ampulla, seminal vesicles, prostate, bulbourethral glands) added during ejaculation serve as a vehicle, stimulate the metabolism of sperm and provide the energy requirements for passage through the uterus (Bart and Oko 1989, Gadella et al. 2001). In contact of female reproductive tract, mainly in the isthmus of oviduct the spermatozoa undergo special changes termed ‘‘capacitation’’ which include reorganization of membrane proteins, metabolism of membrane phospholipids, reduction in membrane cholesterol levels. Capacitation takes 6-8 hours in stallion spermatozoa (Yanagimachi 1994).

The process of spermatogenesis, steroidogenesis and testicular function are regulated by a complex interplay of endocrine, paracrine and autocrine signals. It is well established in many mammalian species, including the horse, that normal testicular function is dependent upon a functional pineal gland and hypothalamic – pituitary - testicular (HPT) axis. The pineal gland secretes melatonin that acts on the hypothalamus to regulate gonadotropin-releasing hormone (GnRH) output. GnRH stimulates secretion of the pituitary hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), which in turn acts at the level of the testis. LH binds to receptors on testicular Leydig cells to stimulate production of testosterone and estrogens while FSH binds to the Sertoli cell to stimulate production of estrogens, inhibin (INH) and activin. These testicular steroid and protein hormones are components of the classic feedback mechanisms involved in the regulation of hypothalamic GnRH and pituitary LH and FSH (Amann 1993).
Recent studies in the horse have demonstrated the potential involvement of other hormones such as opioids, prolactin, growth hormone (GH), thyroid hormone and activin (Gerlach and Aurich 2000, Arai et al. 2006, Roser 2007). Moreover, evidence indicates that local paracrine - autocrine factors within the testes such as growth factors like IGF-1, oxytocin, vasopressin, pro-enkephalins, enkephalins, propiomelanocortin, β-endorphins, cytokines, transferring, modulate endocrine control of testicular function, steroidogenesis and spermatogenesis (Roser 2008). Systemic hormones and local testicular factors work together to initiate and maintain testicular function. Testosterone from the Leydig cells diffuses into the seminiferous tubules and in conjunction with FSH signals the Sertoli cells to produce local factors that initiate and maintain development of the germ cells. FSH is of critical importance in the initiation and expansion of spermatogenesis in mammals during puberty, however the role of FSH in the regulation of spermatogenesis in the adult mammal is still controversial. (Zirkin et al. 1994). Many of the local interactions in the equine testis are unknown. Inhibine and activin have endocrine, paracrine and autocrine functions. Inhibin feeds back on the pituitary to inhibit the release of FSH. Activin has been shown to positively modulate the release of FSH at the level of the pituitary in other species, but its action in the stallion is unknown (Roser 2007). IGF-1 may be involved in testicular development (Hess and Roser 2001, Roser 2008).

Billions of spermatozoa are produced each day. Many of the cells produced are defective and some are eliminated through apoptosis and phagocytosis by the Sertoli cells, whereas others are passed into the ejaculate (Heninger et al. 2004, Card 2005). Sertoli cells, the somatic component of the seminiferous epithelium, are critical for germ cell development during spermatogenesis. High environmental temperature,

other conditions such as fever, orchitis, periorchitis, hydrocele, scrotal hemorrhage, scrotal edema, scrotal dermatitis and improper scrotal descent can interfere with the thermoregulatory mechanism necessary to cool the testis and allow normal spermatogenesis (Varner et al. 1991, Johnson et al. 1997).

2.4 Structure of equine spermatozoa

The spermatozoon consists of the head, neck, and tail and is entirely covered by the plasma membrane. The tail is the longest part of the spermatozoon and consists of midpiece, principal piece, and end piece (Figs 1/A, 1/B, Amann and Graham 1993, Brito 2007). The length of equine spermatozoon is approximately 60 μm (Pesch and Bergman 2006, Brito 2007). Every part of the spermatozoa plays important role in the fertilization process. The plasma membrane surrounds the spermatozoon in total and is characterised by a regional specific glycoprotein and lipid, mainly phospholipids and cholesterol constitution. These so-called surface domains are important for the function of the membrane areas. For example, the part of the membrane at the equatorial segment is responsible for the contact to the oocyte membrane in fertilization (Busch and Holzmann, 2001, Pesch and Bergman 2006). Lipids are arranged as a bilayer. Proteins are intermingled with the lipids as integral or peripheral proteins. The ratio of cholesterol to phospolipids and the nature of phospholipids determine the flexibility of the membrane, which is “fluid” at body temperature (Hammerstedt et al. 1990, Juhász et al. 2000). Plasma membrane of stallion sperm contains approximately 57% phospholipids, 37% cholesterol and 6% glycolipids, such that the stallion sperm differs primarily with regard to its relatively high cholesterol content. Phospholipids are the major lipid components and they are largely composed of polyunsaturated fatty acids (PUFA) (Scott 1973) in particular, docosahexaenoic acid (DHA; 22:6 n-3, an omega-3 fatty acid) and docosapentaenoic acid (DPA; 22:5 n-6, an omega-6 fatty acid). Stallion sperm differ from those of the other mammalian species by having a surprisingly very high content of 22:5 fatty acids in their phosphocholineglycerides and phosphoethanolamineglycerides fractions of phospholipids and relatively few 22:6 fatty acids (Yanagimachi 1994, cited: Gadella et al. 2001).

The spermatozoon head is formed by the acrosome, the postacrosomal lamina, and the nucleus (Fig. 1/A). The anterior two-thirds of the nucleus is overlaid by the acrosome, which is a specialized vesicle formed from a double-layered membrane which contains hydrolytic enzymes essential for spermatozoon penetration of the zona pellucida of the oocyte. The acrosome is covered by the inner and outer acrosomal

membrane and can be divided into apical, principal and equatorial segments. The equatorial segment does not contain enzymes and is not involved in the acrosomal reaction, but the plasma membrane in this area fuses with the plasma membrane of the oocyte. The postacrosomal lamina may have a role in attachment of the spermatozoa to the oocyte. The nucleus compromises most of the spermatozoon head and contains the genetic material in the form of highly condensed DNA. The nucleus is contained by a double-layered nuclear envelope. The base of the nucleus terminates with the implantation fossa, where the outer layer of the double-layered nuclear envelope thickens to form the basal plate, which provides the attachment of the head to the capitulum of the neck. Position of the implantation fossa is often abaxial in the stallion, therefore abaxial position of the tail is considered normal in equine spermatozoa. This results in curvilinear movement of the sperm. The border between the head and neck is clearly defined by a posterior ring (Amann and Graham 1993, Juhász et al. 2000, Brito 2007).

The head of the equine spermatozoon is elliptical shape, slightly thicker at the posterior part. Reported means for dimensions of the stallion spermatozoon head include: 5.33 μm to 6.62 μm for length, 2.79 μm to 3.26 μm for maximum width, 0.43 to 0.52 for length/width ratio, 13.76 μm to 15.64 μm for perimeter and 11.43 μm^2 to 16.28 μm^2 for area. All reports indicate a significant stallion effect (Dott 1975, Bielanski and Kaczmarski 1979, Ball and Mohammed 1995, Gravance et al. 1996, 1997, Casey et al. 1997, Pesch and Bergman 2006, Brito 2007). The variation in the shape of normal stallion sperm heads is considerable, ranging from somewhat thinner and elongated to shorter and broader forms. The correct classification of sperm with extreme head shape morphology may be difficult, and the distinction of tapered and microcephalic sperm heads requires comparison among several sperm to establish what the “normal” sperm head shape for an individual stallion is (Brito 2007). Substantial differences in sperm head shape and size were found between breeds and within breeds in stallions. However preparation of sperm for morphometry analyses was also important, sperm head size as determined from Feulgen-stained spermatozoa was smaller than that determined from live, unfixed spermatozoa (Ball and Mohammed 1995). Differences in sperm head size within breed have been reported in both Warmblood (Ball and Mohammed 1995) and Spanish Thoroughbred stallions (Hidalgo et al. 2008). Similarly, differences between breeds have been observed in Arabian, Warm-blood, Thoroughbred and Morgan stallions (Ball and Mohammed 1995). The results of study Phetudomsinsuk et al. (2008) confirmed the previous observations; morphometric characters of normal sperm heads were significantly different among individual Thai native croosbred (T) or control warmblood (purebred)

stallions, and between T and Purebred stallions. The heads of stallion spermatozoa were analysed by computer-assisted sperm head morphometry (ASMA) in several studies. The mean values for length, width, area and perimeter in the major cluster of sperm head dimensions of fertile stallions (>60% per cycle conception rate) were significantly different from those of the subfertile stallions (<40% per cycle conception rate). The range of values of the major cluster of fertile stallions was length: 4.9-5.7 μm, width: 2.5-3.0 μm, width/length ratio: 0.45-0.59, area: 10.3-12.1 μm^2, and perimeter: 12.9-14.2 μm. On the basis of these values, a significantly higher percentage of normal sperm heads were found in the fertile group than in the subfertile group of stallions (52% versus 19%). The results suggest that a value of < 30% of spermatozoa with normal head morphometry may indicate impaired fertility in stallions, while a value > 40% would be indicative of a fertile stallion. The mean measurements for length, area and perimeter were significantly larger in the subfertile than the fertile group (5.77 vs 5.33 μm, 12.66 vs 11.37 μm^2 and 14.59 vs 13.64 μm respectively). Sperm in subfertile stallions also tended to be more tapered than in fertile stallions (Gravance et al. 1996, Casey et al. 1997). The larger sperm heads found in subfertile stallions may reflect disturbances in spermatogenesis, particularly involving altered chromatin structure. However, it is important to note that subfertile stallions also had lower total sperm number and percentages of motile and normal sperm in the ejaculate than fertile stallions, which likely also influenced fertility (Brito 2007). Révay et al. (2004) measured the head area of bull spermatozoa after viability and acrosome staining using trypan blue and Giemsa stains, followed by X- and Y-chromosome-specific fluorescence in situ hybridisation (FISH). In all bulls, morphologically normal, viable cells with intact acrosomes were significantly smaller than dead cells with damaged acrosomes. No significant difference in the head area between X- and Y-chromosome-bearing viable, acrosome-intact spermatozoa was found in individual bulls. It seems that live/dead status of the sperm also influences head-morphometry and partly can be the reason for higher values of spermatozoa of subfertile males. Arruda et al. (2002) studied the effects of extenders and cryoprotectants on stallion sperm head mophometry using ASMA. The morphometric parameters such as length, perimeter and area were significantly smaller in cryopreserved sperm than in fresh-extended sperm. Changes in dimensions might be due to acrosomal damage or alteration in chromatin condensation associated with cryopreservation, or extender's osmolarity.

The spermatozoon neck is a short linking segment between the head and the tail contains the connecting piece, the proximal centriole, which are the base of the dense fibers and the axoneme (Fig. 1/B). This region is the site, where beat of the tail is

initiated. The tail of the spermatozoon includes the middle piece, the principal piece and the end piece. The length of the tail is in average 54 µm (midpiece: 10 µm, principal piece: 40 µm, end piece: 4 µm). Diameter of the midpiece: 0.9 µm and of the principal piece: ≤ 0,6 µm. The midpiece is the widest part of the tail formed by the axoneme surrounded by the nine outer dense fibers and the mitochondrial sheath, extends from the caudal end of the neck to the annulus (or Jensen's ring). Mitochondrial sheath is the helically arranged mitochondria, which contains enzymes and cofactors for production of ATP. The axoneme and dense fibers of the midpiece continue through the principal piece, but the dense fibers become narrower and terminate at different levels in the distal principal piece. The principal piece is the longest part of the spermatozoon. It contains the axoneme, the dense fibers and a fibrous sheath, which is characteristic of this part. The axoneme consists of a central pair of microtubules surrounded by nine microtubular doublets which are the elements that contract to produce sperm tail movement. Axoneme microtubules extend from the neck region through the midpiece and principal piece into the end piece, where they terminate at slightly different sites. The dense fibers and fibrous sheath do not contract; however, they provide rigidity and flexibility at the same time for the flagellar movement. The end piece is the short terminal segment of the tail containing only the axoneme or single microtubules which are surrounded by the plasma membrane (Bielanski and Kaczmarski 1979, Amann and Graham 1993, Juhász et al. 2000, Brito 2007).

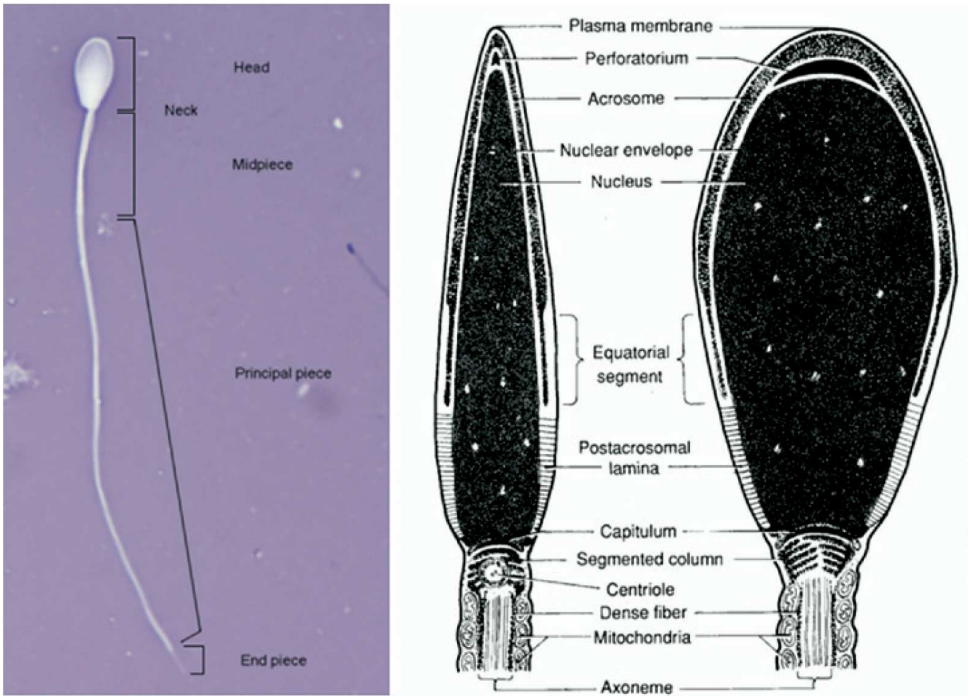


Figure 1/A. Structure of stallion spermatozoon (Source: Amann and Graham 1993, Brito 2007)

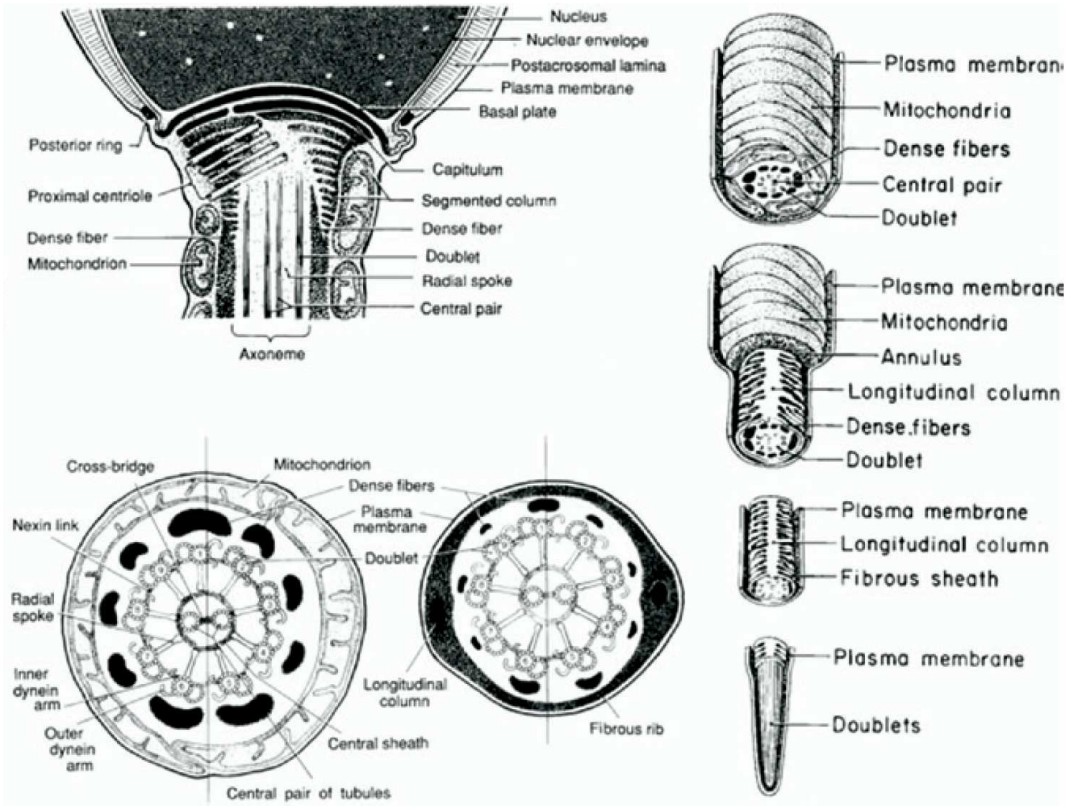


Figure 1/B. Structure of stallion spermatozoon
(Source: Amann and Graham 1993, Brito 2007)

2.5 Morphology of stallion spermatozoa

The morphologic assessment of spermatozoa has a long history that begins with the scientist Van Leeuwenhoek in 1678. He noted ‘‘animalicula’’ in seminal fluid, and was the first to record heterogeneity in spermatozoal head shapes in men and other species. Sperm vary considerably in their morphology between and within species related to sperm competition and cooperation. High inter-male variability and high levels of sperm pleiomorphy within ejaculates are observed in species with low levels of sperm competition (e.g. some monogamous passerine birds in the nature or in domestic animals which are not selected for sperm quality: dog, horse) possibly because of relaxed selection pressure on sperm quality control (Birkhead and Immler 2006). It has been shown that specific morphologic abnormalities of spermatozoa are related to male sub- or infertility. Systematic studies of spermatogenesis and spermatozoal morphology resulted in the development of a number of classification systems (Card 2005).

2.5.1 Classification systems

During morphology evaluation of spermatozoa structure of the sperm is analysed, alterations compared to normal cells and the proportion of abnormal sperm are determined (Pécsi 2007). Abnormalities in spermatozoal morphology traditionally have been classified as ***primary, secondary,*** **or** ***tertiary*** according to their origin. Primary defects are representing a failure of spermatogenesis caused by pathological processes in the seminiferous epithelium. Primary defect is therefore testicular in origin and includes such defects as nuclear vacuoles, pyriform heads, microcephalic sperm, Dag defect, and mitochondrial sheath defect. Secondary abnormalities are created in the excurrent duct system representing a failure of maturation and abnormal epididymal function. An example of a Secondary defect is a distal midpiece reflex (DMR). Tertiary abnormalities develop in vitro as a result of improper semen collection or handling procedures (Blom 1977, Barth and Oko 1989). Using the traditional classification system the assortment and its interpretation can be incorrect because the origin of some spermatozoal morphologic abnormalities is unknown and some defects can be either primary or secondary. Proximal droplets may be either the result of a disturbance of spermatogenesis (primary) or a disturbance of epididymal function (secondary). Detached heads may be due to a defect of the basal plate which connects the sperm head to the midpiece (primary), or it may be due to abnormal epididymal function (secondary) (Barth 1994) or it can be artifact produced by smearing the semen (tertiary) (Varner 2008). High proportion of cells with distal droplet can be the result of epididymal malfunction but may be caused by a lack of a haemolytic factor in seminal fluid which is one of the product of seminal vesicle that enhances cytoplasmic droplet release (Barth and Oko 1989). It is also important to note that definition for primary and secondary sperm defects denotes the origin and not the severity of a defect. Primary defects are not necessarily more deleterious to fertility than secondary defects as a common misinterpretation of this system shows. Furthermore since adverse conditions that cause both types of abnormalities can affect epididymal function and spermatogenesis simultaneously, primary and secondary defects are equally important as indicators of disturbance of testicular function (Barth 1994).

Another classification system reported by Blom labels defects as ***Major*** or ***Minor*** according to their importance to fertility. Major defects include abnormal heads, midpieces, proximal droplets, double forms, which are thought to have a greater impact on fertility. The major defects are mostly those that have been associated with a presumed disturbance of spermatogenesis. Minor defects such as distal droplets or simple bent tail have an unknown role or no consequence for fertility. (Blom 1950,

1973, 1977, Barth and Oko 1989, Barth 1994, Card 2005). Although minor defects are of less importance with regard to fertility, they may cause a serious reduction in fertility when present in very high numbers (Blom 1977, Barth and Oko 1989).

Another concept regarding spermatozoal defects involves determining if the defect is ***compensable*** or ***non-compensable.*** A compensable defect (e.g. knobbed acrosome or bent tails) is one where the defective spermatozoa either do not reach the site of fertilization (uterotubal junction filters a proportion of abnormal sperm), or if the defective spermatozoon reaches the oocyte but not capable of penetrating the zona pellucida and the cortical reaction is not induced. These defects may be compensated by increasing sperm dosage. Defective spermatozoa which are not filtered and which are capable of penetrating the zona pellucida causing cortical reaction but are not supporting further embryonic development and cannot be compensated by higher number of sperm in the insemination dose. These non-compensable spermatozoal defects interfere with fertility and compete with normal spermatozoa for fertilization. An example of a non-compensable defect is a defect in chromatin condensation or diadem defect (Saacke et al 2000, Barth 1994, Card 2005). Consequently it is important to realize that breeding doses with different sperm defects might also have different effects on fertility even when the percentages of normal sperm are the same (Brito 2007). Barth and Oko (1989) proposed limits of different group of sperm categories considering the fertilizing ability of the bull ejaculate: 1. Abnormalities of the nucleus that would allow oocyte penetration, zona reaction, or syngamy (but not fertilization or embryonic development) cannot be tolerated at levels than 15-20% of spermatozoa. 2. Abnormalities of the acrosome or sperm tail do not interfere with the ability of other normal cells to fertilize ova and thus can probably be tolerated at levels up to 25% of spermatozoa. 3. At least 70% of spermatozoa should be normal.

The current trend is to ***record the numbers of specific morphologic defects***, such as knobbed acrosomes, proximal cytoplasmic droplets, swollen midpieces and coiled tails. This method of classification is considered superior to the traditional system because it reveals more specific information regarding a population of sperm, while avoiding false assumptions about the origin of these defects (Varner 2008). Any clustering method is used, normalities and abnormalities should always be consistently identified and categorized (Juhász and Nagy 2003). Simple, practical method which can also be used in routine examination is the classification that defines main morphologic categories according to subdomains of the sperm then divides them further into subcategories (Fig. 2).

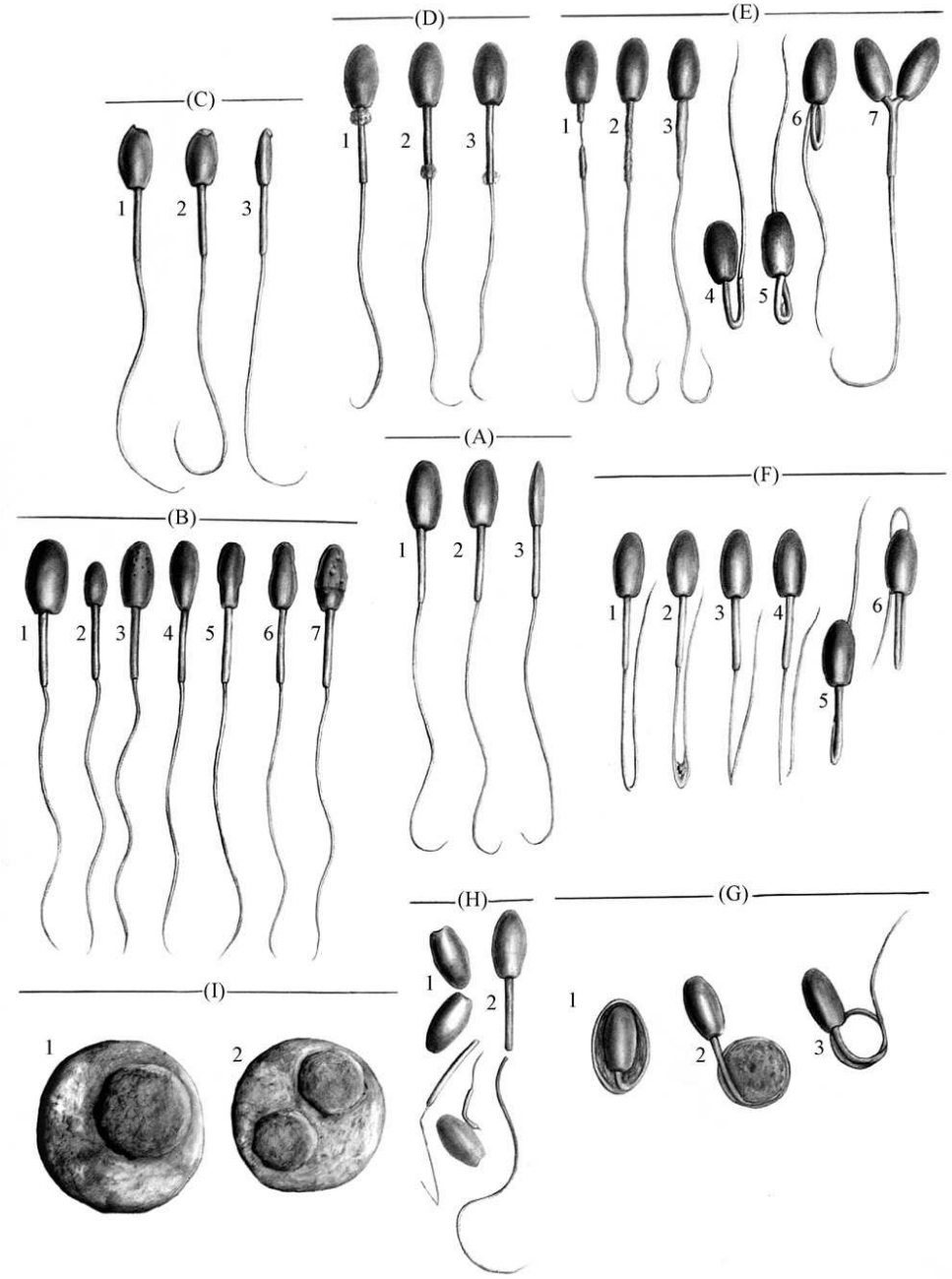


Figure 2. Normal and abnormal sperm morphologic features (Source: Varner 2008)

a) Normal spermatozoa (A)

b) Abnormal head morphology (B)

- macrocephalic (B1)
- microcephalic (B2)
- nuclear vacuoles or crater defects (B3)
- tapered head (B4)

- pyriform head (B5)
- hour-glass head (B6)
- degenerate head (B7)

c) Acrosomal defects /knobbed acrosome/ (C)

d) Proximal cytoplasmic droplets (D1), distal cytoplasmic droplets (D2, D3)

e) Midpiece abnormalities (E)
   - segmental aplasia of the mitochondrial sheath (E1)
   - roughed midpiece from uneven distribution of mitochondria /corkscrew defect/ (E2)
   - enlarged mitochondrial sheath (E3)
   - bent midpiece (E4, E5, E6)
   - double midpiece/double head (E7)

f) Bent tail or hairpin tail (F)
   - bent principal piece (F1–F4)
   - single bend involving the midpiece-principal piece junction /DMR/ (F5)
   - double bend involving the midpiece-principal piece junction (F6)

g) Coiled tail (G)

h) Fragmented sperm /detached heads or tailless heads/ (H)

i) Premature germ cells, round spermatids (I)

In the situation where a spermatozoon has more than one defect, the Society for Theriogenology guidelines suggests the most proximal defect is identified on each spermatozoa (Kenney et al. 1990). In this morphology evaluation system defects are prioritized based on the assumption that certain defects are more important or more deleterious to fertility than others (Brito 2007). The ***differential spermiogram system*** is similar to what occurs when we analyze the white cells in a leukogram. All of the abnormalites are counted regardless of one spermatozoon shows two or three defects. Therefore, if a spermatozoon has a macrocephalic head and a midpiece defect, both are enumerated. The type of head defects and midpiece defects is recorded, too. To enumerate multiple defects simultaneously, a special cell counter is used. When abnormalities are counted on each spermatozoon, only one number is added to the total count when more than one defect are identified by pressing keys representing multiple categories of defects. The percentages of sperm defects when added to the percentage of normal sperm will not tally to 100% (Card 2005, Brito 2007). In the Primary and Secondary defect system, if a spermatozoon has three defects such as a knobbed acrosome, a swollen midpiece and a proximal droplet, one primary defect would be enumerated even though there is another primary (swollen midpiece) and a

secondary (proximal droplet) defect present. Using the Major–Minor system where a spermatozoon has a pyriform head, segmental aplasia of the mitochondrial sheath and a distal droplet, only one major head defect would be enumerated. If a system is used where the defects are prioritized so that only one is chosen per spermatozoon, the examiner will achieve a percentage distribution that will total to 100%. They do not have a record of the real distribution of all the spermatozoal defects in the ejaculate. It is not possible to track changes in individual defects. Primary–Secondary, Major–Minor or differential spermiogram systems will identify the percentage of normal spermatozoa in the sample, which is a constant in all systems. Enumerating all defects for each spermatozoon allows the examiner to determine the potential of the defect to interfere with fertility, and analyse the changes in the patterns or cellular associations of the defects over time. Different defects found together represent more severe disturbances in spermatogenesis and could influence the prognosis (Veeramachaneni et al. 2006). The present Society for Theriogenology forms for stallion breeding soundness evaluation have the following categories listed in the differential spermiogram: normal sperm, abnormal acrosomal regions/heads, detached head, proximal droplets, distal droplets, abnormal midpieces, and bent/coiled tails. The presence of other cells (round germ cells, WBC, RBC, etc.) should also be indicated (Card 2005).

2.5.2 Preparation for morphologic evaluation

The standard evaluation of sperm morphology is performed with phase and/or light microscopy (Kenney et al. 1983) and computer-assisted methods have also been used (Ball and Mohammed 1995, Casey et al. 1997). However, available computer-assisted methods can only evaluate the sperm head, not count morphological abnormalities of mid-pieces, tails and acrosomes (Kavak et al. 2004). The morphology or structure of spermatozoa is typically examined with a light microscope (LM) at 1000 x magnification. Standard bright-field microscope optics can be used to examine air-dried semen smears prepared by specific stains. Giemsa, hematoxylin/eosin, eosin/nigrosin, Papanicolaou are the most widely used stains. Visualization of the structural detail of spermatozoa can be greatly enhanced by fixing the cells in buffered formol saline or a similar fixative, then viewing the unstained cells as a wet-mount with either phasecontrast or preferably, differential-interference contrast (DIC) microscopy (Kenney et al. 1983, Varner 2008). More detailed analysis can be done using transmission and scanning electron microscopy (Dott 1975, Dowsett et al. 1984, Barth and Oko, 1989).

Brito et al. (2011) demonstrated that sperm morphology evaluation results varied, depending on the evaluation method and clinician by analysing a diverse population

represented a wide range of fertility, from normal and fertile to severely sub-fertile stallions. There were significant differences among methods for all sperm morphology categories. Wet-mount preparation with phase-contrast microscopy appeared to be more sensitive for identification of abnormal stallion sperm when compared to eosin/nigrosin- and Papanicolaou-stained smears. The use of wetmount preparations seemed to reduce the introduction of some artifacts (detached sperm heads), but increased others (bent/coiled midpieces). The use of wet-mount preparations and phase-contrast facilitated the observation of acrosome defects, nuclear vacuoles, and cytoplasmic droplets, as demonstrated by the increased proportions of these defects when compared to stained smears. However one potential disadvantage of wet preparations is the observation of improperly oriented sperm, i.e., sperm that do not lie flat on the slide and therefore cannot be properly classified. A common concern with eosin/nigrosin is the hypotonicity of the stain and the possibility of introduction of artifactual tail defects, e.g., bending and coiling. Although the use of Papanicolaou-stained smears is the method recommended by the WHO (2010) for evaluation of human sperm, the results obtained with stallion sperm were extremely poor. Abnormalities of sperm head and size were readily identified, but more subtle defects, including acrosome defects and nuclear vacuoles were difficult to observe with this stain. Moreover, staining of the tail was generally light and contributed to the difficulty of observing rough/swollen midpieces, principal piece defects, and cytoplasmic droplets.

Although reference materials with detailed descriptions and comprehensive documentation of sperm morphology are available for some species (WHO 2010, Barth and Oko 1989), similar materials have not been available for stallions however recent publication of Brito (2007) has attempted to document stallion sperm morphology in samples stained with eosin-nigrosin (Brito et al. 2011).

Percentages for the most frequently used morphologic categories of spermatozoa of stallions with normal fertility according to different publications are presented in Table 2.

Table 2. Means of spermatozoal morpologic characteristics

Morphologic categories	Mean ± SD (%)	Number of Stallions and (samples)	Breed	Reference
Normal morphology	66 ± 15*	398	Dutch warmblood▪	Parlevliet et al. 1994
	61 ± 13	5 (10)	different breeds	Ball and Mohammed 1995
	67.8	168 (531)	9 different breeds	Dowsett and Knott 1996
	59.8 ± 9.4	8 (245)	different breeds	Juhász and Nagy 2003
	57.5 ± 4	6 (12)	Tori breed	Kavak et al. 2004 ▸
	74.4 ± 4	7 (14)	Estonian breed	Kavak et al. 2004
	49.7 ± 1.3	5 (30)	Thai native crossbred	Phetudomsinsuk et al. 2008
	48.1 ±2.8	4 (24)	Warmblood	Phetudomsinsuk et al. 2008
	72.7 ± 1.5	8 (23)	Swedish Warmblood	Einarsson et al. 2009
Head defect	2.8 ± 2.4	398	Dutch warmblood▪	Parlevliet et al. 1994
	5.4 ± 2	168 (531)	9 different breeds	Dowsett and Knott 1996
	3.9 ± 2.1 #	16 (299)	Thoroughbred	Koyago et al. 2008
	3 – 5	8 (245)	different breeds	Juhász and Nagy 2003
	12.6 ± 1.7	6 (12)	Tori breed	Kavak et al. 2004
	13.9 ± 1.5	7 (14)	Estonian breed	Kavak et al. 2004
	10.2 ± 0.5	5 (30)	Thai native crossbred	Phetudomsinsuk et al. 2008
	13.4 ± 1.1	4 (24)	Warmblood	Phetudomsinsuk et al. 2008
	10.2 ± 0.7	8 (23)	Swedish Warmblood	Einarsson et al. 2009
Abnormal midpiece	5.1 ± 4.1	398	Dutch warmblood▪	Parlevliet et al. 1994
	0.35 ± 2.3	168 (531)	9 different breeds	Dowsett and Knott 1996
	4.5 – 6	8 (245)	8 different breeds	Juhász and Nagy 2003
	5.1 ± 1.2	6 (12)	Tori breed	Kavak et al. 2004
	2.4 ± 1.1	7 (14)	Estonian breed	Kavak et al. 2004
	16.5 ± 0.8	5 (30)	Thai native crossbred	Phetudomsinsuk et al. 2008
	23.9 ± 2.1	4 (24)	Warmblood	Phetudomsinsuk et al. 2008
	5 ± 0.9	8 (23)	Swedish Warmblood	Einarsson et al. 2009
Tail defect	2.1 ± 2.1	398	Dutch warmblood▪	Parlevliet et al. 1994
	7.5 ± 2.2	168 (531)	9 different breeds	Dowsett and Knott 1996
	11.5 ± 5.9 #	16 (299)	Thoroughbred	Koyago et al. 2008
	10 – 15	8 (245)	different breeds	Juhász and Nagy 2003
	6.6 ± 1.4	6 (12)	Tori breed	Kavak et al. 2004
	6.4 ± 1.3	7 (14)	Estonian breed	Kavak et al. 2004
	1.6 ±0.1	5 (30)	Thai native crossbred	Phetudomsinsuk et al. 2008
	3.3 ± 0.6	4 (24)	Warmblood	Phetudomsinsuk et al. 2008
	3.7 ± 0.7	8 (23)	Swedish Warmblood	Einarsson et al. 2009
Detached head	1.5 ± 3	168 (531)	9 different breeds	Dowsett and Knott 1996
	2.3 ±0.9	6 (12)	Tori breed	Kavak et al. 2004
	2.3 ±0.9	7 (14)	Estonian breed	Kavak et al. 2004
	1.6 ± 0.3	8 (23)	Swedish Warmblood	Einarsson et al. 2009
Proximal droplets	5.8 ±4.6*	398	Dutch warmblood▪	Parlevliet et al. 1994
	6.7 ± 2.6	168 (531)	9 different breeds	Dowsett and Knott 1996

	2.4 ± 2.6**[#]	16 (299)	Thoroughbred	Koyago et al. 2008
	2.5 – 4	8 (245)	different breeds	Juhász and Nagy 2003
	17.3 ± 2.7	6 (12)	Tori breed	Kavak et al. 2004
	2.9 ±2.5	7 (14)	Estonian breed	Kavak et al. 2004
	11.4 ± 1	5 (30)	Thai native crossbred	Phetudomsinsuk et al. 2008
	5.5 ± 0.7	4 (24)	Warmblood	Phetudomsinsuk et al. 2008
	6.4 ± 1	8 (23)	Swedish Warmblood	Einarsson et al. 2009
Distal droplets	4.3 ± 4.3*	398	Dutch warmblood▪	Parlevliet et al., 1994
	4.8 ± 2.7	168 (531)	9 different breeds	Dowsett and Knott, 1996
	2 – 5	8 (245)	different breeds	Juhász and Nagy 2003
	10.5 ± 0.8	5 (30)	Thai native crossbred	Phetudomsinsuk et al. 2008
	5.8 ± 0.9	4 (24)	Warmblood	Phetudomsinsuk et al. 2008
	3.6 ± 1	8 (23)	Swedish Warmblood	Einarsson et al. 2009

* percentage of live (unstained) spermatozoa (anilin blue-eosin staining)
▪ Three-year-old young stallions
** The rate of spermatozoa with cytoplasmic droplets (proximal + distal droplets)
[#] Measurements from dismount semen (hematoxylin – eosin staining)
▸ Morphological evaluation was performed in wet preparations made from the formol-saline fixed samples
(Hancock 1957) and carbolfuchsin-eosin staining for assesment of head defect

2.5.3 Abnormal morphology of spermatozoa

The pathogenesis and effects on fertility of specific sperm defects have been more extensively studied in bulls than in stallions although several observations related to morphology of sperm from fertile and subfertile stallions have been published. The incidence of sperm ***head defects*** is relatively high and these are usually either the most or second most prevalent defects in the stallion ejaculate (Dowsett and Knott 1996, Dowsett et al. 1984, Love et al. 2000, Jasko et al. 1990). The *pyriform and tapered head defect* is the most common sperm nuclear abnormality. Small numbers of these defects are found in the semen of most bulls, even in bulls of good fertility. Pyriform head defects usually occur as a result of abnormal testicular functions through disturbances in intratesticular heat regulation or endocrine balance (Barth and Oko 1989). The swim-up technique, which is used to separate highly motile spermatozoa from the rest of the sperm population, did not significantly decrease the proportion of pyriform spermatozoa in the insemination droplets (Kawarsky et al. 1995). This finding is in agreement with previous reports that spermatozoa with pyriform heads have motility similar to that of spermatozoa with normal heads (Barth and Oko 1989). These cells have generally normal intact acrosomes. In a study of Kawarsky et al (1995) visual assessment of the oocyte-spermatozoon interaction revealed pyriform spermatozoa binding to the zona pellucida (ZP), penetrating the ZP, and entering the perivitelline space. The rate of sperm penetration and the rates of cleavage and

development embryos beyond the 8-cell stage with spermatozoa from bulls with mostly normal spermatozoa and bull producing over 75% pyriform spermatozoa were not statistically different. Other in vivo studies have revealed differences in fertilization rates using pyriform spermatozoa compared with those of controls. In vitro studies of Thundathil et al. (1999) partly disagreed with Kawarsky et al. (1995). They indicated that sperm with tapered and pyriform heads had reduced ability to bind the zona pellucida, but that the capacity to penetrate the zona and fecundate the oocytes after binding was unaffected. However, defective sperm seemed unable to sustain normal embryonic development after fecundation (Thundathil et al. 1999). Studies in bulls demonstrated that transport of sperm with tapered and pyriform heads was impaired and these sperm were selectively "filtered" throughout the female genital tract, so that only a small proportion of inseminated sperm with these defects were found as accessory sperm (Saacke et al. 1998).

Microcephalic and macrocephalic sperm are probably the consequence of insults to primary and secondary spermatocytes that then have an uneven distribution of nuclear chromatin content after abnormal cell division (Brito 2007). It has been observed that the incidence of macrocephalic heads in the spermiogram of bulls with good fertility is nearly always less than 1 %. It is much more common to observe an increase in microcephalic heads, but the number generally does not exceed 5-7 % of the spermiogram even in severe disturbances of spermatogenesis. The reason for that these cells probably die before reaching the spermatid stage and are phagocytosed by the Sertoli cells (Barth 1994). It is unlikely that microcephalic or macrocephalic sperm are able to participate in oocyte fertilization and embryonic development (Barth and Oko 1989). Giant heads are often diploid or even tri- or tetraploid (Love et al. 2000).

Nuclear vacuoles (also called diadem defect) are primarily found arranged across the equatorial region or at the apex of the nucleus. Vacuoleted spermatozoa have been shown to be transported normally to the oviduct and are able to penetrate oocytes but are incompatible with embryonic development (Barth 1994). The defect is easily missed on smears stained with Giemsa, hematoxylin-eosin, or eosin-nigrosin stains. Phase-contrast microscopy and DIC are suitable tools and Feulgen stain also is sufficient method for detecting this abnormality. The incidence of sperm vacuoles may increases following the stress of injury, illness, feed shortage, abnormal climatic conditions, etc. (Barth and Oko 1989). Nuclear vacuoles are also identified in stallion sperm in fairly high proportion (Janett et al. 2003, Brito et al. 2011). Interestingly Brito et al. (2011) found nuclear vacuoles in the proportion of 6.8 ± 0.6% in 60 semen samples from 34 stallions showing wide range of fertility, from normal fertility to

severe subfertility, whereas Janett et al. (2003) recorded 7.5-9.8 ± 0.5 % of sperm with this defect in 260 semen samples from 10 stallions with unknown fertility.

In stallions, Jasko et al. (1990) observed a negative correlation between the percentage of sperm head defects and fertility and reported that, among sperm morphological categories, the percentage of head defects accounted for the largest proportion of variation in per cycle pregnancy rates. Love et al. (2000) also observed an association between sperm head defects and fertility. Held et al. (1991) reported the case of a 9-year-old Arabian stallion used to breed an undetermined number of mares during 3 years without producing any pregnancies that had 92% abnormal sperm with 75% head defects, 57% of which with single or multiple nuclear vacuoles**.** In this case many seminiferous tubules with mild degenerative changes were observed in one of the testis.

The overall incidence of ***acrosome defects*** detected by light microscopy seems to be low in stallions, but might be high in some individuals. (Love et al. 2000). The most common defect of the acrosome is the *knobbed acrosome*, which consists of an excess of acrosomal matrix and folding of the acrosome over the apex of the sperm head. Membranous vesicles are commonly entrapped in the acrosomal matrix (Barth and Oko 1989, Brito 2007). The appearance of this defect on light microscopy varies from bead-like thickening and protrusion on the sperm head apex to indentation, roughing or flattening of the apex. The knobbed acrosome can be caused by environmental factors (eg. increased testicular temperature, stress, toxins), but can also be of genetic origin which has been described in bulls and in boars (Chenoweth 2005). Genetically affected animals consistently produce great percentages of affected sperm without significant changes in other sperm defects (Barth and Oko 1989, Chenoweth 2005). Knobbed acrosomes of genetic origin have not been reported in stallions. Hurtgen and Johnson (1982) reported data from seven stallions that were identified as having a high percentage of sperm with acrosome defects. Acrosomal abnormalities occur more frequently in conjunction with other sperm abnormalities suggesting impaired spermatogenesis and result in sub- or even infertility in stallion (Hurtgen and Johnson 1982) and bull (Blom and Birch-Andersen 1962, Thundathil et al. 2000). The formation of the acrosome occurs at the same time as some chromatin maturation is occurring in the testes and epididymides. The knobbed acrosome defect may be associated with underlying problems in chromatin maturation. The presence of a substantial percentage of acrosomal defects suggests that additional tests may be required (chromatin assays) to detect if the spermatozoa with knobbed acrosomes have immature chromatin. Mild knobbing or folding of the acrosome may not

influence fertility, but a severely knobbed acrosome with retained vesicular material in the matrix is a more serious form of the defect (Card 2005). The affected spermatozoa have a reduced ability to bind and penetrate the zona pellucida (Blom and Birch-Andersen 1962, Thundathil et al. 2000) and are predisposed for premature capacitation and spontaneous AR (Thundathil et al. 2002, Pesch and Bergmann 2006). Recent studies using electronic microscopy have revealed that the actual incidence of acrosome defects might be much higher than that observed with light microscopy (Brito 2007).

A common ***midpiece defect*** is the *distal midpiece reflex* (DMR), which in light microscopy appears as a bend in the distal region of the midpiece. In bulls, DMR develops in response to environmental insults as sperm migrate to the distal half of cauda epididymis, probably in association with altered ion concentrations. Double bends of the midpiece usually accompany coiling of the principal piece with retention of cytoplasmic material. The difference between the DMR or bent/coiled tails and the Dag-like defect is that, in the former, the midpiece is smooth and complete, whereas in the latter, the bending and coiling involving the midpiece or the entire tail is associated with rough, incomplete mitochondrial sheet usually accompanied by fractures and shattering of the axonemal fibers (Barth and Oko 1989, Brito 2007). DMR defect can be experimentally induced by hypotonic solutions or rapid cooling of semen. There is one major difference between the defects produced in vitro with hypotonic solutions or cold shock and those occurring in vivo during epididymal passage. The majority of defected sperm produced in vitro do not have droplet material trapped in the bend whereas a distal cytoplasmic droplet is nearly always entrapped in the bend in real DMR sperm (Barth and Oko 1989). Similar defects which involve *fractures or double bends* probably originate in the last steps of spermatogenesis and are usually found concurrently with the epididymal forms. DMR defects are common in semen collected from bulls in the late winter and in stressed bulls. Most effected bulls quickly recover to normal sperm production (Barth 1994). Blom (1977) suggested that DMR was a minor defect. He observed that the abnormality could be found in up to 25% of sperm cells from normal fertile bulls. A high proportion of stallion sperm with midpiece abnormalities (25.3%) has been also reported by Voss et al. (1981). However, in this study, the stallions achieved acceptable pregnancy rates of 62.5 to 91.7%. Since affected spermatozoa have reverse motility it is unlikely that they would be able to penetrate the zona pellucida and initiate zona reaction. Therefore this defect is compensable because the defected spermatozoa are not compiting with normal sperm in ovum fertilization.

The ‘‘*dag*’’ *defect* is named after the bull in which it was first identified. It is characterised by a strong folding, coiling and fracture of the distal part of the midpiece with or without a retained distal cytoplasmic droplet (Barth and Oko 1989). In electron microscopy (EM), malformation of the mitochondrial sheath, the loss of single mitochondria and irregular axial fibre bundles are associated with the findings in light microscopy (LM) (Barth and Oko, 1989; Andersen Berg et al. 1996, Pesch and Bergmann 2006). In semen of an infertile Dutch White (Saanenthal) goat buck similar abnormality was found to the Dag-like defect in cattle (Molnár et al. 2001). Light and electron microscopic examinations showed aberrations of the sperm tails. All of the cells had strongly coiled or broken tails, or fractured midpieces. Ultrastructural investigations by transmission electron microscopy (TEM) showed uneven distribution of the mitochondria in the midpiece. Coiled tails were encapsulated by a common membrane, and dislocated axial fibres and different membranous structures were also present (Molnár et al. 2001). In stallion, a defect consisting of a loss of microtubules in the axoneme and a disorganization of midpiece, similar to the ‘‘dag defect’’ is characterised by Hellander et al. (1991) This resulted in subfertility: per cycle pregnancy rate was 24%. Dag-like defect can sometimes be observed in association with other defects in cases of disrupted spermatogenesis. In bulls, a hereditary basis is established and at levels above 50% serious fertility implications are known to exist (Pesch and Bergmann 2006).
Segmental aplasia of the mitochondrial sheet might be observed in a low percentage of stallion sperm in varying degrees; some sperm lack a small part of the sheet, whereas others seem to miss the mitochondrial sheet completely (Brito 2007). Pseudodroplet and ‘‘corkscrew’’ defects are rare midpiece defects. *Corkscrew defect* has been described in bulls (Blom 1959) and in a stallion (Chenoweth et al. 1970). An irregular distribution of mitochondria resembling a corkscrew characterises this defect. *Rainbow shaped* (bowed midpieces) midpieces are in most cases artefacts caused by staining and drying. However in rare cases large percentages of spermatozoa may be affected by abnormal bowed midpieces which result in a stiff circling movement of spermatozoa (Barth 1994). *Abaxial midpieces* are considered to be morphologically normal (Varner 2008).

A spermatozoon with a loop-like *bend in the* ***principal piece*** usually is association with DMR. Generally a cytoplasmic droplet is trapped in the loop. The defect appears to originate in the epididymis under the same circumstances as distal midpiece reflexes (Barth 1994) or during ejaculation when they are mixing with secretums of accessory glands. The cause of this abnormality can be also an abnormal secretion in the genital tract. The normal amount and contents of seminal plasma can prevent

sperm damages (Swanson and Boyd 1962). Hypotonic or cold shock may cause a similar type of bend without a trapped droplet. Urine contamination of the semen may also induce hypotonic shock consequently bent midpiece and principal piece (Barth 1994). Bent tails can become looped tails and looped tails frequently progress to coiled tails. In these cases the affected sperm are moving in circles or backwards at a lower speed than normal sperm. Tail defects, especially abnormal tubule pattern, are known to be important for stallion sub- and infertility (Hellander et al. 1991). Simple coiled or broken tails are among the most common sperm defects (Pesch and Bergmann 2006). DMR, bent and coiled tail defects are considered compensable defects. These sperm are either selectively filtered throughout the female genital tract or unable to penetrate the zona pellucida at the fertilization place (Barth 1994, Saacke et al. 2000). In this aspect fertility of the sperm can be improved with higher number of spermatozoa in the insemination dose. The incidence of specific midpiece and tail defects and their effects on fertility in horses are difficult to ascertain because those are seldom reported separately (Jasko et al. 1990, Pesch et al. 2006b). Love et al. (2000) observed no correlation of midpiece bends and fractures with fertility; however, these authors estimated that a 1% increase in the percentage of other midpiece abnormalities resulted in a 2.9% reduction in per cycle pregnancy rates, whereas a 1% increase in the percentage of coiled tails resulted in a 3.9% reduction in per cycle pregnancy rates.

Duplication of the tail is an uncommon defect that is associated with duplication of the implantation fossa and replication of the distal centriole. Sperm with *multiple heads and tails* might have normal head structure with normal DNA content, but abnormalities of nuclear shape and abnormal DNA condensation in one or more heads might also be observed. These sperm originate from multinucleated spermatids and/or as the result of incomplete cell dissociation during spermatogenic divisions (Brito 2007).

Sperm cytoplasmic droplets are normal remnants of the spermatid residual cytoplasm (derivatives of degenerating Golgi apparatus, endoplasmic reticulum and nuclear membranes) that remain attached to the neck region of sperm after release into the seminiferous tubules. During the maturation process, along the transit through the body of the epididymis, the droplet moves from this proximal neck position to the distal portion of the midpiece. In bulls, approximately 35% of sperm shed the distal droplet in the tail of the epididymis, but the majority of sperm only shed the distal droplet after mixed with secretions from accessory sex glands, therefore cytoplasmic droplets in ejaculated sperm are considered abnormal (Barth and Oko 1989). Sperm

cytoplasmic droplets are often the most prevalent defect in the ejaculate, especially in young peripubertal stallions. While *proximal droplets (PD)* are thought to have a great impact on fertility and therefore are classified as major defects, the role of *distal droplets (DD)* hasn't been clearly known for a long time (Dowsett et al. 1984, Jasko et al. 1990, Dowsett and Knott 1996, Love et al. 2000, Card 2005). Although proximal cytoplasmic droplets may result from impaired epididymal function, research in bulls indicated that cytoplasmic droplets may result from insults to spermatids in any stage of spermiogenesis and even to spermatocytes (Brito 2007). Ultrastructural analysis of the CD shows numerous internal vesicular elements surrounded by an intact plasma membrane. One comparison finds the area of these internal CD membranes equivalents to 54% of the total surface area of the external sperm plasma membrane (Kaplan et al. 1984). Several glycolytic enzymes have been localized to the CD, which suggests a relationship to lysosomal activity (Dott and Dingle 1968).

Proximal cytoplasmic droplets have severe adverse effects on fertility in bulls, and levels as low as 10% may be associated with lowered fertility (Barth and Oko 1989). In vitro studies demonstrated that sperm with proximal cytoplasmic droplets are not capable of binding and penetrating the zona pellucida. Moreover, other genetic defects in morphologically normal sperm which was capable of fertilizing oocytes probably contributed to the impaired embryonic development observed in vitro after the use of semen from bulls producing a large percentage of sperm with proximal droplets (Amann et al 2000, Thundathil et al. 2001). A negative effect of infertile bull spermatozoa with retained CDs on normal bull spermatozoa was also shown during bovine fertilization in vitro (Thundathil et al. 2001). Zona pellucida binding (ZPB) and capacitation of PP spermatozoa are also disturbed in dog according to Peña et al. (2006). Jasko et al. (1990) observed that the negative correlation between the percentages of proximal cytoplasmic droplet with per cycle pregnancy rates was three times greater than the correlation with distal droplets, and only the former variable accounted for a significant percentage of variation in fertility in horses. Persch et al. 2006b indicated a negative correlation between the percentage of cytoplasmic droplets and per cycle pregnancy rates, but did not differentiate proximal from distal droplets in their report. In another study, however, the percentage of proximal cytoplasmic droplets was not associated with fertility in stallions (Love et al. 2000).

The effect of a *retained distal droplets* on fertility is less well defined, although there is some evidence suggesting a negative impact for such semen used in artificial insemination programmes. In boars, the proportion of spermatozoa with distal CDs in stored semen had a negative correlation with pregnancy rates and litter size (Waberski

et al. 1994). Boar sperm with retained CDs have a reduced binding affinity for porcine oviductal epithelial explants in culture (Petrunkina et al. 2001). Defective sperm function is associated with defects in spermiogenesis that lead to the release of immature spermatozoa from the germinal epithelium expressing high concentrations of cytoplasmic enzymes. Increased level of these enzymes associated with the retention of excess residual cytoplasm in the low-density sperm populations after Percoll centrifugation, could lead to the excessive generation of ROS, the induction of peroxidative damage, and a loss of sperm function, relative to the high-density sperm populations (Aitken and Fisher 1994, Huszár and Vigue 1994, Gomez et al. 1996).

Today, retained DD are concerned to be more detrimental to fertility than previously suspected (Kuster et al. 2004, Pesch and Bergmann 2006). Larger amounts of ubiquitinated proteins were present in extracts from sperm cells from an ejaculate with an abnormally high percentage of retained DDs (52% DDs) compared to a morphologically normal sample (6% DDs) (Kuster et al. 2004). *Ubiquitin*, a small peptide, is an universal marker for proteolysis found in all tissues and organisms. It marks proteins for recycling and identifies misfolded or damaged proteins for degradation in the intracellular space (Hershko 1998). Ubiquitination of the retained CDs has important implications when coupled with the knowledge that PDs and DDs have been associated with depressed fertility in vitro and in vivo. In mammals, some of the paternally derived organelles, such as mitochondria, are degraded in the lysosomes of the oocyte after fertilization, while others, such as the centrosome and male pronucleus, become vital zygotic components (Yanagimachi 1994). It has been theorized that following natural fertilization, the ubiquitin present on the surface of spermatozoa from subfertile ejaculates is carried over to the oocyte cytoplasm, where it could potentially target vital paternal organelles for destruction by the proteolytic processes of the oocyte, effectively interfering further embryonic development. In support of this theory, a relatively high correlation coefficient (r = –0.432) was obtained by comparing Sperm Ubiquitin Tag Immunoassay (SUTI) to cleavage rate after in vitro fertilization in human infertility patients, even though fertilization rates were poorly correlated (r = 0.046) (Sutovsky et al. 2001). *Lipoxygenases (LOXs)* are a family of enzymes capable of peroxidizing phospholipids. A member of the LOX family of enzymes, 15-LOX, participates in the degradation of mitochondria and other organelles within differentiating red blood cells, the reticulocytes. The study of Fischer et al. (2005) provides biochemical and immunocytochemical evidence for the presence of 15-LOX in the sperm cytoplasmic droplet. The 15-LOX and various components of the ubiquitin-proteasome pathway were detected in sperm CDs of mammalian species, including the human, mouse, stallion and wild babirusa boar.

They concluded that 15-LOX is prominently present in the mammalian sperm CD and thus may contribute to spermiogenesis, CD function or CD removal. The results of Kuster et al. (2004) and Fischer et al. (2005) support a model in which the ubiquitin-dependent proteolytic pathway acts in synergy with the 15-LOX pathway for the degradation of the CD. In the proposed model for organelle membrane degradation inside sperm CDs, the 15-LOX degrades the lipid membrane components and the ubiquitin–proteasome pathway is responsible for targeting the protein components of the CD for proteolytic degradation. In the result of structurally alteration of CD by activities of the 15-LOX and ubiquitin–proteasome pathways, the CD could descend from the connecting piece to the annulus and finally release from the sperm axoneme to be liquefied and phagocytosed by the cells of the epididymal epithelium or rejected after ejaculation (Hermo et al. 1988).

Detached sperm heads are commonly observed in low percentages (<5%) in the ejaculate, but might be present in very high numbers in cases of sperm accumulation in the excurrent tract (Dowsett et al. 1984, Jasko et al. 1990, Dowsett and Knott 1996, Love et al. 2000, Card 2005). Detached heads might result from abnormal spermiogenesis or from sperm senescence in the tract (Brito 2007).

Other cells that might be found in the ejaculate include blood cells, round germ cells, and Sertoli cell mantles. Proper classification of these cells requires the use of different staining techniques that could include Giemsa or Diff-Quick. Round germ cells are occasionally observed in small numbers in stallions. Most round germ cells in semen are spermatids (many of which binucleate) and some are secondary or even primary spermatocytes (Brito 2007). Increased percentages of round germ cells are observed in peripubertal stallions and in cases of testicular degeneration when germ cells are prematurely shed in the seminiferous tubule lumen (Card 2005).

Cumulative incidence of certain sperm abnormalities (e.g. bent midpiece and tails, bowed midpieces) may draw the attention for some sperm processing failures as hypoosmotic solutions, cold shock or inproper smearing (Juhász and Nagy 2003).

In optimal cases the morphologic evaluation of fertile stallions results in around or more than 60% normal spermatozoa. Percentage of head defects is between 3-5%, neck abnormalities are 1-1.5%, rate of midpiece defect is 4.5-6%, and proportion of tail defects is not higher than < 10-15%. Incidence of proximal cytoplasmic droplets in average 2.5-4%, rate of distal cytoplasmic droplets is 2-5%. (Juhász and Nagy 2003). There is a wide variation in sperm morphology among breeding stallions, but in

general, the average stallion has approximately 50% morphologically normal sperm according to Card (2005). The present Society for Theriogenology Breeding Soundness Examination (BSE) Stallion Guidelines have no standard for percentage of normal spermatozoa or the percentage of specific defects in the ejaculate, but state that approximately 1 billion progressively motile morphologically normal spermatozoa should be present in in each of 2 ejaculates collected 1 hour apart. Stallions with less than 40% morphologically normal spermatozoa may achieve acceptable pregnancy rates if breeding pressure is low or spermatozoal numbers are increased per breeding dose so a minimum threshold number of normal spermatozoa are present. The normal spermatozoa compensate for many of the compensable abnormal spermatozoa. Stallions with ≥60% morphologically normal spermatozoa frequently require fewer spermatozoa per insemination dose (Card 2005). Hungarian Standard for breeding stallion semen (7034/1999) has strict guidelines. This proposes ≤ 30% sperm with any morphologic aberrations, if less than half of these abnormal cells have primary defect.

Breed differences in sperm morphology were previously reported by Pickett 1993b, Dowsett and Knott 1996. There was a significant difference between breeds in percentage of morphologically normal spermatozoa /N/ and proximal droplets /PD/ (N: 57.5 ± 4.1% and 74.4 ± 3.8%, PD: 17.3 ± 2.7% and 2.9 ± 2.5% in Tori and Estonian stallions, respectively) (Kavak et al. 2004). For morphologically abnormal sperm, high numbers of sperm were presented with an abnormal midpiece in both Thai native /16.5%/ and Purebred stallions /23.9%/ (Phetudomsinsuk et al. 2008).

Sperm morphology can vary considerably during the four ***seasons***. In winter, the average percentages of morphologically normal sperm (74.3%) were higher than during the breeding season in May (65.9%). Tail defects (broken, bent or coiled flagella) were in the range between 7.65 and 13.70%. The differences of this proportion in fresh semen were significant between stallions but not between the seasons. A remarkable percentage of spermatozoa showed protoplasm droplets at the midpiece. The means of this value amounted to 10–23% in May and 12–15% in December. The relatively high proportion in May seemed to indicate a diminished maturation in the epididymis as a consequence of the high frequency of ejaculation during breeding (Blottner et al. 2001). During the 1 year experiment all semen quality parameters (volume, concentration, and motility, and the number of morphologically normal sperm and sperm with major defects /abnormal heads, acrosome defects, nuclear vacuoles, proximal droplets, abnormal midpieces/) showed a clear seasonal pattern in the study of Janett et al. (2003). Sperm with normal morphology was

significantly lower in summer than at any other time of the year and higher values were found for major defects in summer than in spring and autumn.

Love et al. (2000) found that some morphologic defects (e.g. cytoplasmic droplets, bent tails) appear to have a minor effect on fertility of stallions bred by natural cover, whereas other defects (e.g., detached heads, abnormally shaped heads, abnormally shaped midpieces, coiled tails, and premature germ cells) have a deleterious effect on fertility. After the morphological evaluation of sperm from 160 fertile stallions and additionally 30 subfertile and 29 fertile stallions and the relations between morphology results and fertility, the authors found that fertility per cycle varied with the percentage of total sperm defects, head defects, proximal doplets and midpiece defects and no relation was detected between fertility and the percentage of knobbed acrosomes, distal droplets, principal and end-piece defects. Subfertile stallions presented a higher percentage of abnormal forms than fertile stallions (52 ± 20 % vs. 30 ± 12 %) (Clément et al. 2001). There is a lack of experimental evidence on the tolerance level of various defects, and their effect on fertility, however more than 10% premature germ cells, greater than 30% head and/or midpiece defects, > 25% proximal cytoplasmic droplets and <30% morphologically normal spermatozoa are reasons for concern (Colenbrander et al. 2003, Card 2005 and Brito 2007). Using LM, Pesch et al. (2006b) found a significant increase of morphological deviations from 24.5% in fertile stallions (pregnant mares ≥70%, n = 29) to 34.5% in subfertile stallions (pregnant mares 10–69%, n = 14) and 73.5% in infertile stallions (pregnant mares < 10%, n = 3). Using TEM, they found a significant increase of detached acrosomes from 6.1% in fertile to 7.6% in subfertile and 21.4% in infertile group. Deviations in tubule pattern were also increased (but not significantly) from 2.7% in fertile and 2.8% in subfertile to 11.4% in infertile stallions as well as multiple tails from 1.9% in fertile to 2.0% in subfertile and 8.9% in infertile group.

2.6 Evaluation of the spermatozoa

A variety of techniques and protocols are available for evaluation of the spermatozoon. Basic requirements for the laboratory assays are objectivity (lowest bias possible), repeatability (production of similar results every time) and accuracy (evaluated on each particular sperm attribute in a precise manner). Not all laboratory tests for semen analysis pass these requirements. Accuracy is probably the most complicated problem to solve, merely because spermatozoa are terminal, highly differentiated cells, whose multitude of attributes that are of relevance for fertilization cannot be easily assayed by one single test. This is the reason why a combination of

tests, each measuring one or more of these attributes, provides better relationships to fertility, compared with a test measuring a single attribute (Rodríguez-Martínez 2003). Good sperm quality is essential for the success of artificial insemination. For a spermatozoon to fertilize an oocyte it must have at least the following attributes: metabolism for production of energy, progressive motility, enzymes located in the acrosome, proper structure, lipid and protein composition of plasma and acrosomal membrane and normal morphology (Amann and Graham 1993, Nagy 2002). Over the past decades, a number of laboratory tests have been developed to determine properties of sperm function. These include quantitative sperm motion parameters, capacitation, basal and induced acrosome reactions, nuclear and mitochondrial sperm DNA but few have been adopted into routine clinical use.

Traditionally, quality of equine sperm has been determined by estimation of **total and progressive motility**. This can be done either visually or with computer assisted sperm analysis (CASA). CASA was introduced more than twenty years ago (Jasko et al. 1988), and since then, it is used regularly in the semen evaluation process in many laboratories. This technique is objective and evaluates the motility according to the given criteria (Juhász et al. 2000). Most research on evaluation of sperm quality has included CASA analysis, as well as other attributes of the sperm function. Unfortunately, motility of the spermatozoa is poorly correlated with fertility in many studies. This seems reasonable because motility is only one attribute of the sperm (Squires 2005). Love et al. (2003) evaluated the relationship between sperm motility and sperm viability using the fluorochromes, SYBR-14/PI and the mitochondrial membrane probe, JC-1. They evaluated samples immediately after collection or after 24 h storage at 5°C. There was a high correlation ($r = 0.98$) between membrane integrity and total sperm motility. Although motility is known to have a high importance in fertilizing ability of the spermatozoa, in itself, it is a poor predictor of sperm fertility (Nie et al. 2002). A very low motility would probably be an indication not to use the semen, but a good motility does not necessarily indicate that the fertilizing capacity of spermatozoa has been maintained (Katila 2001a).

The maintenance of normal function of the plasma membrane is a crucial prerequisite for sperm **viability** as well as for reactivity at the site of fertilisation. An increased proportion of spermatozoa with damaged membranes is indicative for a reduced fertility of the stallion (Zhang et al. 1990). Several staining methods have been developed to detect disruption in the plasma membrane. Simple light-microscopic live-dead stains (aniline blue-eosin, eosin-nigrosin, eosin-fast green, bromphenol blue-nigrosin) are more widely used for the determination of cell viability. Integrity of the

plasma membrane is shown by the ability of a viable cell to exclude the dye, whereas the dye will diffuse passively into sperm cells with damaged plasma membranes (Colenbrander et al. 1992). Glycerol can interfere with the staining properties of these dyes making them less reliable for the evaluation of cryopreserved semen (Wilhelm et al. 1996). The viability stain indicates “dead cells” and the contrast stain gives background behind the “live cells”. These staining methods give information from only the sperm head membrane.

Simultaneous information on the **viability and acrosome status** of spermatozoa is important for distinguishing true and false acrosome reaction, as well as for the study of cell lesions after cryopreservation and other treatments. Aalseth and Saacke (1986) combined eosin-fast green staining with differential-interference-contrast (DIC) microscopic acrosome evaluation. For simple light microscopic evaluation a "triple-stain" technique was developed. Trypan blue was used for marking dead cells and acrosomes were stained by rose Bengal or Giemsa and the third stain was Bismarck brown which extruded Rose Bengal from the sperm-head (Talbot and Chacon 1981, Didion et. al. 1989, Dudenhausen and Talbot 1982, Kusunoki et al. 1984, Varner et al. 1987). The methods, including incubation and centrifugations were too difficult for routine application and differentiation was not clear due to the fading of trypan blue and to the similar colours of trypan blue and Giemsa (Cross and Meizel 1989, Kovács and Foote 1992).

A more simple technique, a **trypan blue-neutral red-Giemsa staining** method for simultaneous evaluation of acrosome integrity, sperm membrane, and overall morphology has been described for bull, boar and rabbit spermatozoa by ***Kovács and Foote (1992)***. It was reported later that stain-permeable ("dead") sperm tails also could be distinguished (Nagy et al. 1999). Since its introduction, this technique has been applied successfully to many other mammals including sheep (Sarhaddi et al. 1995), goat (Molnár et al. 2001), horse (Kovács et al. 2000), yak (Nagy et al. 2000), red and fallow deer (Nagy et al. 2001), mouse (Somfai et al. 2002a), water buffalo (Presicce et al. 2003), mouflon, dog, cat, two-toed sloth, argali (Kovács et al. 2007b), fossa (Kovács et al. 2007a), Asian elephant and white rhino (Behr et al. 2007; 2009a,b; Hermes et al. 2009; Saragusty et al. 2009).
Twelve classes of spermatozoa can be distinguished according to the membrane status of their domains (head, tail and acrosome). The percentage of cells with intact membranes and no morphological aberrations is a practical index of semen quality (Nagy et al. 1999). Simultaneous evaluation of the viability and acrosome integrity of sperm permits differentiation of true acrosome reaction from degenerative acrosome

loss after cell death (Kovács and Foote 1992, Assumpcao et al. 2000, Costa et al. 2010). This staining method showed acceptable repeatability and good agreement with flow cytometric measurements using fluorescein isothiocyanate-conjugated peanut agglutinin/propidium iodide (FITC-PNA/PI) staining of bull spermatozoa (Nagy et al. 2003a).

Koehler (1985) ascertained that the differences between results of motility assessment and convencional viability staining could be explained with that in the former case the functional integrity of the sperm tail, whereas in the latter case, the structural integrity of sperm head membrane were evaluated (Nagy 2002). Using trypan blue (TB)-Giemsa staining the proportion of cells with unstained tails corresponded to both the percentage of motile spermatozoa and the reaction to the hypo-osmotic swelling test (HOST). Sperm cells with an intact head membrane, but with a stained, membrane-damaged midpiece and tail, are considered immotile (Nagy et al. 1999).

After freezing and thawing of stallion semen, the number of spermatozoa with intact, unstained head membranes, but damaged, stained tail membranes, is increased significantly. Percoll gradient centrifugation separated the ejaculates into a more motile fraction with a higher percentage of sperm with intact membranes and a less motile fraction containing more sperm cells with stained tails. This observation confirmed that spermatozoa with a stained tail are immotile and likely explains the low fertilization rates with frozen/thawed semen (Domes and Stolla 2001). Therefore, unambiguous differentiation of the intact/damaged sperm tail membrane is very important for evaluating semen quality.

In the initial study Kovács and Foote (1992) stated that the procedure had not given satisfactory results for stallion spermatozoa due to some reasons: The area of the head of stallion spermatozoa is about half of bull or boar sperm head. Evaluation at 400x magnification was satisfactory in the case of these species but not in stallion. The cell concentration of stallion semen is lower, therefore the samples were less diluted with physiological saline before staining. Because of less dilution more trypan blue was bind to the solved proteins of the seminal plasma and egg yolk of the extender, resulting in more disturbing background. Later Kovacs et al. (2000) applied the technique to equine spermatozoa using frozen semen samples with more dilution and evaluated at 1000x magnification. Since then, some special characteristics and problems have been observed in stallion semen staining. One problem with the method was the length of the procedure (overnight Giemsa staining). Another problem was differentiation of intact/damaged sperm tails mainly in the case of frozen and thawed samples.

The use of **fluorescent dyes and flow cytometry** has provided the researcher and clinician with powerful tools to evaluate several sperm attributes. These procedures have been utilized to evaluate sperm viability, acrosome status and stages of capacitation, mitochondrial status and DNA integrity. With flow cytometry a large number of sperm can be evaluated in a relatively short period of time (Squires 2005). A combination of 2 fluorescent DNA stains: propidium iodide (PI) with carboxyfluorescein diacetate (CFDA) (Garner et al. 1986; Harrison and Vickers 1990) or with carboxydimethylfluorescein diacetate (CDMFDA) (Ericsson et al. 1993; Magistrini et al. 1997), or with SYBR-14 (Garner et al. 1994), can be used to assess **sperm viability**. Other frequently used fluorescent dyes are ethidium bromide (EB) and Hoechst 33258 (Eliasson and Treichl 1971). The most commonly used method to detect **acrosome integrity** is staining with fluorescein-conjugated lectins, such as Pisum Sativum Agglutinin (PSA), Peanut Agglutinin (PNA) /Farlin et al. 1992, Casey et al. 1993, Cheng et al. 1996/ or Concanavalin A (ConA) /Blanc et al. 1991/ coupled with fluoresceinisothiocynate (FITC) /Magistrini et al. 1997, Katila 2001a/. FITC-PNA is more reliable utilised for equine spermatozoa. Chlortetracycline assay (CTC) is used to detect **capacitation** and acrosome reactions of the spermatozoa (Varner et al. 1987, 1993). Unfortunately, it does not appear to be reliable when semen has been diluted with milk-based extenders. Another marker demonstrated to be useful for detection of capacitation in stallion spermatozoa is merocyanine 540, an impermeant lipophilic probe which permits evaluation of the architecture and disorder of lipids in the outer leaflet of the plasma membrane bilayer (Rathi et al. 2001, Gadella et al. 2001) Other probes, such as fluorochrome-conjugated Annexin V is being used increasingly to monitor membrane asymmetry (Gadella et al. 1999, Varner 2008). **Mitochondrial activity** can be evaluated by Rhodamine 123 (R123) /Evenson et al. 1982, Graham et al. 1990/, JC-1 (5,5',6,6'- tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide) /Gravance et al. 2000/, Mitotracker Green FM (Garner et al. 1997) or Mitotracker Deep Red 633 (Hallap et al. 2005).

Triple (or quadruple) Fluorophore Stain Combinations: /PI, FITC- PSA, rhodamine 123, Graham et al. 1990/; /Carboxy-SNARF-1 (SNARF), PI and FITC-PNA, Peña et al. 1999/; /SNARF, PI and FITC-PSA, Kavak et al. 2003a/; /SYBR-14, phycoerythrin-conjugated peanut agglutinin (PE-PNA), PI, Nagy et al 2003b/; /PI, FITC-PSA and MitoTracker Green FM, Celeghini et al. 2004/; /SYBR-14, FITC-PNA and PI, Kirk et al. 2005/; /Merocyanine 540, Yo-Pro 1 and Hoechst 33342, Hallap et al. 2006/; /PI, FITC-PSA and MITO; PI, Hoechst 33342, FITC-PSA and CMXRos; PI, Hoechst 33342, FITC-PSA and JC-1, Celeghini et al. 2007/; /LIVE/DEAD Reduced Biohazard Viability Kit Red, Hoechst 33342 and Alexa Fluor 488 PNA,

Nagy 2007/ can be used to assess different features simultaneously on individual spermatozoa of various species.

In order to evaluate cell acrosomal and mitochondrial function frozen-thawed semen has to be freed from milk and egg yolk components, debris cells and other particles before successful flow cytometric evaluation (Peña et al. 1999), because these particles have scatter properties similar to those of sperm cells that trouble the elimination of nonsperm events by scatter gating. Using the PI/ FITC-PSA double-staining protocol, complete removal of yolk particles from thawed sperm suspensions is required for accurate analyses of sperm integrity (Nagy et al. 2003b). The acrosomal probe FITC-PNA exhibited a high binding affinity to the components in skim milk based sperm extenders (Kirk et al. 2005). In these cases washing or Percoll® density gradient centrifugation are needed to separate spermatozoa from the particles prior to staining and evaluation. However, washing procedure is most likely coupled to induction of sperm deterioration. Due to the separation procedures, the proportion of the different cell types receiving is not the same as it was in the sample right after thawing. Percoll® gradients are also used to remove dead cells from a sperm sample in the laboratory practice (Rodriguez-Martinez et al. 1997). Kirk et al (2005) intended to achieve separation of equine spermatozoa from the diluent's particles without necessarily changing the sperm population. They found that centrifuging sperm through a 36%/63% discontinuous Percoll® gradient at 700 x g for 6–7 min did not significantly alter the percentage of live or live acrosome damaged cells in the sperm population. The other practical innovation was reported by Nagy et al. (2003b). In their study phycoerythrin-conjugated peanut agglutinin (PE-PNA) was used. The SYBR-14/PE-PNA/PI triple-staining technique was applicable for assessing the integrity of a frozen bull sperm specimen after thawing without any separation method. The abundant egg yolk particles did not interfere with this triple-staining method (Nagy et al. 2003b).

DNA stability can be assessed also using flow cytometry. The Sperm Chromatin Structure Assay (SCSA) measures the stability of DNA within the sperm nucleus, which uses the metachromatic properties of acridine orange to distinguish between denatured (red fluorescence = single stranded) and native (green fluorescent = double stranded) DNA in sperm chromatin. This assay, introduced by Evenson in 1980, and has been applied to spermatozoa from a number of species, including horses (Love and Kenney 1998). It was used to evaluate the cause of subfertility in stallions, as well as to assess the damage that occurs during cooling and/or freezing and thawing. Love and Kenney (1994, 1998) reported on the relationship between sperm chromatin and

fertility in the stallion (Colenbrander et al. 2003). Assays other than the SCSA are available to measure spermatozoal DNA fragmentation/chromatin disruption, including a TUNEL assay, an in situ nick translation (NT) assay, sperm chromatin dispersion (SCD) assay, and an electrophoresis-based Comet Assay (Baumber et al. 2003a, Chohan et al. 2006). Although these assays have not been used to the same extent as the SCSA in the equine laboratory practice, they are commonly applied in the human field (Varner 2008).

Although flow cytometry is extremely useful for evaluating the effects of various cooling and freezing treatments on sperm damage, the limitations of the technique include expense of the equipment, as well as technical training necessary to properly operate the equipment (Squires 2005). There is a difficulty and disadvantage and a relevant shortcoming of these techniques: In most cases they claim sperm washing or separation procedures and further incubations with the stains which can alter the original sperm quality parameters especially in equine semen in which spermatozoa are very sensitive to time-consuming processes. The shortcoming of the methods is that sperm morphology could not be assessed with them. Unfortunately there is no reliable computer aided automatised method which is able to perform complete morphology evaluation neither of all spermatozoa nor separately morphology of membrane-intact, viable sperm.
Viability assessment by flow cytometry or fluorescence microscopy is generally not accessible to practitioners in the field, and the cost of the equipment puts it out of reach for most producers. While keeping in mind its limitations, a relatively inexpensive light microscope provides a practical and realistic alternative for on-farm use (Merkies et al. 2000).

Tail membrane function may be also evaluated using the hypoosmotic swelling test (HOST). When exposed to a hypoosmotic solution, sperm with an intact, functional membrane swell to establish an osmotic equilibrium, this is seen as a characteristic swelling and coiling of the sperm tail (Neild et al. 1999). The HOS test is simple to perform and, for man, has been reported to correlate highly with other predictive tests for fertility, such as hamster oocyte penetration (Jeyendran et al.1992) and in-vitro fertilization (IVF) results (van der Ven et al. 1986). Kuisma et al. (2006) detected both negative and positive correlations, suggesting that this test is not suitable for evaluation of frozen-thawed stallion semen. In their experiment plasma membrane integrity with light microscopy correlated with many other parameters, including motility. This is in disagreement with the study of Samper (1992) who noted membrane integrity to show extremely poor correlation with motility, particularly in

preserved semen. Disadvantege of the HOST is that it disturbes the simultaneous morphologic evaluation (Jeyendran et al. 1984, Nagy 2002).

In the past years a series of **functional assays** has been developed to determine the structural, morphological and functional integrity of the spermatozoon. These functional tests include the parameters of cell volume regulation, sperm ability to undergo capacitation and acrosome reaction (AR) by exogenous stimuli, sperm-oviduct binding capacity, the ability to bind and penetrate the zona pellucida (ZP) or to fertilize in vitro (IVF) /Colenbrander et al. 2003, Rodríguez-Martínez 2003/. **Cell volume regulation** is an important physiological function crucial for the functional regulation of sperm at the time of ejaculation and within the female tract. As the epididymal fluid is hyperosmotic in relation to that of seminal plasma, sperm suffer an osmotic shock at ejaculation and react with recovery of the cell volume to maintain functionality. The response to osmotic stress is a determinant for the adaptive ability of the sperm cell and has been linked with natural fertility (Töpfer-Petersen et al. 2006). Osmotic resistance of spermatozoa is also important during the cryopreservation process (Ball and Vo 2001, Pommer et al. 2002). After deposition in the female genital tract, spermatozoa undergo the capacitation process, which is prerequisite for the induction of the acrosome reaction. The **response of sperm to capacitating conditions** is extremely variable between individuals, ejaculates and even within one ejaculate (Töpfer-Petersen et al. 2006). Sub-fertility in some stallions has been correlated to an inability of their spermatozoa to undergo the AR in response to progesterone stimulation (Meyers et al. 1995). Similarly, Rathi et al. (2000) found that the percentage of spermatozoa in an ejaculate with exposed progesterone receptors on their plasma membrane after incubation in capacitating conditions, was highly correlated with the fertility of the donor stallion (Colenbrander et al. 2003). The mammalian oviduct has been shown to act as a functional sperm reservoir responsible for the selection of the fertilization-competent sperm population, modulation of sperm capacitation, and regulation of sperm transport. The ability of **sperm to bind to the oviductal epithelium** appears to be a highly individual property which could be used for diagnostic purposes (Thomas et al. 1994). A direct association between poor sperm-oviduct binding and low fertility has already been found in pilot studies (Töpfer-Petersen et al. 2006).

Pesch et al. (2006a) determined **concentrations of several enzymes**, and macro- and microelements then evaluated correlations between these and conventional semen evaluation variables. Lactate dehydrogenase (LDH) was concluded to be a significant factor in sperm function and metabolism, as LDH concentrations were strongly

correlated with semen volume, sperm concentration, live/dead ratio and pathomorphology. The concentrations of the enzymes acid phosphatase (AcP), alkaline phosphatase (AP), aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT) were negatively correlated with semen volume and positively to sperm concentration, which could indicate a testicular or epididymal origin of these enzymes. GGT and LDH concentrations were also correlated with total sperm motility and progressive motility. Fe, Zn and Cu concentrations are negatively correlated with semen volume, with Fe and Zn concentrations correlating also to sperm concentration.

2.7 Cryopreservation of equine spermatozoa

2.7.1 History and present of sperm cryopreservation and its utilization

The first report about semen cryopreservation and artificial insemination dated back to the Italian priest and physiologist Spallanzani (1776): Human, stallion and frog sperm stored on snow for 30 minutes and rewarmed with motility recovery. More than 70 years ago, motile human spermatozoa were recovered after being frozen to -269ºC in liquid helium. Sixty years ago, spermatozoa were the first mammalian animal cells to be successfully cryopreserved at –79ºC (Smith and Polge 1950), demonstrating the cryoprotective properties of glycerol. In the following year the first calf was born as a result of artificial insemination with spematozoa cryopreserved by the method of Smith and Polge (Stewart 1951, Polge and Rowson 1952). Since then offspring of fifty four mammalian species have been produced with cryopreserved spermatozoa (Leibo, 2006). The first equine pregnancy using frozen semen was reported in 1957 by Barker and Gandier.

Now the total number of artificial inseminations with frozen semen in cows is 78 million per year, in horses 0.1 million AI/ year internationally (Central European Management Intelligence /CEMI/ data from 2006). Cryopreserved bull semen has been used commercially in dairy cattle for decades and conception results are now comparable or better than with natural mating.

Stallion semen cryopreservation, despite its impact on the horse industry, is not an established technology. During the last years, a number of modifications have been proposed to the freezing process, however, a large population of stallions still have poor semen quality and fertility after frozen-thawed. Only 30–40% of stallions produce semen that is constantly suitable for cryopreservation with acceptable pregnancy results after AI, and a consistent variation on sperm freezability has been also observed among breeds (Alvarenga et al. 2005). The stallions showed different susceptibilities to stress of dilution, freezing and thawing, independently from initial quality. Stallions have shown a particularly high degree of individual variation with

respect to the cryosurvival of their sperm (sperm survival varies between 70% - <5%). In dairy cattle, bulls have been selected by the AI industry for many years based on the ability of their sperm to withstand the stresses of standard cryopreservation protocols. In the dairy industry, each bull stud, essentially utilizes a single cryopreservation technique, and bulls that produce sperm that do not cryopreserve well using that technique are replaced by other bulls. However, replacing stallions is unlikely to prove acceptable to the equine industry, where specific genotypes are desired. Instead, to increase the number of stallions that can be effectively utilized for cryopreserved semen production, it is likely that more than one method for cryopreserving sperm will be necessary. No such selection has been applied to stallions and as a result there is a wide variation in semen freezability among individuals (Loomis and Graham 2008). Such differences could be genetic in origin, and genetic selection of stallions for successful freezing could be a possibility. On the other hand, the difference might be non-genetic and in this regard it would be particularly desirable to be able to apply assays of sperm function before and after freezing which correlate well with either semen freezability or stallion fertility. Unfortunately, such assays are not currently available (Katila 2001a; Kuisma et al. 2006). Sperm from some stallions often tolerate cryopreservation better when certain aspects of the protocol are customized. In the study of Loomis and Graham (2008) during the period 2000–2006, 332 different stallions were presented for semen cryopreservation. Of those stallions, 84.6% produced spermatozoa that exhibited ≥30% post-thaw progressive motility in >25% of the ejaculates frozen using one of their various cryopreservation protocols tested. Even if more stringent criteria were applied to determine a stallion's suitability for a commercial freezing program (≥30% post-thaw progressive motility in ≥50% of the ejaculates frozen), 74.7% of the stallions would still be considered suitable. These data are in agreement with the report of Vidament (2005), summarizing 20 years of field results with frozen semen in France using their modified freezing protokoll which we also used in Experiment II. (the protocol is described in the section of Materials and Methods). Although the developing sperm freezing techniques result in more and more stallions involved to the semen freezing processes the other big problem with the use of frozen–thawed equine semen remains a significantly reduced lifespan of the spermatozoa within the mare's reproductive tract, most likely caused by premature induction of the acrosome reaction in a significant proportion of the spermatozoa, necessitated both by the need to centrifuge the semen for separation of the seminal plasma prior to adding the freezing extender (Morris et al. 2003) and by the cooling and freezing processes themselves. Thus, to achieve fair conception rates when inseminating mares with frozen–thawed semen, increased care must be taken to inseminate close to the time of

ovulation that needs circumspect, time consuming, costly management of the mares which impedes widespread application of the use of frozen semen by the horse industry (Allen 2005).

2.7.2 Cryobiology of equine spermatozoa: principles, factors affecting on semen quality and freezability, species differences and individual variations

Since the first foal was born after AI with cryopreserved equine semen, many aspects of semen cryopreservation in the horse have still remained empirical and relatively little information is available on the basic cryobiologic and biophysical stresses imposed during freezing and thawing.

There is considerable variation in the ***lipid composition of the sperm plasma membrane*** in different mammalian species. The plasma membrane of stallion sperm differs primarily with regard to its relatively high cholesterol content (37% of total lipids, in boar sperm this ratio is 24%) (Yanagimachi 1994, cited: Gadella et al. 2001). Semen lipids play a major role in motion characteristics, sensitivity to cold shock and fertilizing capacity of sperm. It is important to note that the distribution of long chain polyunsaturated fatty acids in stallion sperm is more similar to boars than that of the bulls. Bulls produce sperm that are more resistant to cold shock and freeze well, whereas sperm from boars and stallions have very low tolerance to cold shock and in general, freeze poorly. Sperm of bulls have higher levels of 22:6 fatty acids, whereas sperm from stallions and boars have much higher levels of 22:5 fatty acids (Parks and Lynch 1992). The variation on membrane fluidity could be an explanation for the variability on sperm freezability observed between individual stallions. The major variable is the amount of cholesterol in the sperm plasma membrane between different males within a species and even between different ejaculates from a single male. Furthermore, the cholesterol content seems to be related to the rate of capacitation possibly because cholesterol must be depleted from the plasma membrane during this process (Yanagimachi 1994, cited: Gadella et al. 2001). The sperm plasma membrane serves as the main physical barrier to the outside environment and is a primary site of freeze-thaw damage. Such damage includes membrane destabilization due to lateral lipid rearrangement, loss of lipids from the membrane, and peroxidation of membrane lipids as a result of formation of reactive oxygen species (De Leeuw et al. 1990). Lipid-based cryoprotectants such as egg phosphatidylcholine or soy phosphatidylcholine may provide a physical barrier to freeze-thaw damage and prevent membrane phase separation by influencing lipid packing at the membrane surface. These exogenous lipids from the freezing extender does not incorporate into the sperm membrane but strongly associates with the membrane surface (Ricker et al. 2006).

Cryopreservation requires exposure of spermatozoa to extreme variations in temperature and osmolality. Post-thaw survival of cryopreserved spermatozoa exhibits a maximum at a presumptive optimum ***cooling rate***, and the optimum cooling rate is also dependent on the warming rate, the optimum rates presumably being due to sperm permeability properties. The optimum cooling and warming rates may also be significantly dependent on the specific cryoprotective additive and buffer solution in which the spermatozoa are cryopreserved (Leibo 2006). During freezing, the most critical time for sperm damage is the period of extracellular ice crystal formation. The solution at this stage is cooled down to between -6 and -15 °C (Pickett and Amann 1993, Caiza de la Cueva et al. 1997). Both too slow and too rapid freezing were found to be associated with lethal cryoinjury. If freezing progresses at very slow rates, the dehydration will take place over a longer time period resulting in high degree of shrinking associated with fatal cellular disruption. (Mazur et al. 1972). However, the cooling rate must be slow enough to allow water to leave the cells by osmosis in sufficient quantity. The cellular damage that spermatozoa encounter at rapid rates of cooling has often been attributed to the ***formation of intracellular ice***. However, no direct evidence of intracellular ice has been presented. In the study of Morris et al. (2007) they concluded that cell damage to horse spermatozoa, at cooling rates of up to 3000 °C/min, is not caused by intracellular ice formation rather the cells are subjected to an ***osmotic shock*** when they are thawed. The observed differences in the viability and motility measurements suggest that different mechanisms of cellular injury may be occurring at ‘‘slow’’ and ‘‘rapid’’ rates of cooling (Morris et al. 2007). Sperm cells are generally frozen at quite rapid rates in the range of 15–60 °C/min, which have been empirically determined as giving the best survival rates (Watson 2000). Optimal cooling rates for stallion sperm are about 29 °C/min in the absence of cryoprotective agents and about 60 °C/min in their presence, as calculated at subzero temperatures (Devireddy et al. 2002). Stallion spermatozoa are extremely sensitive to chilling injury also when cooled from 37 °C to approximately 8 °C at rates > 0.3 °C/min. The ***cold shock*** effect includes abnormal patterns of swimming (circular or backwards), rapid loss of motility, acrosomal damage, plasma membrane damage, reduced metabolism and loss of intracellular components (Moran et al. 1992).

The capacity for spermatozoa to respond with cell volume adjusment is determined by several factors including membrane phospholipid composition, water permability (*L*p), lipid phase transition temperature, Na+/K+ ATPase activity, ion channels, and cytoskeletal elements (Pommer et al. 2002). Cells with a higher *L*p will reach equilibrium faster (Devireddy et al. 2002). Spermatozoa subjected to hyperosmolal

environment (up to 450 mosmol/kg) appear to have intact membranes (viable cells) and are capable of preserving their mitochondria, as demonstrated by a high mitochondrial membrane potential (MMP). However when sperm were subjected to hypotonic solutions, MMP and viability markedly decresed. Thus the thawing process may be more detrimental to spermatozoa than the freezing process (Ball and Vo 2001, Pommer et al. 2002).

Oxidative stress is defined as the imbalance between pro-oxidative and antioxidative molecules in a biological system. This imbalance can lead to damage to the structure of cells and macromolecules such as plasma membrane components, proteins, and DNA (Aitken et al. 1999). Because of the high content of polyunsaturated fatty acids (PUFA) in the plasma membrane, mammalian sperm are sensitive to oxidative stress (Parks and Lynch 1992, Aitken 1995). In several experiments the effect of sperm freezing/thawing and storage (Kankofer et al. 2005) on production of ROS and effectivness of various antioxidants (Aurich et al. 1997; Ball et al. 2000, 2001; Baumber et al. 2000, 2003a; Sarlós et al. 2002; Gadea et al. 2005) added to the semen were evaluated. While the uncontrolled generation of reactive oxygen species (ROS) by defective spermatozoa can have detrimental effects on sperm function, controlled production of ROS plays physiologically relevant roles in signalling events controlling sperm capacitation, the acrosome reaction, hyperactivation and sperm–oocyte fusion (Baumber et al. 2000). In stallion semen, ROS are generated mainly by immature, damaged and abnormal spermatozoa and by contaminating leukocytes. Although lipid peroxidation is well characterized for mammalian sperm, equine spermatozoa appear relatively more resistant to membrane peroxidation than sperm of other domestic animals (Baumber et al., 2000; Neild et al., 2005). Cryopreservation of equine sperm, however, increased lipid peroxidation particularly over the region of the sperm midpiece (Neild et al. 2005, Ball 2009).

Factors affecting on semen quality and freezability

Differences in sperm membrane composition, biochemistry and metabolism between both species and individuals within a species may be responsible for differences in membrane permeability to water and cryoprotectants. Glycerol toxicity could be also one reason for the variation on stallion sperm freezability. Hammerstedt and Graham (1992) reviewed cellular effects caused by glycerol that included changes in cytoplasmic events due to increased viscosity by intracellular glycerol, altered polymerization of tubulin, alteration of microtubule association, effects on bioenergetic balances and direct alteration of the plasma membrane and glycocalyx.

Increasing cryoprotectant permeability, either by altering membrane composition or by using alternative cryoprotectants may improve cryosurvival rates of sperm that normally survive freezing poorly. Indeed, increasing the cholesterol content of stallion (Moore 2005b) increases their osmotic tolerance. In addition, for stallion sperm that do not survive cryopreservation well using standard procedures, changing the cryoprotectant to a smaller more permeable cryoprotectant such as formamide or dimethyl formamide can improve cryosurvival (Squires et al. 2004; Alvarenga et al. 2005). Fertility trials have also been performed that showed a significant improvement on fertility of stallion semen frozen with dimethyl-formamide (DMF) when compared with glycerol (Medeiros et al. 2002, Moffet et al. 2003, Medeiros 2003).
Due to the great variation in semen quality between and within stallions, factors affecting quality should carefully be controlled. Routinely used medicines like Eqvalan or Quadrisol (Janett et al. (2001, 2005) , stress situations as training or competition (Dinger et al. 1986, Lange et al. 1997, Janett et al. 2006), seasonality (Janett et al. 2003) or nutrition like feeding a nutriceutical rich in docosahexaenoic acid /DHA/ (Brinsko et al. (2005b) can influence the fresh and frozen sperm quality.

Current freezing protocols for stallion semen involve a two-step dilution procedure in which semen is first diluted with a primary extender, centrifuged and then diluted a second time prior to freezing in an extender containing cryoprotectants. The first dilution employs either saline/sugar extenders or skim milk extenders with or without egg yolk used to dilute fresh semen. The dilution rate is either 1:1 or the semen is diluted to a concentration of ~50 million spermatozoa/ml. The success of centrifugation depends on duration (10–15 min) and centrifugation force (350–700×g). Despite the development of an ever increasing range of freezing extenders, each claiming some improvement or benefit over the other (Martin et al. 1979, Loomis et al. 1984, Heitland et al. 1996, Ecot et al. 2000, Allen 2005). In spite of the elevated research on the alternative cryoprotectans, glycerol at a concentration of 3–5% has been the major penetrating cryoprotectant routinely used to freeze stallion semen. The yolk of fresh chicken or duck eggs at a concentration of 10–20% v:v has remained the preferred source of protein in the freezing mixture. Sugars (usually combination of fructose and glucose, alternatively raffinose or trehalose) are often added to media which act as non-penetrating cryoprotectants (Squires et al. 2004). The most commonly used freezing containers are 0.5-ml straws. The centrifuged, extended semen is usually cooled to 4°C before freezing which takes place in liquid nitrogen vapour by suspending the rack of pre-filled straws a few centimetres above the liquid nitrogen in a specially adapted freezing bath (Boyle 1999), or in a computer-controlled automated freezing machine (Allen 2005). Using controlled rate freezers

different freezing curves can be set, for example the recommendation of Digitcool devise (IMV CryoBio-System, L'Aigle, France) for equine sperm cryopreservation is the following: from 4 to -10 C at 10 C/min, from -10 to -100 C at 20 C/min, from -100 to -140 C at 60 °C/min. The samples were then plunged into liquid nitrogen.

Some alternative methods such as unique freezing technique /UFT/ (Vartorella 2003, Goolsby et al. 2004), ultra-low temperature freezers (Álamo et al. 2005), 'Multi-Thermal-Gradient' (MTG) technology (Zirkler et al. 2005, Saragusty et al. 2007) also have been utilised and showed comparable results than conventional liquid nitrogen methodology. These techniques may be suitable to replace the tradional method. A sublethal environmental stress, through the application of a high hydrostatic pressure (HHP) impulse (30 MPa pressure for 90 min) before cryopreservation significantly improved the post-thaw motility, viability and fertility of frozen bull sperm (Pribenszky et al. 2007, Kútvölgyi et al. 2008). However the preliminary study showed that the response for different pressure/time combinations is more individual in stallion sperm and it was not found substantial improvement in most of the examined attributes, only the VCL parameter of CASA after using 5MPa pressure for 60 minutes, increased after freezing/thawing (Horváth et al. 2007).

2.7.3 Cryodamages and their evaluations

More than 50% of all spermatozoa are damaged by freezing process (Leibo 2006). Formation of ice crystals and the osmotic stress present during freezing and again at thawing are the two major factors related to cryoinjury. However, in recent years a number of other factors related to cryodamage have been characterized: phase transitions in the plasmalemma, oxidative damage, DNA damage, toxicity of cryoprotectants, premature aging, and capacitation-like changes (Watson, 2000). **A**n apoptosis-like phenomenon has been also identified (Anzar et al. 2002, Martin et al. 2004). In general, the most susceptible structures of the spermatozoa to preservation procedures seem to be the membranes (Parks and Graham, 1992). Cryopreserved mammalian semen is generally acknowledged to have an impaired fertility by comparison with fresh semen. The reduction arises from both a lower viability post-thaw and sublethal dysfunction in a proportion of the surviving subpopulation.

- ***Mebrane damages– viability***

Changes in plasma membrane structure and integrity appear to be an important component associated with reduced fertility of frozen–thawed spermatozoa. Sperm membrane destabilization occurs when the membrane undergoes a phase transition

from the fluid phase to the gel phase as temperatures decrease (Squires 2005). There are numerous light- and fluorescence microscopic staining methods to determine viability of spermatozoa. These mainly give information from the integrity of the head and acrosome membranes, but not from the tail membrane. The HOS test is thought to have an advantage over the sperm viability stains because it is not only indicative of whether the plasma membrane is intact but also an indicator of whether it is osmotically active (Colenbrander et al. 2003). Neild et al. (2003) have used various fluorochromes to evaluate membrane damages during the freeze-thaw process for equine sperm. Sperm viability and capacitation state were simultaneously evaluated using chlortetracycline (CTC) and Hoechst 33258 dye. Membrane function was also evaluated using HOST. Sperm were analysed immediately after collection, after dilution and centrifugation, after re-dilution and equilibration at room temperature, after cooling to 5 °C, after super-cooling to −15 °C and after thawing. The results show that freezing-thawing induces cell damage and a relative increase in live capacitated/acrosome reacted cells. The most pronounced functional damage to membranes of sperm occurred after thawing. An unique aspect of this study was the ability to evaluate capacitation and acrosomal integrity in conjunction with viability, however only the sperm head membranes were possible to assess. The trypan blue (TB)-neutral red-Giemsa staining method was applied for simultaneous evaluation of sperm head and tail membrane integrity, acrosome status, and overall morphology (Kovács and Foote 1992, Nagy et al. 1999, Kovács et al. 2000). After freezing and thawing, a high proportion of spermatozoa with intact head membranes but damaged tails are observed. These cells are considered as immotile (Nagy et al. 1999).

- ***Decrease in motility***

Post-thaw motility of cryopreserved stallion sperm shows poor correlation with fertility, indicating that subcellular damage can affect fertility without concomitant impact on motility. Decline of motility shows species- and individual differences after cryopreservation of spermatozoa. In the semen of the 30–40% of stallions which ‘freeze well’, post-thaw sperm progressive motility and total motility figures of 40–60% and >70% respectively, but in the semen of 30–40% of stallions that ‘freeze badly’, postthaw sperm progressive motilities as low as 10–15% occur commonly (Jasko et al. 1992, Allen 2005). In the study of Ortega-Ferrusola et al. (2009) a shift from a more linear to a less linear pattern of movement and a significant drop in sperm velocities was observed, rather than a dramatic loss in sperm motility after thawing.

- ***DNA fragmentation***

DNA damage is another well-known cytopathic effect of ROS. In equine sperm, exposure to increasing concentrations of ROS resulted in a dose-dependent increase in DNA fragmentation as detected by the Comet Assay. This DNA damage was blocked in the presence of catalase or reduced glutathione (GSH) but not in the presence of SOD, which indicates that hydrogen peroxide (H_2O_2) was the major ROS responsible for DNA damage in these cells (Baumber et al. 2003a). During storage of equine spermatozoa, there is a measurable increase in DNA fragmentation as detected by the comet assay with both cooled (Linfor and Meyers 2002) and frozen storage (Baumber et al. 2003a). In contrast there was no significant difference in the sperm DNA fragmentation index (sDFI) of sperm evaluated initially after collection compared to those tested immediately after chilling or cryopreservation evaluated by sperm chromatin dispersion test (SCD). However, within 1 h of incubation at 37 °C, both chilled and frozen-thawed spermatozoa showed a significant increase in the proportion of sDFI; after 6 h the sDFI had increased to over 50% and by 48 h, almost 100% of the sperm showed DNA damage (López-Fernández et al. 2007).

Unfortunately, the addition of antioxidants (α-tocopherol, reduced glutathione, ascorbic acid) or enzyme scavengers (catalase, superoxide dismutase) to cryopreservation extenders did not reduce the level of DNA fragmentation, did not improve spermatozoal motility, acrosomal integrity, viability, or mitochondrial membrane potential subsequent to freezing and thawing of equine sperm cells. (Baumber et al. 2005). Spermatozoa have limited or no ability to repair DNA damage, and studies indicate that although fertilization may occur, the rate of subsequent embryonic development is reduced and the rate of early embryonic death is increased in situations in which fertilization is initiated by DNA-damaged sperm (Morris et al. 2002).

- ***Apoptosis***

In the recent years an apoptosis-like phenomenon has been identified during the cryopreservation process (Anzar et al. 2002; Martin et al. 2004). This explains not only cellular death but also the different degree of subtle cellular damage that most of the surviving population of spermatozoa experiences after thawing. The major function of mitochondria is supplying cellular energy, but the second major function of the mitochondria is the regulation of cell death (Ott et al. 2007). In addition, this subcellular structure is the major source of reactive oxygen species. Mitochondria-generated ROS play an important role in the release of cytochrome C and other proapoptotic proteins, which can trigger caspase activation and apoptosis. In relation to this, mitochondria have been identified as the most sensitive sperm structure to

cryopreservation (Peña et al. 2003). All of these changes result in reduced longevity of the cryopreserved spermatozoa within the female reproductive tract. The kinematics of the appearance of apoptotic markers was studied by flow cytometry and immunoblot assays in equine spermatozoa subjected to freezing and thawing. Caspase activity, low mitochondrial membrane potential, and increases in sperm membrane permeability were observed in all of the phases of the cryopreservation procedure (Ortega-Ferrusola et al. 2008). Ortega-Ferrusola et al. (2009) studied in equine semen the value of these apoptotic markers as predictor of sperm freezeability. Their findings show differences in the expression of apoptotic markers among stallions; moreover in fresh semen these differences were also observed. After cryopreservation frequently shown morphologic changes in the sperm midpiece that is characterized by moderate to marked swelling of the mitochondria suggesting that sperm mitochondria are a significant site of cryodamage with uncoupling of normal oxidative metabolism, generation of ROS and induction of degenerative processes such as apoptosis (Brum et al. 2008).

- ***Capacitation-like changes, early acrosome lost***

Following cryopreservation in modified Kenney's medium, capacitation-like changes were observed evaluated by chlortetracycline (CTC) fluorescence staining. There was a significant increase in the proportion of spermatozoa displaying “capacitated” pattern (64.8%) and acrosome reacted (AR) pattern (32.8%) with a corresponding decrease in the proportion of spermatozoa displaying the “uncapacitated” pattern (2.5%). There was a major decrease in the proportion of uncapacitated spermatozoa corresponding to an increase of capacitated spermatozoa following removal of seminal plasma after centrifugation and resuspension in freezing medium. (Schembri et al. 2002). Neild et al. (2003) found that freeze-thawing induces cell damage and a relative increase in live/capacitated and live/acrosome reacted cells. However, it was not possible to determine whether the changing CTC patterns reflect a true capacitation phenomenon or an intermediate destabilized state of the sperm cell membrane. In another study (Kavak et al. 2003a) a very low percentage of the cells showed early capacitation sign after thawing detected Merocyanine 540/Yo-Pro-1 probe using flow cytometry. Wilhelm et al. (1996) has used PI and phycoerythrin-conjugated PSA lectin for evaluation of acrosomal status of frozen-thawed stallion spermatozoa, and found that 87–88% of live spermatozoa had intact acrosomes. Cryopreserved and capacitated sperm share several characteristics such as plasma membrane reorganization, increased intracellular calcium levels, generation of reactive oxygen species, and acquisition of fertilization capacity (Bailey et al. 2000). Seminal plasma of man and stallion contains cholesterol-rich vesicules secreted by

prostate (prostasome) which block cholesterol efflux from the membrane thus delay the capacitation until appropriate time (Cross and Mahasreshti 1997). Removal of seminal plasma and herewith prostasomes by centrifugation before freezing may influence this physiological process. Generation of reactive oxygen species can promote equine sperm capacitation and tyrosine phosphorylation, suggesting a physiological role for ROS generation by equine spermatozoa (Baumber et al. 2003b). Thomas et al. (2006) found that the regulation of phospholipid scrambling, the capacitation-like alterations in the plasma membrane and protein tyrosine phosphorylation following cryopreservation are not identical to those in in vitro capacitated equine spermatozoa thus capacitation and ‘‘cryocapacitation’’ are not equivalent processes.

- ***Morphology***

After freeezing and thawing, ultrastructural changes were observed in the acrosome, in the outer fibres of the midpiece, and in the axoneme of the principal piece (Christensen et al. 1995, Katila 2001a). Results of SEM showed spermatozoa with typical fenestrations and ruptures of the plasma membrane in the acrosomal region, some spermatozoa with abnormal necks and some specimens with frequently separated head and flagella. Large areas of rough or disrupted acrosomal surface and with ruffled membranes occurred in frozen samples (Blottner et al. 2001).

- ***Functional changes, in vitro „fertility ability" tests: Binding to oviductal epithelial cells and zona pellucida***

Spermatozoa that have been altered during the process of freezing and thawing have a reduction in their ability to attach to the oviductal epithelial cells (OEC)**,** hence making a smaller reservoir in the mare's reproductive tract (Lefebvre and Samper 1993**,** Dobrinski et al. 1995, Samper 2001). The mean number of spermatozoa bound to equine OEC and zona pellucida (ZP) and percentage of acrosome-intact spermatozoa were lower for frozen-thawed than for fresh spermatozoa. The motility of spermatozoa attached to OEC was lower in cocultures of OEC with frozen-thawed spermatozoa than with fresh spermatozoa at each time point between 0.5 and 48 hours, probably reflecting lower sperm motility in the insemination dose (Dobrinski et al. 1995).

- ***Early embryonic loss***

Many authors suggest that frozen–thawed spermatozoa are associated with an increased incidence of early embryonic mortality. The potential mechanisms can now

be studied more effectively. DNA damage may indicate functional damage to the nuclear structures. Possible importance of sperm RNA to the events before the embryonic genome is activated cannot be disregarded (Watson 2000).

- ***Cryodamages, laboratory assessment methods and their correlation to the frozen equine sperm's fertility***

The results of the studies evaluating correlation of frozen equine sperm quality parameters and fertility are contradictory. The fertility of frozen semen is influenced by a number of factors including semen quality, stallion selection, freezing technique, insemination dose, mare selection, mare status and management. The fertility of frozen semen in commercial programmes has been reported to range between 32% and 73% per cycle (Samper 2001) and between 56% and 89% per season (Loomis 2001). Katila (2001a) has extensively reviewed the different techniques available to assess sperm quality in stallion. Her primary conclusion was that sperm motility and viability were the best parameters. However, within the same research group, a review by Kuisma et al. (2006) later claimed that the fertility of frozen-thawed semen samples from stallions was unpredictable using current laboratory methodologies.

It's not easy to find correlation between fertility of frozen stallion semen and laboratory tests. It is difficult and expensive to inseminate an adequate number of mares to achieve statistically significant differences. In the early studies there were contradictory results from relation of frozen/thawed equine sperm evaluation methods and fertility parameters: Significant, but low correlations have been demonstrated between the foaling rate and subjective motility of sperm incubated for 2 h and 4 h at 37°C (Katila et al. 2000b) and hypoosmotic swelling test after 0 and 3 h of incubation (Katila et al. 2000a)**.** Significant correlations have been reported between the pregnancy rate (based on 40 mares) and viability of propidium iodide-stained sperm assessed by flow cytometry (Wilhelm et al. 1996) as well as for glass wool and Sephadex filtration tests (Samper et al. 1991). Using CASA system, motility had a low (0.45) but significant correlation with the first-cycle pregnancy rate of 177 mares inseminated with frozen semen from 9 stallions (Samper et al. 1991). In another study there was no correlation between fertility and subjective post-thaw motility or percentage of sperm moving >30 μm/sec (RAP) analysed by CASA (Bataille et al. 1990). In another French study, 766 mares were inseminated with frozen semen, but none of the criteria measured by CASA (VCL, LIN, ALH, MOT, RAP) had a significant correlation with fertility (Palmer and Magistrini 1992). It is clear that freezing and thawing processes cause sublethal changes, premature cryocapacitation and acrosome reaction of spermatozoa, damage membranes and kill cells. Not all of

these changes are reflected in motility. In spite of that, motility estimation by light microscope is the most commonly used method to evaluate frozen-thawed stallion sperm.

Analysis of single-sperm parameters was not highly correlated with stallion fertility in the experiment of Wilhelm et al. (1996). However, with a statistical model that included data on percentage of viable sperm (flow cytometric estimates measured by PI-PSA), percentage of motile spermatozoa and percentage of hamster oocytes penetrated, these tests were highly correlated with stallion fertility /r = 0.85; P = 0.002/ (Wilhelm et al. 1996). Kuisma et al. (2006) detected both negative and positive correlations between HOST and fertility, suggesting that this test is not suitable for evaluation of frozen-thawed stallion semen. In their experiment plasma membrane integrity with light microscopy correlated with many other parameters, including motility. This is in disagreement with the study of Samper (1992) who noted membrane integrity to show extremely poor correlation with motility, particularly in preserved semen. HOST combined with eosin stain for evaluation membrane physical and functional integrity and trypan blue-Giemsa staining for evaluation of sperm plasma membrane and acrosome integrity together was a valuable fertility predictive test could be used for the prognosis of the potential fertility of frozen-thawed bovine semen samples used for IVF or AI according to Tartaglione and Ritta (2004). In the study of Kirk et al. (2005) evaluating only a single parameter did not adequately explain differences among stallions in fertility, however, combining results of assays that measured multiple sperm attributes improved the ability to evaluate the fertilizing potential of frozen-thawed spermatozoa. The four-variable model, which included: (1) motility at 90 min; (2) straightness measured by CASA at 90 min; (3) percentage of live cells evaluated by flow cytometry using propidium iodide and SYBR-14.; and (4) mitochondrial membrane potential measured by flow cytometry, using mitochondrial probe, JC-1, explained the majority of the variation in first cycle fertility between stallions (r^2 = 0.93) (Kirk et al. 2005). The ultimate goal of multi-parametric sperm analysis was to be able to distinguish sperm samples that have potentially good fertilizing potential from those likely to have poor fertility. Katila (2001a) and Colenbrander et al. (2003) also emphasized the need to combine several tests for fertility evaluation of frozen-thawed stallion semen.

„Sperm cryobiology is still a puzzle" (Leibo 2006). Future attempts to optimize sperm cryopreservation may be more useful if attention is paid to the individual characteristics of males instead of pooled sperm specimens of a species (Leibo 2006).

2.8 Sperm separation for in vitro embryo production

Many valuable stallions produce poor quality semen, including bad sperm freezability, low number of sperm and low percentage of viable spermatozoa. Availability of semen of some very valuable stallions is reduced, because they are dead or it is not possible to collect semen from them anymore. In these cases sperm can be used for in vitro embryo production. Standard in vitro fertilization (IVF) is largely unsuccessful in horses, primaly because it is difficult to adequately stimulate horse sperm to penetrate the zona pellucida in vitro. Therefore fertilization is performed by intracytoplasmic sperm injection (ICSI) (Roasa et al. 2007). ICSI can be applicable also when semen quality is insufficient for standard insemination, use of sex-sorted spermatozoa, a failure of natural fertilization of oocyte in the mare or using oocytes from ovaries of some valuable mares post-mortem. In vitro embryo production is possible in the horse for both routine and research applications. Oocytes may be collected from excised ovaries post-mortem, or from either immature follicles or stimulated pre-ovulatory follicles in the live mare (Hinrichs 2010).The first ICSI foal was produced by Squires et al. (1996). This technique is now being offered commercially in several laboratories around the world (Squires 2005).

The onset of clinical assisted reproduction in human medicine required the isolation of motile spermatozoa. Under in vivo conditions, potentially fertile spermatozoa are separated from immotile spermatozoa, debris and seminal plasma in the female genital tract by active migration through the cervical mucus. During this process, not only progressively motile sperm are selected, but spermatozoa also undergo physiological changes called capacitation, which are prerequisites for the sperm's functional competence with regard to acrosome reaction (Yanagimachi 1994, Henkel and Schill 2003). The introduction of assisted reproduction, especially of IVF, during the 1980's, led to the development of a wide range of different sperm separation methods. An ideal sperm preparation technique for assisted conception requires the capacity of accumulating in a relatively small volume the largest number of morphologically normal, mature, viable sperm with good motility and intact DNA and this extract of the ejaculate must be free of seminal plasma, leukocytes, bacteria, and other debris and should reduce ROS. There are four basic approaches for sperm separation: (1) dilution and washing (centrifugation and resuspension), (2) sperm migration (swim-up procedures, migration-sedimentation), (3) selective washing of subpopulations (density gradient centrifugation, e.g. Percoll®, PureSperm®, Nycodenz®), and (4) techniques with adhesive substances to eliminate dead spermatozoa and debris (e.g. glass wool (GW), glass beads, Sephadex and Leucosorb) (Rodriguez-Martinez et al

1997, Sieme et al. 2003). Henkel and Schill (2003) summarized all the sperm separation techniques which had been using in the human ART procedures until they prepared the manuscript. They pointed out the advantages and disadvantages of the different methods and also discussed the ways of further developments of the techniques.

Initially, starting from simple washing of spermatozoa, separation techniques, based on different principles like migration, filtration or density gradient centrifugation evolved. For all migration methods, the self-propelled movement of spermatozoa is an essential prerequisite, while for density gradient centrifugation and filtration techniques the methodology is based on a combination of the sperm cells' motility and their retention at phase borders and adherence to filtration matrices, respectively. The ***migration techniques*** can be subdivided into ***swim-up*** (SU), under-lay and migration-sedimentation methods. SU method originally described by Mahadevan and Baker (1984) As swim up separation is based only on the ability of active movement of spermatozoa from the pre-washed cell pellet into an overlaying medium, morphologically abnormal spermatozoa and spermatozoa with damaged DNA will be present along with the normal spermatozoa. However SU is easy to perform and usually recovers a very clean fraction of highly motile spermatozoa, the method has disadvanteges also: restricted to ejaculates with high sperm count and motility, the yield of motile spermatozoa is limited, spermatozoa can be massively damaged by reactive oxygen species, significant decrease of the percentage of normally chromatin-condensed spermatozoa. (Henkel and Schill 2003) A more sophisticated and most gentle migration method is ***migration-sedimentation.*** However, its yield is relatively small and the technique is therefore normally only limited to ejaculates with a high number of motile spermatozoa (Tea et al. 1984, Zavos et al. 2000, Henkel and Schill 2003).

Centrifugation on a discontinuous density gradient (DGC) is a technique used to separate many different types of cells. Spermatozoa have a different density from epithelial cells, leucocytes, bacteria and cell debris, and therefore can be separated from the other components of the ejaculate. Seminal plasma remains at the top of the gradient. Motile spermatozoa will orient themselves in the direction of the centrifugal force and will pellet faster than immotile spermatozoa, careful selection of centrifugation time and speed allows the motile spermatozoa to be separated from immotile ones. Immature and senescent spermatozoa, and those with damaged DNA, are also trapped in the upper layers of the gradient or the interfaces, leaving a sub-population of motile, and hopefully fertile, spermatozoa in the pellet (Morrell 2006).

DGC separates usually clean fraction of highly motile spermatozoa. In this method sperm from ejaculates with a very low sperm density can be separated, the yield of separated spermatozoa is good, leukocytes, bacteria and debris can be eliminated to a large extent, reactive oxygen species are significantly reduced. Disadvantage of the methods: production of good interphases between the different media is a bit more time-consuming, DGC is an expensive method and there is a potential risk of endotoxins mainly using Percoll® (Henkel and Schill 2003).

In the last decade, work in several clinics world-wide has shown that density gradient preparation of spermatozoa, in conjunction with swim-up, can remove viral infectivity from human semen samples when semen came from donors infected with HIV, hepatitis C or hepatitis B, as reviewed by Englert et al. 2004. Recent studies with virally-infected animal semen have shown that it may be possible to remove some animal pathogens in a similar manner, for example equine arteritis virus from stallion semen (Geraghty et al. 2004, Morrell 2006).

During ***glass wool filtration*** (GW), motile spermatozoa are separated from immotile sperm cells by means of densely packed glass wool fibres (Van der Ven et al. 1988). The principle of this sperm separation technique lies in both the self-propelled movement of the spermatozoa and the filtration effect of the glass wool. This can also be used for patients with oligo- and/or asthenozoospermia. Like density gradient centrifugation, glass wool filtration also provides the advantage that the sperm separation can directly be performed from the ejaculate. However leukocytes are eliminated to a large extent and reactive oxygen species are significantly reduced using this method, the filtrate is not as clean as it is with other sperm separation methods and remnants of debris are still present (Henkel and Schill 2003).

Recently, species-specific glycidoxypropyltrimethoxysilane (GPMS)-coated silica colloid formulations for use with animal spermatozoa have been developed at the Swedish University of Agricultural Sciences (SLU). Here only one layer of colloid is used, instead of the two or more layers commonly used for a gradient. ***Single layer centrifugation*** (SLC) was successfully used for separation of small and large volume of fresh, chilled or frozen-thawed sperm in equine, porcine and bovine species and can be an alternative method to density gradient (Macías García et al. 2009, Morrell et al. 2009a,b,c,d; Thys et al. 2009).

The complex sequence of biological steps involved in reproduction in vivo is only partially reproduced in current IVF procedures. In fact, events playing a key role in vivo such as male gamete selection can only be partially mimicked in vitro. To

understand the role played by the mammalian oviduct in sperm storage and selection several ***in vitro sperm-oviductal cell co-incubation systems*** have been developed. Particular sperm subpopulations have been reported to be selected by in vitro cultured oviductal cells through cell-cell adhesion, in different species. The isthmus of oviduct acts as a sperm reservoir thus ensuring sperm survival until ovulation (Yanagimachi 1994). In vitro experiments showed that sperm co-cultured with oviductal explants or monolayers undergo a slow, spontaneous and progressive release that may mimick the in vivo sperm release occurring in close association to ovulation. In the bovine, in vitro selected sperm have been demonstrated to be endowed with a superior zona pellucida binding and fertilization competence (Gualtieri and Talevi 2000, Talevi and Gualtieri 2004). Studies showed that adhesion to oviductal epithelial cells and oviduct secretions are able to prolong the sperm motility, viability, and fertility (McNutt and Killian 1991, Grippo et al. 1995, Lefebvre et al. 1995). Co-incubation of equine spermatozoa with equine oviductal epithelial cells (OEC) monolayers resulted in attachment of a subpopulation of spermatozoa to the monolayer. These spermatozoa are a selected subpopulation of the initial inseminate, containing a higher proportion of morphologically normal, motile cells than the inseminate (Thomas et al. 1994). Adhesion to the oviduct allows the selection of sperm characterized by an uncapacitated status (Thomas et al. 1995, Talevi and Gualtieri 2004).

The most widely used sperm separation technique has been the ***Percoll®-based density gradient*** (PG) for all methods of assisted reproduction (IUI, GIFT, IVF, ICSI, etc.). It was introduced by Hyne et al. (1986) for human in vitro fertilization. The typical methodology for the density gradient centrifugation comprised continuous or discontinuous gradients from which discontinuous gradients are used generally. Percoll® consists of colloidal silica particles coated with polyvinylpyrrolidone (PVP) that select spermatozoa according to their density, which seems to be related to their maturation stage and their integrity. Spermatozoa with chromatin integrity are denser and are deposited in the area of greater density. In addition, motile spermatozoa deposit faster than nonmotile cells with the centrifugal force, because of the alignment of their movements with this force. Because of their simplicity, rapidity and excellent yields, they have become very popular in various medically assisted conception procedures. Activated caspases, decreased mitochondrial membrane potential, altered plasma membrane permeability and increased DNA fragmentation, all indicators of an apoptotic-like process. Apoptotic-like changes were examined in different subpopulations derived after density gradient centrifugation of human and equine semen. Spermatozoa isolated from the low-density interface had a significantly greater proportion of apoptotic-like changes than ones from the high density fraction detected

(Barroso et al. 2006, Brum et al. 2008). Percoll® density-gradient fractionation clearly separates spermatozoa from foreign material such as extender particles, cells and bacteria. The morphological selection of spermatozoa in the prepared population varies, with most tail, and midpiece defects being primarily excluded (Rodriguez-Martinez et al. 1997). Percoll® is considered to be completely non toxic to cells and to have essentially no free PVP. Avery and Greve (1995) suspected that some Percoll® batches could have had an excess of free PVP, exceeding the reported 1 to 2%, and that the spermatozoa could have been coated with PVP during the Percoll®-treatment, a coating which would not have an affect on the initial motility of the spermatozoa, but which might result in a low sperm penetration rate of the oocyte. Another problem was that some batches of Percoll® had endotoxic effect so it was discarded for use in assisted reproduction technics in human medicine. While Percoll® as a density medium was removed from the market in 1996 for clinical use in the human because of its risk of contamination with endotoxins (Pharmacia Biotech Inc. 1996), this separation method (after a thorough post-Percoll washing step) still has remained to use widely in ARS techniques in domestic animals. Percoll® density gradient separation for bovine sperm was described by Parrish et al. in 1995. Percoll® gradients technique is generally used for separating equine spermatozoa for ICSI (Landim-Alvarenga et al. 2008). Since Percoll® was stopped to use in the human practice other media like IxaPrep®, Nycodenz®, SilSelect®, PureSperm® or Isolate® have been developed in order to replace Percoll®. BoviPure®, a sperm separation product was formulated specifically for use with bull sperm was found a good alternative media in bovine IVF programs (Samardzija et al. 2006).

There are numerous studies for comparing different sperm separation techniques in human and also in animal science. The results are often controversial and depend on many aspects of the experiments (species; preparation, quality and quantity of the semen; amounts and concentrations of the separating media etc.).

2.8.1 Comparison of swim up and density gradient centrifugation

Several studies have previously been carried out to compare the effectiveness of swim-up and Percoll® separation on human spermatozoa with very varied results (Menkveld et al. 1990, van der Zwalmen et al. 1991, Chan et al. 1991, Morales et al. 1991, Englert et al. 1992, Lachaud et al. 2004. Parrish et al. (1995) studied these sperm separation methods in bull semen and reported better motility of bull spermatozoa by Percoll® than swim up procedure but the penetration and cleavage rates after IVF of bovine oocytes using the swim up technique were higher than those of Percoll® treatment. Rodriguez-Martinez et al. (1997) perceived this beneficial

effect of Percoll® separation only on frozen–thawed semen having spermatozoa with a low post-thaw motility (27–30%) or a low rate of intact membrane. Swim-up and Percoll® separation techniques were compared also to harvest viable sperm in bovine (Somfai et al. 2002b) and in bufallo (Mehmood et al. 2009). Somfai et al. (2002b) observed higher rate of viable sperm with intact acrosome evaluated by Kovács-Foote staining (Kovács and Foote, 1992) and also better recovery rate after Percoll® separation than that after swim-up of frozen–thawed bull sperm. In the study on buffalo semen swim-up separated sperm showed a higher motility, while percent recovery of motile sperm was higher with Percoll® separation. Swim-up method rendered a significantly greater number of sperm with intact membrane assessed using the hypoosmotic swelling (HOS) compared with Percoll® gradient whereas acrosome integrity of the sperm determined by staining with Coomassie Blue did not differ between the two separation methods. Swim-up separated sperm gave a higher cleavage rate and cleavage index (Mehmood et al. 2009). In the study of Stokes et al. (2004) bovine oocytes were injected by equine or bovine sperm separated by standard two-layer Percoll® density gradient or Swim up technique. Pronuclei formation, cleavege and blastocyst development did not significantly differ if PG or SU was used for sperm preparation.

2.8.2 Comparison of different sperm preparation techniques

There are many studies evaluating other methods, e.g. glass wool filtration, Silane-coated silica bead (PureSperm®), Sephadex column filtration (SpermPrep®) and compairing those to Percoll® and/or swim up procedures performed by in vitro (Gabriel and Vawda 1993, Centola et al. 1998, Chen and Bongso 1999, Hinting and Lunardhi 2001, Mendes et al. 2003, Sieme et al. 2003, Lee et al. 2009) or in vivo (Nie et al. 2003) experiments. However, in some aspects the newer separations were better, but the types of samples (fresh, chilled or frozen; normozoospermic, oligozoospermic, asthenozoospermic), designs of experiments and the results were different between laboratories. There is no single separation technique, showing constant superior result.

2.8.3 Preparation of low quality, small volume and low concentration sperm for ICSI

ICSI is an extreme example in low-dose insemination, because only a single spermatozoon is injected into the oocyte. Recently horse breeders have requested that semen be frozen with few numbers per straw for subsequent sperm injection. Studies are being conducted on frozen semen with few numbers of spermatozoa per straw for

subsequent sperm injection, and also to determine the effect of thawing, re-dilution and refreezing of semen on sperm quality and on embryo development after ICSI (Squires 2005). McCue et al. (2003, a manuscript, unpublished) thawed frozen stallion sperm (0.5-ml straws at a concentration of 400 million per ml) and either re-froze at the same concentration or diluted to 40 x 10^6, 4 x 10^6, 4 x 10^5 and 4 x 10^4 sperm per ml with additional extender and re-froze in 0.25 straws. Thawed and Re-frozen/thawed semen was evaluated for motility visually and by CASA and stained with PI for determination of sperm viability. Total motility was 92% pre-freeze, 64% after first freeze, and 46% after second freeze. Viability was 31% after first freeze and 19% after second freeze. Dilution prior to re-freezing resulted in similar motility to those samples re-frozen without further dilution (McCue et al. 2003 manuscript, unpublished and also cited in Squires, 2005). Choi et al. (2006) demonstrated that thawing one semen straw, diluting 1:100 and refreezing does not lower blastocyst formation rate after ICSI. The studies show that it is possible that one straw provides nearly a thousand additional straws for subsequent sperm injection. Other possibilities include cutting a piece of the straw under liquid nitrogen, thawing the semen and then refreezing the extra sperm that are not needed for the ICSI procedure (Squires, 2005). These techniques would allow one to conserve genetic material for a long time period and extend the use of valuable semen several orders of magnitude compared with its use in conventional breeding methods (Squires, 2005).

ICSI procedure requires separated, cleaned, intact spermatozoa. Standard sperm separation methods are not always effective with low numbers of total and viable sperm especially using sperm frozen in the three lower concentrations mentioned above in McCue et al. (2003). In humans for oligozoospermic (sperm concentration: 10-20 x 10^6/ml) and asthenozoospermic samples (sperm concentration: <5 x 10^6/ml), the regular Percoll® gradient centrifugation yielded low rates of sperm recovery. Therefore, a discontinuous mini-Percoll gradient (0.3 ml of each of 95%, 70% and 50% Percoll®) was developed and resulted in better recovery of clean, motile (Ord et al. 1990) and also morphologically normal and HOS active spermatozoa (Smith et al. 1995) In a clinical study separation by mini-Percoll increased the rates of implantation and clinical pregnancy (Egbase et al. 1997).

In human sperm preparation washing procedure usually takes longer than in animals, because cleaning is very important and because they use usually low speed of centrifugation. In the human experiments Percoll® gradient centrifugation took 25-45 min at 300 x g power. Then the pellet was washed out in two steps for 10 min at 500 x g (Johnson et al. 1996, Egbase et al. 1997). In addition in the study of Johnson et al.

sperm was centrifuged twice at 400 x g for 5 min before sperm separation. These are very time-consuming processes. Stallion spermatozoa (mainly frozen semen) are very sensitive to protracted procedures like the ones used in humans; however not sensitive for higher centrifugation speed (Dell'Aqua et al. 2001, Hoogewijs et al. 2010 and personal experiences).

2.8.4 Improving of efficiency of sperm separations using treatment of spermatozoa

Commonly there are two different main approaches to increase the effectiveness of sperm separation. One is modifying and developing separation methods and the other is adding chemical stimulators to the media to improve functional capacity of spermatozoa for successful fertilization. Many substances including serum, peritoneal fluid and follicular fluid or other chemically defined pharmacological substances like kallikrein, progesterone, adenosine analogues or methylxanthin derivates have been proposed to stimulate human sperm functions (Henkel and Schill 2003). Recent studies are focusing also to the problem of immotile human sperm preparation from sperm of oligozoospermic, oligoasthenozoospermic men and also from testicular and epididymal biopsy. Pentoxifylline (PX) and hyaluronic acid (HA) are successfully used for initiating and inducing motility and viability in these cases.

Pentoxifylline is a methylxantine derivate and non-specific inhibitor of phosphodiesterase (PDE). Therefore increases intracellular levels of cAMP. It increases sperm motility, progressive motility. PX also may play a role at induction of capacitation and acrosome exocytosis (Tesarik et al. 1992), but the motion characteristics didn't show that effect after PX treatment of human sperm samples (3 mM PX dissolved in control medium, and incubated for 20 min at room temperature), because only curvilinear velocity (VCL) increased but neither elevated lateral head displacement (ALH) nor reduction of the linear motion - which are revealing for capacitated spermatozoa - were found evaluated by CASA (Yogev et al. 2000). In other studies the authors found an increase in hyperactivated motility, average path velocity (VAP), VCL and ALH in hamster and human sperm after PX treatment (Jayaprakash et al. 1997, Calogero et al. 1998).

Non-specific inhibition of the PDE's would obviously result in both, stimulation of motility and acrosome reaction depending on the conditions and most importantly on the time of stimulation and the concentration of pentoxifylline in the medium. Over-stimulation could definitely result in a too early acrosome reaction. For ICSI, where spermatozoa bypass all physiological barriers because they are directly injected into

the oocytes, the relevancy of this problem seems to be conflicting (Fisch et al. 1998, Henkel and Schill 2003).

In human medicine, pentoxifyllin is widely used to initiate motility in the case of immotile testicular or epididymal spermatozoa or for astheno-zoospermic men for ICSI (Tarlatzis et al. 1995, Terriou et al. 2000, Kovacic et al. 2006)). Sperm preparation with PX resulted in higher fertilization rates and more viable pregnancies (Tarlatzis et al. 1995, Kovacic et al. 2006). Although sperm samples treated by adding pentoxifylline as a supplement to the cryoprotectant didn't improve motion characteristics after post-thaw (Stanic et al. 2002), the treatment after thawing increased significantly the total and progressive motility of human (in 3 mM PX) (Stanic et al. 2002) and also of equine spermatozoa (in 3.5 or 7 mM PX) (Gradil and Ball 2000). It was also reported that PX supplementation before or after cryopreservation does not alter the plasma membrane evaluated by HOS test (Stanic et al. 2002). The addition of 3.5 or 7.0 mM pentoxifylline appeared to increase the motility of chilled spermatozoa compared to controls (Gradil and Ball 2000). Esteves et al. (2007) confirmed the results above.

Hyaluronic acid is a non-sulfated glycosaminoglycan which is physiological component of the cumulus and of the female and male reproductive tracts. It has also been used in human medicine in a special swim-up method called Sperm Select System (Select Medical Systems, Williston, VT). The medium contains 1mg/ml highly purified HA. In the technique sperm swim up directly into the HA solution. HA significantly increases sperm motility but also induce acrosome exocytosis because increases the Ca^{2+} influx into spermatozoa measured by Triple Stain Technique (Slotte et al. 1993). In another study the hyaluronate method produced greater percentages of motile, viable, and morphologically normal sperm, with lower proportions of premature acrosome reactions, higher sperm velocity, and greater linearity (Zimmerman et al. 1994). In the experiment of Sybracia et al. (1997), semen was incubated in media containing 0.25 mg/ml HA. HA improved the retention of sperm motility in cryopreserved/thawed human spermatozoa even after the removal of HA from the incubation medium. However cryopreservation of spermatozoa in the presence of HA did not improve the recovery of motility (Sybracia et al. 1997). In contrast HA supplementation appeared to preserve post-thaw boar spermatozoa viability and maintained membrane stability after cryopreservation (Peña et al. 2004). The presence of HA has been identified in the epithelium of the pig preovulatory spermatozoa reservoir. Important aspects of sperm function such as motility and capacitation appear to be mediated through HA (Suzuki et al. 2002).

Hyaluronic acid was used successfully in combination with swim-up for separating motile spermatozoa from frozen bovine semen. (Shamsuddin et al. 1993, Shamsuddin and Rodriguez-Martinez 1994). In the earlier study spermatozoa were collected after 1 h of self-migration at 39°C in a medium consisting of equal volumes of Tyrode's albumin lactate pyruvate (TALP) and phosphate-buffered saline at a final concentration of 1 mg/ml HA (HA medium). The selected spermatozoa were used to inseminate in vitro matured oocytes The beneficial effect of the HA medium was more evident in the group of low-fertility bulls (Shamsuddin et al. 1993). In the latter study a swim up procedure has been developed where spermatozoa from frozen-thawed bull semen were allowed to swim up through a medium containing 1 mg/ml HA to the upper Fert-TALP solution. Motility, the proportions of spermatozoa with intact plasma membrane and acrosome increased after HA-swim-up method (Shamsuddin and Rodriguez-Martinez 1994).
The addition of HA to cryopreservation extenders did not effect the post thaw membrane integrity, motility or acrosomal integrity of stallion spermatozoa (Ottier and Curtis 2005, Mari et al. 2005). However there was a significant increase in the number of sperm bound to oocytes in the stallion with low fertility in the presence of HA (Ottier and Curtis 2005).

Recently a reserchер group developed a novel method using immobilized HA coated Petri dishes or glass slides for human spermatozoa selection (Huszár et al. 2003, Jakab et al. 2005, Huszár et al. 2006, Yagci et al. 2010). They demonstrated that HA shows a high degree of selectivity for sperm with high DNA integrity. In the HA-bound sperm the chromosomal disomy and diploidy is reduced 4- to 6-fold compared with semen sperm fractions.

2.8.5 Effect of acrosome on the success of ICSI

In the recent years more and more publications have been published studied the effect of acrosome membranes and materials on the development of embryos following ICSI. The acrosome vesicle of a sperm cell has different enzymes, which can damage the oocyte when introduced in the ooplasm during ICSI (Tesarik and Mendoza 1999). This does not happen in IVF, because only the acrosome-reacted sperm cells are able to fertilize. Some authors have observed that oocytes injected with acrosome-intact spermatozoa delay the onset of male chromatin decondensation and male pronucleus formation (Katayama et al. 2002). Immediately before normal fertilization, both the sperm plasma membrane covering the acrosome and the contents of the acrosome are shed as a result of the acrosome reaction (Yanagimachi 1994). Thus, the acrosome and

its contents never enter the oocyte under normal conditions.. In view of potentially harmful effects of acrosomal enzymes on embryo development, the removal of acrosomes before ICSI is recommended for animals with large acrosomes. It is very important to keep sperm nuclei undamaged during or after the removal of the acrosomes. Excess exposure of demembranated spermatozoa to harsh reagents or prolonged maintenance of demembranated spermatozoa in an inappropriate medium may damage sperm nuclei, thus resulting in the failure, rather than the improvement, of ICSI (Morozumi and Yanagimachi 2005). The most commonly used reagents for removing acrosomal membranes are lysolecithin, Triton X-100 and calcium ionophore. There are many publications associated with various species and type of sperm with different results about using spermatozoa with presence or absence of acrosome for ICSI (Goto et al. 1990, Gómez et al. 1997, Kasai et al. 1999, Nakai et al. 2003, Katayama et al. 2005, García-Roselló et al. 2006, Malcuit et al. 2006, Morozumi et al. 2006, Roldan 2006, Tian et al. 2006, Seita et al. 2009, Gianaroli et al. 2010, Nakai et al 2011a, 2011b). There are few publications in equine species and these are contradictory about effectivity of using chemicals for acrosome reaction and plasma disruption or activation of fertilized oocytes. (Matsukawa et al. 2002, Matsukawa et al. 2007, Bedford et al. 2004). Oocyte activation rates after ICSI in equine species have been largely inconsistent and generally low among laboratories (Choi et al. 2002*)*. The development of effective and repeatable methods for ICSI is associated with to use Piezo drill to perform the sperm injection. Sperm are subjected to pulses while within the pipette that may aid in membrane disruption and facilitate release of sperm-borne oocyte activation factors. No exogenous activation treatment is needed after sperm injection using frozen/thawed spermatozoa (Choi et al. 2002). Therefore a simple mechanical membrane disruption within the pipette is currently performed during sperm injection in equine species (Hinrichs 2010). The effect of existence of acrosome and their contents after ICSI procedure is still the subject of extended studies in many in vitro laboratories.

2.9 Fertility of the stallion

2.9.1 Fertility parameters, subfertility

Measuring stallion fertility is not an exact science and all of the indices used have obvious shortcomings. Many factors such as management of the stallion (nutrition, housing, semen collection, semen processing and storage) and his mares (optimal time of insemination, reproductive status and conditions of the mares) may have a large impact on the success of insemination and overall fertility of the stallion. The fertility of stallions is most accurately defined based on pregnancy of mated/inseminated

mares. For objective assessment of fertility, however sufficient number of mares is required. Reproductive performance of the stallion cannot be estimated if only a few mares are mated/inseminated and the mares haven't been pregnant for many years (Juhász and Nagy 2003). The fertility of stallions can be characterized by three parameters: *foaling rate, pregnancy rate per season and per cycle pregnancy rate***.**

The per season and per cycle ***foaling rates*** (the number of foals produced as a percentage of the mares mated) are often considered the ultimate measures of stallion fertility, they are influenced strongly by non-stallion factors such as the age and reproductive status of mated/inseminated mares and the intensity and quality of veterinary management (Sullivan et al. 1975, van Buiten et al. 1998, Morris and Allen 2002, Colenbrander et al. 2003). For the precise determination of foaling percentage data of the given stallion from several breeding seasons are taken into account (in the case of 25 mares per year inseminated approx. ten years). To respect the complicating elements above this parameter is less suitable for rapid examination and monitoring changes of stallion fertility (Juhász and Nagy 2003). Using the first cycle foaling rate instead of the all cycles or seasonal rates, will remove some of the factors attributable to mare subfertility, but the foaling rate remains a retrospective measure for which data are only available in the year after the stallion has begun to mate (Colenbrander et al. 2003). According to data from France the foaling rate is around 63% in stallions with average fertility (Juhász and Nagy 2003).

Per season pregnancy rate (end of season pregnancy rate; seasonal pregnancy rate) is calculated of the pregnant mares at the end of the season divided the total number of inseminated mares. The value is strongly influenced by the reproductive status, veterinary and housing management of mated/inseminated mares (Juhász and Nagy 2003). Seasonal pregnancy rates should be above 85% in well - managed herds. Stallion which has this parameter below 70 % is considered subfertile, however the notable exception is the Thoroughbred breed where the seasonal pregnancy rate is around 50-55% (Card 2010).

A quicker and most practical way of assessing fertility is to examine the ***per cycle pregnancy rate*** (number of cycles resulted in pregnancy/total number of cycles in which insemination obtained). Detected pregnancy by ultrasound or later by rectal palpation, healthy foal born, early embryonic loss and abortion are also counted as a positive pregnancy result in this system. If a mare is inseminated in several cycles, data of each cycle are recorded separately (Juhász and Nagy 2003). This parameter has the advantages that the result is available in a shorter interval and gives indication of fertility during the breeding season; this can be a useful early warning system for a

developing fertility problem. However, if the figures are to give an accurate indication of stallion fertility, data must be collected from a relatively large number of normal young mares. The day 14–18 pregnancy rate will also exclude later pregnancy losses where it is possible, if unusual, for a stallion to be a major source of pregnancy loss because of sperm chromosome or chromatin abnormalities (Kenney et al. 1991, Colenbrander et al. 2003). This parameter is less influenced by breeding management of the mares and suitable for monitoring changes of stallion fertility (Juhász and Nagy 2003). Ideally to avoid a larger impact of subfertile mares on a stallion's apparent fertility it is preferable to include only 1st cycle pregnancy rates. Nevertheless because the average stallion breeds relatively few mares (10 or less) a per cycle pregnancy rate is a reasonable compromise (Card 2010). The ***non-return rate*** is another parameter often used as an early index of fertility in farm animal species in which pregnancy examination can only be performed reliably after the time of the next expected oestrus, and in horses when mares are not routinely returned for early pregnancy diagnosis (eg. Shetland ponies, van Buiten et al. 1999). The non-return values suffer from the obvious disadvantage that mares may not be returned for mating for reasons other than becoming pregnant. Otherwise the non-return rate gives similar information to the pregnancy rate. *Per cycle pregnancy rate (fertilization rate)* for stallions is lower (43–60%; Woods et al. 1987; Brück et al. 1993; Morris and Allen 2002) than for domestic livestock species e.g. rams (80–90%; Menzies 1999) and boars (85–90%; Colenbrander et al. 1993) because at the latters, reproductive performance is an integral part of productivity, selection of males for good fertility is much more rigorous. This parameter is considerably more variable between individual stallions (35–90%; Morris and Allen 2002). And while experimental studies demonstrate that fertilization rates can reach high levels in mares mated at the appropriate time with semen from a stallion of proven fertility (>90%; Ball et al. 1989), the discrepancy between experimental fertilization rates and pregnancy rates in the field appears to be explained primarily by deficiencies in stallion fertility and management, and only partially by pregnancy loss (Jasko 1992, Colenbrander et al. 2003). In the case of insemination with fresh stallion semen the per cycle pregnancy rate is in average 45-55% (Card 2010) or 50% ((Juhász and Nagy 2003) in a non-selected population of mares. Using chilled or frozen semen the value of 40-45% is satisfactory (Juhász and Nagy 2003).

Comparing frozen stallion sperm parameters to seasonal pregnancy rate is less reliable than per cycle fertility, especially when field results are being utilized, since seasonal fertility is affected by more variables. For instance, multiple inseminations using increased numbers of spermatozoa may have been necessary to achieve acceptable seasonal fertility for some ‘‘lower fertility’’ stallions (Kirk et al. 2005).

In general, an end of season pregnancy rate of 80% and a foaling rate of 70–80% are considered satisfactory, and can be achieved within four cycles per mare by a stallion with a per cycle pregnancy rate as low as 35%, assuming normal rates of pregnancy loss (Colenbrander et al. 2003).

Reduced fertility (subfertility) of the stallion is stated if per cycle pregnancy rate was significantly lower than 50% using fresh semen for inseminating mares. Significance of the differences is influenced by the number of cycles used for insemination. The stallion which inseminated mares in 25 cycles is only considered safely as subfertile if the per cycle pregnancy rate was lower than 30%. Result of < 40 % per cycle pregnancy rate based on 100 cycles is significantly different from the average 50%. Ratio of subfertile stallions is approximately 4.5-7.5% among breeding stallions depending on horse type; in coldblood horses this rate is higher than warmbloods (Juhász and Nagy 2003). Most stallions loose commercial viability if they have <30% per cycle pregnancy rate. Stallions are generally considered not suitable for commercial use if their per cycle pregnancy rate (PR) falls below 30%. The reason for this is that mares are re-bred many more times and most mare owners are reluctantly to breed their mare more than twice or 3 times, because she will foal later in the subsequent year. If a stallion was breeding at 30% per cycle PR after 3 breedings he would leave 35 out of a 100 mares non-pregnant. In consideration of the long gestation length of the mare, and the fact that there is pressure to get the mares pregnant early so they do not foal later and later every year, the time lapsed due to lower per cycle pregnancy rate becomes a significant problem. Stallion which has 0-5 % PR and < 10% seasonal pregnancy rate, is considered infertile (Card 2010).

For comparing fertility of individual stallions, or groups of stallions it is need to take into account the followings: When 60% versus 70% per cycle pregnancy rates are compared, the difference is not significant even when based on 100 mares for each stallion. Important rule is that if the difference in 1st cycle pregnancy rate is ≤10 percentage units, it will not be statistically significant unless the two values each are based on >190 mares. Comparing 45% versus 60%, only if each 1st cycle average was based on >95 mares is the difference significant. Moreover, calculations based on cumulative pregnancy rates over four cycles tend to mask a possible difference between two stallions if both stallions have a cumulative pregnancy rate >60% (Amann 2005).

2.9.2 Relationship between sperm quality and fertility

Finding a laboratory test reliable enough to predict the potential fertility of a given semen sample or a given sire for artificial insemination (AI) is still considered utopistic, as indicated by the modest correlations seen between results obtained in vitro and field fertility. Male fertility is complex, and depends upon a heterogeneous population of spermatozoa interacting at various levels of the female genital tract, the cumulus cells, zona pellucida and the oocyte. For this reason, laboratory assessment of semen must include the testing of most sperm attributes relevant for fertilization and embryo development, not only in individual spermatozoa but within a large sperm population as well (Rodríguez-Martínez 2003).

The problem about semen evaluation is that semen samples with very high or very low quality may be relatively predictable, but for medium quality, no single sperm attribute exists that is highly and accurately correlated with fertility in vivo (Amann and Hammerstedt 1993, Colenbrander et al. 2003, Pesch and Bergmann 2006). It is crucial to understand that the main objective of semen evaluation is to identify infertile and subfertile stallions or ejaculates (Amann and Hammerstedt 1993).

Classical andrologic parameters in relation to fertility can be categorised into three groups: 1. Quantitative and qualitative parameters of sperm (concentration, total sperm number, progressive motility after collection and dilution with extender, progressive motility after 24 and 48 hours storage at +4°C, live/dead ratio, and proportion of morphological abnormal sperm) which are evidenced related to the future fertility; 2. Parameters decribing function of accessory sex glands (pH, volume of the ejaculate) which are not proven connected to future pregnancy results; 3. Characteristics of sexual behavior and libido of the stallion (time required for preparing and for semen collection/mating, number of jumps), in this cases it is not proven and could not be excluded either the correlation to fertility (Juhász and Nagy 2003).

Evaluation of spermatozoal ***motility*** in both raw and extended forms is considered to be a fundamental laboratory test for assessing the fertilizing capacity of spermatozoa in an ejaculate. Evaluation of raw semen provides an indication of how well spermatozoa perform in their natural fluid milieu (Varner 2008). Jasko (1992) performed the most comprehensive study of the relationship between conventional semen quality parameters and fertility. Although they found a weak correlations between the percentages of motile ($r = 0.40$), progressively motile ($r = 0.46$) and morphologically normal ($r = 0.36$) sperm with fertility, they also reported that

variation in these characteristics accounted for only 20% of the total variation in fertility (Jasko 1992).

Attempts to correlate the percentage of morphologically normal or abnormal spermatozoa with fertility have given conflicting results. Thus Jasko et al. (1990), Hellander et al. (1991) reported that the rate of spermatozoa with normal ***morphology*** correlates positively with fertility to various degrees, while others (Voss et al. 1981, Dowsett and Pattie 1982) did not find any relationship between sperm morphology and fertility. Many studies show that pathomorphology is highly correlated with fertility in stallion (Jasko et al. 1990, 1991; Parlevliet and Colenbrander 1999). An examination of sperm morphology alone can never justify the statement that the potential fertility of an ejaculate is high, but is reasonable to state that potential fertility is low when a high proportion of spermatozoa have abnormalities (Dott 1975). A wide range of morphological sperm abnormalities may be acceptable for normal stallions (Kavak et al. 2004). However, highly fertile stallions in regular use usually have a low frequency of morphologically abnormal spermatozoa (Rousset et al. 1987, Einarsson et al. 2009). The sperm head has been reported to be larger in semen taken from subfertile stallions than in that from fertile stallions (Gravance et al. 1996, Casey et al. 1997, Brito 2007): mentioned in details in the session of "Structure of equine spermatozoa" above. Some incidences of morphologic abnormality in human (Lewis-Jones et al. 2003), equine (Brito et al. 2010) and bull (Révay et al. 2009, 2010) sperm may be associated to inherited, congenital defect.

Significant correlations (r = 0.68) have been reported between the pregnancy rate and ***viability*** of propidium iodide-stained frozen-thawed stallion sperm assessed by flow cytometry (Wilhelm et al. 1996). Neild et al. (2000) found no significant connection between the hypoosmotic swelling test (HOST) and fertility but a tendency for the HOST to correlate with the number or services per pregnancy. Colenbrander et al. (2003) concluded in their study that it was possible to identify a small population of subfertile stallions using HOST that could not been identified by conventional semen analysis.

Vidament et al. (1999) defined ***minimal requirements*** of sperm quality of stallions used for artificial insemination (AI) or national breeding based on systematic examination of large number of stallions in French National Studs. These limits separate the lower 10% from the upper 90% of the population (Table 3). Minimal values are much higher in warmblood than in coldblood stallions. One stallion is considered to have sufficient fertility and suitable as a breeding stallion if the value in each parameter of his sperm is higher than the minimum value. Based on this system

80% of subfertile stallions can be identified, however the results of the other 20% of the stallions are above the limits. It is important to note that 26% of the stallions showing proven fertility has at least one examined parameter in which the value is lower than the minimum. Therefore, special care must be taken in the case of high-genetic value breeding stallions with lower sperm quality. Semen evaluations repeated after a few months, and some breeding or AI trials should be taken (Vidament et al. 1999, Juhász and Nagy 2003). General principles related to insemination of a mare that time interval between AI and ovulation is should be ≤ 48 hours (fresh semen) or ≤ 6-12 hours (frozen/thawed semen) and the insemination dose needs to contain minimum 300-500 million motile sperm (Householder et al. 1981).

Table 3. Minimal requirements of sperm quality in Warmblood and Coldblood stallions (Source: Vidament et al. 1999, Juhász and Nagy 2003)

Parameter	Warmblood stallions	Coldblood stallions
Concentration (10^6/ml)	≥ 73	≥ 28
Total sperm number (10^9 sperm/ajaculate	≥ 2.5	≥ 1.9
Progressive motility (%) after dilution	≥ 55	≥ 31
Progressive motility (%) after 24 hours at + 4°C	≥ 15	≥ 3
Progressive motility (%) after 48 hours at + 4°C	> 5	> 0
Rate of morphologic abnormalities (%)	≤ 36	≤ 47

Stallion sperm which is used for chilled- or frozen-sperm-AI should be met to stricter criteria (Table 4). According to these requirements 75% of the warmblood stallions which are sufficient for using their fresh semen for AI or for mating mares, are suitable for chilled-semen AI. Per cycle pregnancy rate of selected stallions is highly correlated to progressive motility of sperm used after 24 hours storage at + 4°C. This test is proposed to perform in the beginning of the season in those stallions of which semen are planned to use as chilled-transported sperm for AI (Vidament et al. 1999, Juhász and Nagy 2003).

Table 4. Minimal quality requirements of fresh stallion sperm used for chilled- or frozen-sperm-AI (Source: Vidament et al. 1999, Juhász and Nagy 2003)

Parameter	Warmblood stallions	Coldblood stallions
Concentration (10^6/ml)	≥ 120	≥ 120
Progressive motility (%) after dilution	≥ 70	≥ 60
Progressive motility (%) after 24 hours at + 4°C	≥ 40	≥ 30
Progressive motility (%) after 48 hours at + 4°C	≥ 30	≥ 20

Althogh in traditional classifications of sperm morphologic defects the primary abnormalities which are originated by testicular malfunction are thougt to be more important to fertility and the major defects are also mostly the same as primary defects, recently the function of epididymis and the secretums of accessory glands have got more and more significance since these organs ensure the complete maturation of the sperm which is prerequisite of normal fertilization. Many biochemical characteristics of spermatozoa are modified during the epididymal transit including the nucleus chromatin condensation, changes in phospholipids and cholesterol plasma membrane composition, and modifications of plasma membrane surface proteins composition. Disturbances of these processes may result in noncompensable defects, because all the spermatozoa transported through the male genital tracts are affected. Many organs of the male reproductive tract are known to secrete membranous particles called exosomes. ***Exosomes*** are involved in the selective transfer of proteins to spermatozoa, playing a major role in the production of fully functional male gamete. Prostasomes in men and prostasome-like vesicles in stallions are responsible of the post-ejaculatory modifications occurring in the sperm cells. It seems that these exosomes have the ability to protect spermatozoa in the lower and upper female genital tract against bacteria, reactive oxygen species, phagocytosis, or premature capacitation so that they reach the fertilization site in a state that will ensure their function (Minelli et al. 1998). Many ***proteins*** are associated to epididymosomes, particles of epididymis: The role of gluthation peroxidase-5 (GPX5) is a protection of sperm against premature acrosome reaction is hypothesized. Ubiquitin is another protein associated with epididymal vesicles. This protein secreted by the epididymal epithelium is transferred to spermatozoa and is thought to be involved in the elimination of defective spermatozoa. P25b, P26h and P34H sperm surface proteins in different species are involved in the binding of spermatozoa to the zona pellucida (Parent et al. 1999)**.** Aldose reductase, sorbitol dehydrogenase, a cytokine named macrophage migration inhibitory factor (MIF) modulate sperm motility during the transit along the male reproductive tract (Sullivan 2005). Lipocalin-type prostaglandin D2 synthase and the angiotensin-I-converting enzyme in semen were strongly correlated with fertility in the stallion, whereas the total protein concentration in seminal plasma (SP) was not related to fertility (Barrier-Battut et al. 2005). Brandon et al. (1999) found 14 protein bands in stallion semen, and 4 of these proteins Horse Seminal Protein (HSP)-1 was positively, HSP-2, HSP-3 and HSP-4 were negatively correlated with breeding scores of the stallions. Kareskoski et al. (2011) found that HSP-1 and HSP-2, are the most abundant proteins in all fractions of the stallion ejaculate. These heparin-binding proteins were hypothesized to modulate

capacitation. Most of the proteins found in seminal plasma, except for HSP-4, are attached to the surface of spermatozoa at the time of ejaculation (Calvete et al. 1994).

Sperm from stallions classified as fertile on the basis of breeding history (n = 6, pregnancy rates > 70%) had higher percentages of *progesterone-induced acrosome reactions* in comparison with stallions classified as subfertile (n = 4, no record of live foals or pregnancy rates <10%). These data suggest that some cases of stallion sperm dysfunction may have a molecular etiology and these ***tests of sperm function***, such as response to in vitro capacitation and agonists of the acrosome reaction, may be useful in evaluation and therapy of stallion subfertility (Meyers et al. 1995). In the study of Rathi et al. (2000), it was assessed whether there is a correlation between stallion fertility, defined on the basis of first cycle foaling rate and first cycle "non-return rate", and the proportion of spermatozoa with exposed *progesterone receptors* on their plasma membranes. A high correlation was observed between the proportion of spermatozoa with exposed progesterone receptors and stallion fertility ($r > 0.70$; $P < 0.01$). This result indicates that exposure of progesterone receptors is a potential parameter for predicting stallion fertility. Varner et al. (2000) identified four stallions with previously unexplained infertility, whose spermatozoa do not respond to A23187 to the same degree as fertile stallions. Exposure of fertile stallion semen to the calcium ionophore resulted in >80% of the spermatozoa becoming acrosome reacted, whereas <20% of spermatozoa from the stallions with unexplained subfertility or infertility underwent the acrosome reaction under the same conditions. Acrosomal status was evaluated by transmission electron microscopy. Bosard et al. (2005) assessed *acrosome response rate* (ARR) (intact to reacted) following exposure to A23187, between stallions with normal fertility and unexplained subfertility. The results indicate a difference in ARR of sperm between fertile and subfertile stallions, even though sperm motility and morphologic parameters were similar between groups. Acrosomal dysfunction appears to be associated with an elevation in the *ratio of cholesterol to phospholipid* in stallion sperm. In the study of Brinsko et al. (2005a) confirmed this hypothesis. Cholesterol-to-phospholipid ratios in the sperm of fertile stallions ranged from a low of 0.6:1 to a high of 1.2:1, whereas cholesterol-to-phospholipid ratios in the sperm of subfertile stallions ranged from 0.97:1 to 1.96:1.

Lipid peroxidation in the seminal plasma seemed to be a general indicator for sperm damage. In the non-breeding season positive correlations between lipid and protein oxidation levels in both sperm and seminal plasma and several defects in sperm function were found, but only for subfertile animals, thus suggesting that lipid and protein oxidation may aid in the identification of subfertile stallions during the non-

breeding season (Morte et al. 2008). Sperm chromatin implies sperm DNA and associated nucleoproteins. The relationship between ***sperm chromatin stability*** and male fertility has been reported for several species. Kenney et al. (1995) reported higher rates of sperm chromatin denaturation evaluated sperm chromatin structure assay (SCSA) in semen from subfertile than fertile stallions (32% vs 16%) and a negative correlation between denaturation score and seasonal pregnancy rate. In a group of fertile stallions, Love and Kenney (1998) were able to demonstrate variations in SCSA that correlated moderately with both seasonal and per cycle fertility rates (Colenbrander et al. 2003). While there are many potential causes for disruption of sperm chromatin structure, clinically there appear to be two manifestations in the stallion. The first is a result of a traumatic or stressful incident to the testes, such as a kick or any event that would result in fluid accumulation around the testes. This has the effect of insulating the testes, resulting in a heat stress condition. In this case, changes in spermatozoal motility and morphology can be seen, as well. Depending on the severity of the heat stress, the morphology and motility will be affected first, while the chromatin appears more resistant to mild forms of heat stress. As the adverse effects resolve, the chromatin returns to normal. Therefore, it appears that chromatin disruption is reversible when the inciting cause is removed. The second form of chromatin disruption involves those stallions which appear to have a high percentage of chromatin that is inherently abnormal with unknown cause. Sperm chromatin may deteriorate progressively as stallion's age, or may be manifest as an inherent trait at the onset of their breeding career (Varner et al. 2000).

Fazeli et al. (1995) used the ***hemi-zona assay*** to demonstrate that the number of sperm binding to a hemizona was significantly higher for fertile than for subfertile stallions, even though no significant differences in conventional semen parameters were apparent between the two groups of stallions compared.

It is useful to keep in mind that in a commercial fertility trial, female factors, herd (or management), and timing of AI or natural breeding have a much greater effect on the probability of pregnancy than the stallion per se. Typically, such factors contribute >5x more to the outcome than the male (Amann 2005). The caudal isthmus has been proposed as a sperm reservoir in the mare. The pattern of ***transport*** and ***survival of spermatozoa in the mares reproductive tract*** are different between fertile and subfertile stallions, between fertile and some infertile mares, and between fresh and frozen-thawed semen. Possible explanations for these differences include a selective phagocytosis of damaged or dead spermatozoa, impaired myometrial activity in subfertile mares, bio-physiologieal changes of spermatozoa during cryopreservation,

and the removal of seminal plasma during cryopreservation of equine semen. The total number of spermatozoa that reach the oviduct at 4 hours after insemination with fresh extended semen is significantly greater from fertile stallions compared to subfertile stallions. More than 90% compared to 25% motile sperm were recovered from the oviducts of mares inseminated with semen from fertile and subfertile stallions, respectively (Scott et al. 1995, Troedsson et al. 1998). Seminal plasma is presumably important in the transport of sperm, since it contains oxytocin and prostaglandins. Seminal plasma inhibits PMN-chemotaxis and -phagocytosis and thereby may protect sperm in the mare's uterus (Katila 2001b). Sperm transport is affected by both mare and stallion factors. It is impaired in mares with dysfunctional myometrial contractility, mares inseminated with semen from subfertile stallions, and mares inseminated with cryopreserved semen (Troedsson et al. 1998).

Recent studies have shown that male fertility does not only depend on the absolute number of viable, motile, morphologically normal sperm that can be inseminated in a female. Rather, a more important parameter appears to be the functional competence of sperm cells – since this cannot be evaluated using a single variable, researchers have proposed that semen samples should be subjected to ***multi-parametric analysis*** (Rodriguez-Martinez 2006). Laboratory tests which could evaluate several attributes of the sperm simultaneously are needed. Combining results of assays that measured multiple sperm attributes improved the ability to evaluate the fertilizing potential of stallion spermatozoa (Wilhelm et al. 1996, Kirk et al. 2005). The use of fluorescent dyes and flow cytometry is an excellent tool to evaluate several sperm features /viability, acrosome status, mitochondrial status, DNA integrity and stages of capacitation/ (Squires 2005). However, assessment by flow cytometry or fluorescence microscopy is generally not available to practitioners in the field because of high cost of the equipment (Merkies et al. 2000). For clinicians in the field but also for researchers, ***combinations of light microscopic methods*** are fully available. HOST combined with eosin stain and trypan blue-Giemsa staining together could be used for the prognosis of the potential fertility of bovine semen samples used for in vitro fertilization or artificial insemination (Tartaglione and Ritta 2004). Domes (2003) found that a combination of three parameters of stallion semen (progressive motility, total number of spermatozoa with progressive motility per dosis and percentage of spermatozoa with intact plasma membrane assessed by TB-Giemsa staining) provided a more reliable prognosis of the single cycle pregnancy rate. This statement was based on the comparison of per cycle fertility rate between the 8 best quality- and the 8 worst quality semen samples ranked on the combination of the results of these 3 parameters from total of 80 semen samples used for single insemination.

2.9.3 Reproductive disorders of stallions; diagnosis, prognosis and possible treatment

There are a number of conditions associated with poor fertility such as mismanagement, anabolic steroid treatment, infectious and non-infectious pathology of reproductive organs, behavioral and endocrine disorders (McKinnon and Voss 1993, Roser 2001). The differential diagnosis of a fertility problem in stallions includes narrowing a list of differentials to determine if a horse has psychological, mechanical, or reproductive problems. Reproductive problems include: Poor intrinsic fertility, Testicular degeneration, Partial ejaculation, Spermiostasis, Sexually transmitted diseases (STD), Hemospermia, Urospermia, Trauma, Torsion, Tumours, and other infections (Ball 2008, Card 2010). Many of these problems if diagnosed timeously and treated appropriately will result in a successful return to fertility. However, some disorders such as progressive testicular degeneration cannot be treated (Murchie 2005).

Reduced fertility of the stallion may originate in behavioural abnormalities, testicular abnormalities, scrotal and penis pathologies, rarely accessory gland disorders or endocrine diseases. Approximately a quarter of all fertility problems in the stallion are caused by **behavioral disorders**. (e.g: poor libido, injury during breeding or associate pain with breeding, failure to ejaculate, lack of sexual rest, loss of breeding vigour due to health related problems like joint diseases or recurrent airway obstruction /RAO/) The possible solution is changing in stallion handling, medication of background diseases, e.g. for musculo-sceletal problems (Murchie 2005). **Dysfunction of accessory glands** is very rare in stallions. Of these seminal vesiculitis is observed in some cases but quite uncommon; however, it is a noteworthy disorder because of its persistent nature and interference with fertility. The disease usually does not manifest in clinical signs. Spermatozoal quality may appear unaffected when examined immediately post-ejaculation, but spermatozoal longevity is usually reduced. Culture and cytologic evaluation of expressed secretions of the vesicular glands aid in diagnosis of seminal vesiculitis (Varner et al. 2000). **Penile pathologies** are relatively common causes of fertility problems in stallion (McKinnon and Voss 1993). Abnormalities such as balanoposthitis and trauma can be disruptive during the breeding season. Some diseases have a temporary effect whilst others such as phimosis and paraphimosis can have a more serious and permanent negative impact on fertility (Murchie 2005).
Regarding to semen evaluation as a possible diagnostic way, testicular, scrotal abnormalities and endocrine disruptions are more important in this point of view.

Testicular pathology can have a significant negative impact on a stallion's fertility causing disruption in steroid hormone production, spermatogenesis and libido. Testicular abnormalities range in severity from progressive testicular degeneration to minor testicular trauma. Diagnosis of the different testicular disorders is based on palpation, ultrasonographic examination, semen evaluation and in some cases biopsy and histology. ***Testicular degeneration*** is a major cause of stallion subfertility and infertility (Blanchard and Varner 1993, Blanchard et al. 2000). It is an acquired condition initiated by a variety of inciting causes varying only in degree of severity. Testicular degeneration may have a multitude of causes including thermal injury, vascular pathologies, trauma, systemic or local infections, malnutrition, dietary deficiencies, toxins, x-ray exposure, medications, testicular neoplasia, age-related degeneration or idiopathic (Murchie 2005, Card 2010). These various factors may elicit the same temporary or permanent response in the testis: degenerating germ cells become more common, multinucleate giant germ cells form by coalescence of spermatocytes or spermatids, the ratio of germ cells to Sertoli cells is reduced, and spermatozoa production is adversely affected (Johnson et al. 1997). Testicular degeneration may unilateral or bilateral and can be transient or permanent. Diagnosis of testicular degeneration is based on physical and ultrasonographic examination of the testicles, semen and hormone analysis (Murchie 2005, Card 2010). The spermatozoa in the ejaculate show the events that occurred in the past 2 months that influenced spermatogenesis, when the spermatozoa were being formed, and their subsequent transport and maturation through the excurrent duct system (Amann 1993, Card 2005). In testicular degeneration the typical changes in semen include low spermatozoa concentration and a high percentage of morphological defects, specifically a high number of immature germ cells, head defects and midpiece defects. Serum concentrations of FSH and LH may be elevated whilst estrogens, inhibin and testosterone may be low (Murchie 2005). Histologically the stallion would have vacuolization of the seminiferous epithelium and loss of normal cellular arrangements in the seminiferous tubules. Owing to a special survival mechanism, the testis has an amazing ability to completely recover spermatogenic capability some time after the insult (Johnson et al. 1997). Elevated testicular temperature and endocrine disruption are probably the most common causes of mild testicular degeneration resulting in the production of abnormal sperm in the absence of any other clinical signs (Brito 2007). Causes of increased testicular temperature include high ambient temperature, fever, intensive exercise, scrotal edema or dermatitis, hydrocele, tunic adhesions, and hernias. Idiopathic testicular degeneration usually affects older stallions and produces detectable changes on testicular size and consistency (Turner 2002). The condition is often progressive and may result in infertility (Card 2010). Treatment of testicular

degeneration is usually unrewarding. Inciting causes that contribute to the condition should be diagnosed and treated appropriately (Murchie 2005). In most cases changes in management of these stallions are the only hope of improving their fertility. The stallion's health and comfort should be optimized. His book may be biased with young and foaling mares to increase his chance of success (Card 2010). ***Testicular trauma*** as a result of a kick from a mare may cause orchitis, periorchitis, hematoma, testicular rupture, scrotal lacerations, infections, abcessation, etc. Diagnosis is based on physical and ultrasonographic examination of the scrotal contents and treatment should be begun accordingly. Trauma and its consequences significantly disrupt normal thermoregulatory mechanisms and usually result in some degree of testicular degeneration. ***Testicular neoplasia*** include: teratoma, Leydig-cell tumors, Sertoli-cell tumors, embryonal carcinoma, and teratocarcinoma. Stallions with testicular neoplasia usually present with a painless enlargement of the affected testis. Definitive diagnosis can only be made on histological evaluation. If testicular neoplasia is strongly suspected, removal of the affected scrotal testis and its cord is the treatment of choice ((Murchie 2005).

Scrotal pathology usually has a negative impact on spermatogenesis due to the adverse effects on thermoregulation. Scrotal abnormalities such as hydrocele, hernia and torsion of the spermatic cord are relatively common causes of fertility problems in stallions. ***Hydrocele*** may be caused by trauma, inflammation, varicocele, hernia, enlarged internal inguinal rings, high ambient temperature or may be idiopathic. Diagnosis is based on palpation and ultrasonographic examination of the scrotal contents. Slight hydrocele does not significantly affect testicular function and depending on the initiating cause may be treated conservatively. Castration with resection of the vaginal cavity has been suggested as a therapy in severe cases. ***Scrotal hernias*** may be congenital or acquired and are usually associated with large inguinal rings (De Vries 1993, Murchie 2005). Surgical reduction of the entrapped intestinal content is indicated in all cases. Less than 180 degrees ***torsion of the spermatic cord*** does not cause any clinical signs and does not appear to have a significant effect on fertility. Greater then 180 degree torsion causes vascular occlusion which results in serious testicular compromise, scrotal edema and acute clinical signs of colic. Surgical correction is indicated in all cases (Murchie 2005).

Young stallions may also have **poor intrinsic fertility**, which may be generally identified during breeding soundness examination. These stallions have no history of illness or trauma. Poor intrinsic fertility is usually constitutive and reflects an individual stallion's intrinsic genetic ability (Card 2005). Characteristics include:

small soft testis, small total scrotal width < 7 cm, low testicular volume, poor spermatozoa morphology and low motility. They may have a specific morphologic defect that is present in a high percentage of sperm (Card 2010). Intrinsic factors which influence spermatogenesis result in a spermiogram with only seasonal fluctuations, and the features of the spermiogram do not change or improve substantially over time.

Similar to other **endocrine**-regulated tissues, the testis is subject to a hierarchy of controls. An alteration in the intracellular system during development or in adult life may be responsible for some cases of idiopathic subfertility or infertility in the stallion (Roser 2008). No difference was detected in intra-testicular concentrations of estradiol, estrogen conjugates and testosterone among the fertile and idiopathic subfertile and infertile groups of stallions, however levels of inhibin tended to be lower in subfertile stallions and significantly lower in infertile stallions, suggesting that intra-testicular INH may be a good marker for the detection of an early decline in fertility (Roser 1995, Roser 2007). Plasma concentrations of INH, estradiol and estrogen conjugates, but not testosterone were significantly lower, and gonadotropins were significantly higher in the idiopathic infertile stallions. Between fertile and idiopathic subfertile stallions no changes in plasma levels of these hormones were observed (Stewart and Roser 1998, Roser 2008). Decreases in concentrations of inhibin have been noted in stallions early in the course of testicular degenaration, prior to detectable changes in steroid hormones (Ball 2005). There is some evidence to suggest that measurement of IGF-1 in seminal plasma may be an indicator of fertility (Macpherson et al. 2002).. Endocrine therapy in subfertile stallions has received considerable attention over the years. However studies on the use of GnRH or GH therapy have not shown reliable benefits and improved seminal parameters (Ball 2005).

3. OBJECTIVES OF THE DISSERTATION

The objectives of the dissertation were: (1) to improve, validate and adapt the complex staining technique which evaluates sperm head and tail membrane integrity, acrosomal status and morphology for stallion spermatozoa, (2) to use this valuable method to evaluate sperm quality during and after two prominent sperm manipulation procedures (cryopreservation and sperm separation) and (3) to apply the technique to define detectable anomalies of semen from stallions with reduced fertility; consequently draw an attention to the complexity and wide range of use of the staining technique in laboratory experiments as well as in quality control or determination of possible fertility potential of the fresh or processed equine sperm.

Objectives of the four studies:

1. After freezing and thawing, a high proportion of spermatozoa with unstained heads and stained tails are observed. As these cells are considered immotile (Nagy et al. 1999), unambiguous differentiation of intact and damaged sperm tail membrane is important for evaluating semen quality. Using the standard TB/Giemsa method for staining stallion sperm, differentiation of intact or damaged sperm tails was problematic, mainly in frozen/thawed samples. The aim of the **Experiment 1** was to improve this complex method using another viability stain, Chicago sky blue 6B (CSB) which molecule is very similar to TB and optimizing each steps of the staining procedure to distinguish more accurately the different cell types, especially in stallion sperm.

2. The ratio of intact, viable spermatozoa is the most important parameter of the quality of frozen semen. However, for the further development of cryopreservation technologies, determination of freezability of individual stallions and usability of frozen semen, also important to define accurately the localization of cell injury during the cryopreservation process for which, each part of the sperm need to be assessed. The objective of the **Experiment 2** was to analyse of the injuries of stallion spermatozoa during the whole freezing procedure with evaluation of viability (membrane integrity of the subdomains of sperm), acrosomal status, and morphology of stallion spermatozoa using TB - Giemsa or the modified CSB - Giemsa complex staining.

3. Standard sperm separation methods are not always effective with low numbers of total and viable sperm. The aim of **Experiment 3** was to compare mini-Percoll and

swim up method alone and combination with PX and HA supplementation for frozen equine sperm separation to determine the most effective technique in the special case of poor quality semen when small volume and low numbers of sperm is available. Sperm recovery rate; membrane integrity of sperm head, tail and acrosome and morphology were evaluated after the treatments. We tried to find the answer whether PX or HA may improve the yield and has any effect on the proportion of different cell types of the separated spermatozoa.

4. The aim of the study in **Experiment 4** was to compare semen samples of stallions with reduced fertility to samples of proven fertile stallions based on evaluation of both membrane integrity and morphology using the complex viability and acrosome staining method. We investigated also whether some alterations would be detected either in viability or morphology evaluation in the “subfertile’ stallion samples related to their decreased fertilization potency. In case reports clinical assessments of semen samples and their diagnostic relevance and also possible solutions to the problems and recommendations are discussed.

4. MATERIALS AND METHODS

- The studies of **Experiment 1, 2, 4** were performed between 2001-2008 in Hungary in co-operation with some Equine Artificial Insemination Stations in the country and in the spermatologic laboratory of Research Institute of Animal Breeding and Nutrition, Herceghalom. **Experiment 3** was investigated in 2004 at the Colorado State University, Department of Biomedical Sciences, Animal Reproduction and Biotechnology Laboratory, Fort Collins, Co. USA. Fresh, diluted, centrifuged, chilled/stored, frozen or separated semen samples from forty stallions (36 animals in the main experiments, and altogether 40 in the main and preliminary studies) of wide range of different breeds were analysed during the whole PhD work.

4.1 Semen samples, main procedures and experimental design

Experiment 1. Improvement of assessment of stallion sperm quality by Chicago sky blue and Giemsa viability and acrosome staining method

The aim of the study reported here was to improve Kovács-Foote staining method to distinguish different cell types better, especially in stallion sperm. We searched for other supravital stains in the "acid disazo dye" group with the aim of finding a dye with more affinity for the proteins of the tail of membrane-permeable stallion spermatozoa. We selected Chicago sky blue 6B (CSB) to compare to trypan blue (TB). The chemical structure of CSB and TB are similar (Fig. 3). The water soluble biological stain CSB has been also sold as Niagara blue 6B, pontamine sky blue, and direct blue 1 (Lillie et al. 1977) and has been widely applied in biological science.

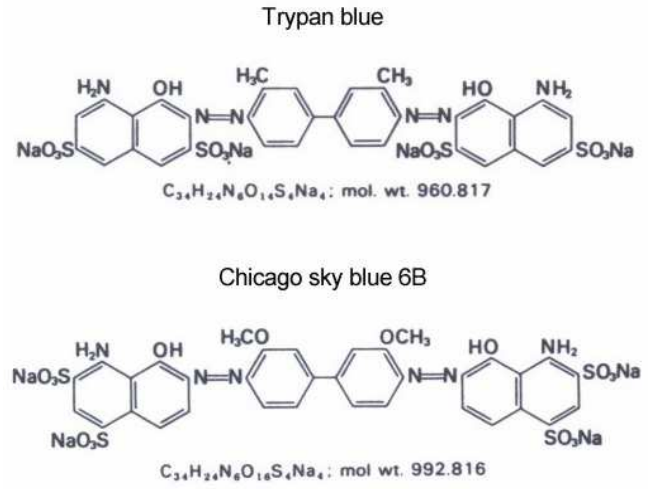


Figure 3. Chemical structures of trypan blue and Chicago sky blue 6B (Lillie 1977)

- In a preliminary study, I evaluated different fixation (2, 3, 4, 6 min) and Giemsa staining times (1-4 h, and overnight) following TB and CSB staining. Three different concentrations of CSB (0.26%, 0.16%, 0.13%) also were tested to choose the best combination of the parameters of the staining's steps.

- In the main experiment altogether thirty semen samples: fresh, diluted, centrifuged or frozen and thawed from 10 stallions were used for repeatability and methodology comparisons. Smears were made from each of the 30 samples to compare the live/dead ratio for smears stained by 0.16% CSB and 0.27% TB for evaluating the toxicity of CSB. Twenty samples from 15 stallions were used for densitometry.

Experiment 2. Analysis of the injuries of stallion spermatozoa during the whole freezing procedure

- In the second experiment 10 fertile stallions were involved. All of the stallions were used as a breeding stallion for artificial insemination. Three-four ejaculates were frozen from 10 stallions (n=33), the collection dates performed randomly throughout between the years 2001-2004. Semen of Stallion 9 was used for more additional freezing (altogether 17 collecting days between Sept. 2003 – Jan. 2004).

Table 5. Semen collection dates	
07.09.2001	18.02.2003
19.09.2001	24.06.2003
01.10.2001	25.06.2003
20.10.2001	02.09.2003
19.11.2001	05.09.2003
22.11.2001	13.09.2003
23.11.2001	14.09.2003
12.12.2002	16.09.2003
18.12.2002	26.10.2003
20.12.2002	08.11.2003
15.01.2003	15.11.2003
07.02.2003	14.02.2004
10.02.2003	19.02.2004
12.02.2003	25.03.2004

Table 6. Stallions
Stallion 1: 10-year-old Dutch Warmblood
Stallion 2: 19-year-old Holsteiner
Stallion 3: 8-year-old Swedish Warmblood
Stallion 4: 20-year-old Hungarian Sport horse
Stallion 5: 14-year-old Quarter horse
Stallion 6: 9-year-old Holsteiner
Stallion 7: 16-year-old Arabian
Stallion 8: 5-year-old Arabian
Stallion 9: 11-year-old Arabian
Stallion 10: 14-year-old Lipizzaner

- **Freezing procedure**

Freezing process followed the advised protocol of Vidament et al. (2000) using modified INRA 82 extenders (Vidament et al 2000, 2001; Table 7). After collection, the gel-free fraction of the ejaculate was diluted in centrifugation extender (E1): (INRA82 + centrifuged egg yolk, 2%, v/v) in v:v 1:2 or 1:3 rates at 37 °C. After cooling to room temperature diluted semen was centrifuged for 10 min at 600xg in a 50 ml conical centrifugation tube. After centrifugation sperm pellets were resuspended in INRA 82 extender containing 2% egg yolk and 2,5 % glycerol (E2) to obtain 100×10^6 spermatozoa/ml at room temperature (22 °C). Semen was equilibrated for 60 min from 22°C to 4°C with -0.3°C/min cooling rate, then an additional 60 min at 4°C before freezing. Semen was packaged in 0.5 ml straws and freezing was performed by keeping 0.5 ml straws at 4 cm above liquid nitrogen for 10 min then plunging the straws in liquid nitrogen. Thawing of 0.5 straws was done at 37 °C for 30 sec using a waterbath.

Table 7. Composition of the basic extender (INRA 82) with 20 mMoles Hepes (Vidament et al. 2000)

Components		**1 liter**
Modified HF20		
	Glucose, anhydrous	25 g
	Lactose, 1 H_2O	1.5 g
	Raffinose, 5 H_2O	1.5 g
	Citrate Na, 2 H_2O	0.25 g
	Citrate K, 1 H_2O	0.41 g
	Hepes	4.76 g
	Water (apyrogenic)	QSP 0.5 L
Skim milk UHT		0.5 L
Gentamicin sulfate		50 mg
Penicillin G		50 000 UI
pH		6.8
mOsm/kg		*310*

- **Evaluation**

Viability, acrosome status and morphology of the fresh, centrifuged, and frozen-thawed spermatozoa were evaluated by TB/Giemsa or CSB/Giemsa staining using ***light microscopy***.

Scanning and transmission electronmicroscopic investigations were carried out as well from sperm of Stallion 9.

Experiment 3. Use of pentoxifylline and hyaluronic acid for stallion sperm separation

- In the third experiment poor to medium quality frozen semen (cryopreserved in 0.5-ml straws in 200 x 10^6/ml sperm concentration, using EZ-Freezin-LE extender, progressive motility after thawing ≤ 30%) from 3 stallions was used, 3 replicates each. Two straws were thawed at 38 °C for 30 minutes and mixed. From this sperm suspension one hundred µl semen was allocated to each of 7 separation treatments.

- **Sperm separation**

Mini-Percoll: Three aliquots (100 µl) were incubated at 38°C in an atmosphere of 5% CO_2 for 20 min in 0.25 ml Hepes-buffered chemically defined handling medium (HCDM) (P-NT: non-treated; P-PX: 3.5 mM PX (P 1784 Sigma, St Louis, MO); or P-HA: 1 mg/ml HA final concentration) before Percoll®-centrifugation and one aliquot was centrifuged through Percoll® without incubation (P-CON). Composition of HCDM medium is described in the Tables of Appendix.
Our mini-Percoll discontinuous density gradients were prepared by carefully layering 0.4 ml of 90%, and 0.5 ml of 45% Percoll® solutions in a 1.5-ml microcentrifuge tube starting with the highest-density solution at the bottom. Incubated or not-incubated (P-CON) sperm was layered on top of the Percoll® gradients and centrifuged at 600 x g for 5 min. Then a 30-µl pellet was aspirated from the bottom and washed in 1 ml HCDM at 300 x g for 5 min.

Swim up: At the same time 3 aliquots of 100 µl sperm were placed in 1 ml HCDM - without or with supplementation - for swim-up (SU-NT: non-treated; SU-PX: 3.5 mM PX; or SU-HA: 1 mg/ml HA final concentration) at 38°C in an atmosphere of 5% CO_2 for 30 min in a 5 ml round-bottomed tube. After incubation 0.65 ml supernatant was collected and centrifuged in 1 ml HCDM at 300 x g for 5 min.

The experimental design of sperm separation treatments is shown in Fig. 4.

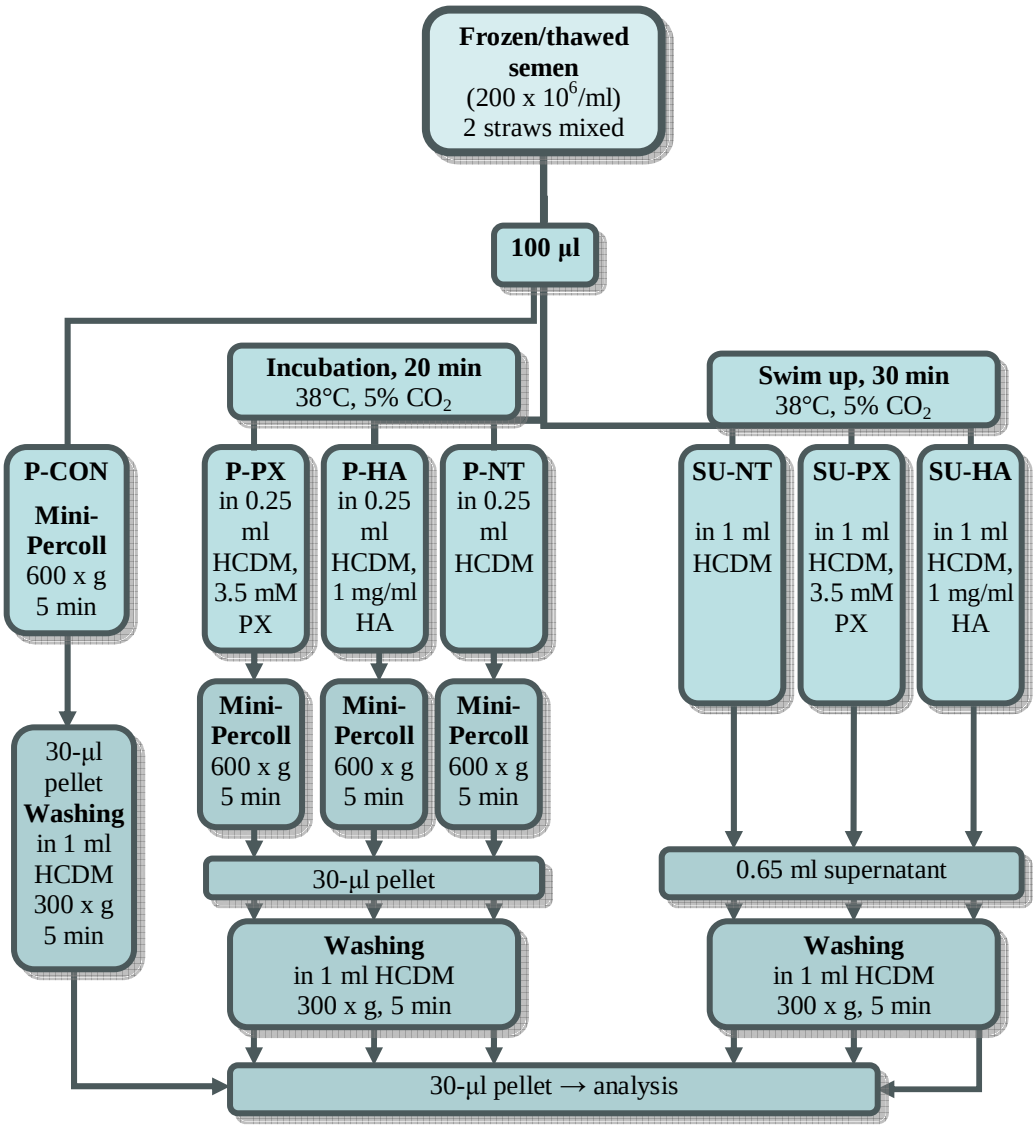


Figure 4. Experimental design of the sperm separartion treatments

- **Evaluation**

In every treatment, the final 30-µl pellet after washing aspirated from the bottom was analysed. For evaluating sperm concentrations 5 µl samples were taken from each final sperm suspension after treatment then diluted in 95 µl distilled water. Concentration was determined using a hemacytometer. ***Recovery rate*** was calculated as a percentage of original concentration (200 million/ml) of the frozen semen. ***Sperm head, tail and acrosome membrane integrity and morphology*** were evaluated with ***CSB-Giemsa staining***. Equal drops (10 µl) of viability stain and undiluted sperm pellet remained after treatments were mixed on a slide and made two thin smears. The

smears were air dried nearly vertically at room temperature, fixed, then stained in Giemsa solution. The slides were cover slipped and evaluated at 1000 X magnification with immersion objective counting 300 sperm per sample to determine the percentage of different cell types.

Experiment 4. Viability, acrosome integrity and morphology evaluation of sperm samples from subfertile stallions

- In the forth experiment, semen samples of 14 fertile and 10 subfertile stallions were analysed. Stained smears were prepared from fresh ejaculates of 10 fertile and 10 subfertile stallions and from chilled semen of 5 fertile and 4 subfertile stallions after 1 day storage at 4°C. Non Fat Dry Skim Milk (NFDSM)-Glucose Extender derived from the formula provided by Kenney in 1975 was used as a diluent for cooled storage of semen. In the case of one subfertile stallion (Stallion „G") the regression of sperm characteristics were analysed at four different times during 1 day chilled storage (0, 10, 18 and 24 hours after collection). The effect of two different extenders on the sperm quality of Stallion „H" was studied after 24 and 48 hours chilled storage.
Ages and breeds of the stallions were different (Table 8-9). All of the stallions were used as a breeding stallion at different Breeding Stations in Hungary. Stallions were categorized as "fertile" or "subfertile" by veterinarians of Breeding Stations based on the rates of pregnant or non-pregnant mares inseminated with sperm of the given stallion during the breeding season.

Table 8. Fertile stallions
Stallion 1: 20-year-old Hungarian Sport horse
Stallion 2: 19-year-old Holsteiner
Stallion 3: 6-year-old Hanoverian
Stallion 4: 5-year-old Arabian
Stallion 5: 8-year-old Swedish Warmblood
Stallion 6: 10-year-old Dutch Warmblood
Stallion 7: 9-year-old Dutch Warmblood
Stallion 8: 10-year-old Hungarian Sport horse
Stallion 9: 16-year-old Hanoverian
Stallion 10: 14-year-old Quarter horse
Stallion 11: 11-year-old Trotter horse
Stallion 12: 10-year-old Dutch Warmblood / same as Stallion 6
Stallion 13: 14-year-old Dutch Warmblood
Stallion 14: 6-year-old Hungarian Sport horse
Stallion 15: 9-year-old Trotter horse

Table 9.	Subfertile stallions
Stallion „A”:	11-year-old Nonius
Stallion „B”:	9-year-old Belgian coldblood
Stallion „C”:	22-year-old Shagya-Arabian
Stallion „D”:	22-year-old Thoroughbred
Stallion „E”:	9-year-old Shagya-Arabian
Stallion „F”:	24-year-old Thoroughbred
Stallion „G”:	10-year-old Trotter horse
Stallion „H”:	5-year-old Arabian
Stallion „I”:	13-year-old Trotter horse
Stallion „J”:	16-year-old Trotter horse

Additionally in some stallions with lower fertility, one or more parameters of the sperm were altered compared to the acceptable values during routine examinations. For example reduced motility, irregular movement, low sperm concentration in the raw semen or low pregnancy results after using chilled-transported semen were observed. Ejaculates of each stallion from more collecting days were evaluated.

- **Quantitative and motility measurments of the semen samples**

The gel free volume of the ejaculate and sperm concentration were determined after semen collection using a Bürker chamber, Makler chamber or spectrofotometric method; motility analyses were performed by subjective evaluation or using Minitüb CASA system depending on the generally used technique in the different breeding stations.

- **Evaluation**

Membrane integrity and morphology of the sperm subdomains were analysed by the complex viability and acrosome staining method.

4.2 Sperm evaluation method

Viability staining, fixing and acrosome staining

Dilution of the semen samples: Stallion spermatozoa are sensitive to pH, osmolality and temperature changes. Therefore, phosphate-buffered saline (PBS) containing 0.06% K_2HPO_4 anhydrate and 0.825% NaCl (pH: ~ 6.8) was used for semen dilution at the same temperature as the sperm specimen was. Dilution rate depends on the status of the semen samples, spermatozoa concentration and extender type. For example

sperm concentration in the pellet after separation (**Experiment 3**) was fairly low (in the range of 1-62 million/ml at the various treatment and stallions), therefore to count enough sperm for the correct evaluation after further dilution with PBS was almost impossible in many cases. However proteins of seminal plasma and extender or glycerol did not disturb the staining with their binding affinity to the viability dye, the solutions were clean enough to use them without additional dilution for making smear. Fresh sperm and cooled semen - diluted in 1:1 or 1:2 rate with NFDSM-Glucose or egg-yolk-skim-milk-based (EY-SM) extender-, was diluted 1:4 with PBS before viability staining. Both the centrifuged and frozen/thawed semen processed at a final concentration of 100-200 million cells/ml in freezing extender containing egg-yolk and glycerol, diluted 1:9 with PBS just before making smear.

The ***viability test stain*** contained *0.16% Chicago sky blue 6B* (Sigma-Aldrich St. Louis, MO, USA, C-8679). The working solution was made from a 2.6% stock solution in distilled water -which is isotonic-, diluted 1:15 with PBS or *0.27% trypan blue* working solution was prepared from 0.4% stock solution (Sigma T-8154) diluted 2:1 with PBS. Both staining solutions are isotonic, have neutral pH, and are stable for a year in eye-drop bottles at room temperature. In the **Experiment 1** the two viability stains were compared. Semen samples in the **Experiment 2** and **4** were collected partly during that period when the works on improving and validation of the modified complex staining method were being performed. Therefore in some cases only smears stained by TB were available and from the most sperm samples, smears stained with both viability dyes were made because these specimens were also used for the densitometry and methodology comparative study. In these cases I used the CSB-stained samples for the two experiments mentioned above. In the **Experiment 3** semen samples stained by CSB were analysed.
The ***fixative*** was composed of 86 ml 1 N HCl plus 14 ml of 37% formaldehyde solution and 0.2 g neutral red (Sigma N2880); it is stable for a year at room temperature and may be used repeatedly. The ***acrosome stain*** was 7.5% Giemsa stock solution (Sigma GS-500) in distilled water prepared freshly before use.

Staining procedure

Equal drops (20 µl) of viability stain and diluted semen were mixed gently on a slide flatly with the corner of another slide without scratching and touching the glass surface. Liquid layer is formed between the two slides and the droplets get mixed up with a slightly movement of the slides. The slides were attached parallel to each other and pulled to make two smears. The smears were air dried nearly vertically at room temperature. After drying, slides were fixed for 4 min. Both sides of the slides were

rinsed with tap water and distilled water, then stained in Giemsa solution in an uncovered staining jar (not more than 14 slides per jar with 16 spaces) at 25-40° C for 2-4 hrs. Slides were rinsed with tap water, then differentiated in distilled water for 2 min, air dried in a nearly vertical position, and cover slipped with Entellan (Merck 1.07960, Darmstadt, Germany). Slides were evaluated at 1000 X magnification using oil-immersion objective and a yellow filter for better live/dead differentiation.

Viability evaluation

- Three hundred cells were counted on each slide and classified into ***five categories*** (Fig. 5) in **Experiment 1** and **Experiment 3.**

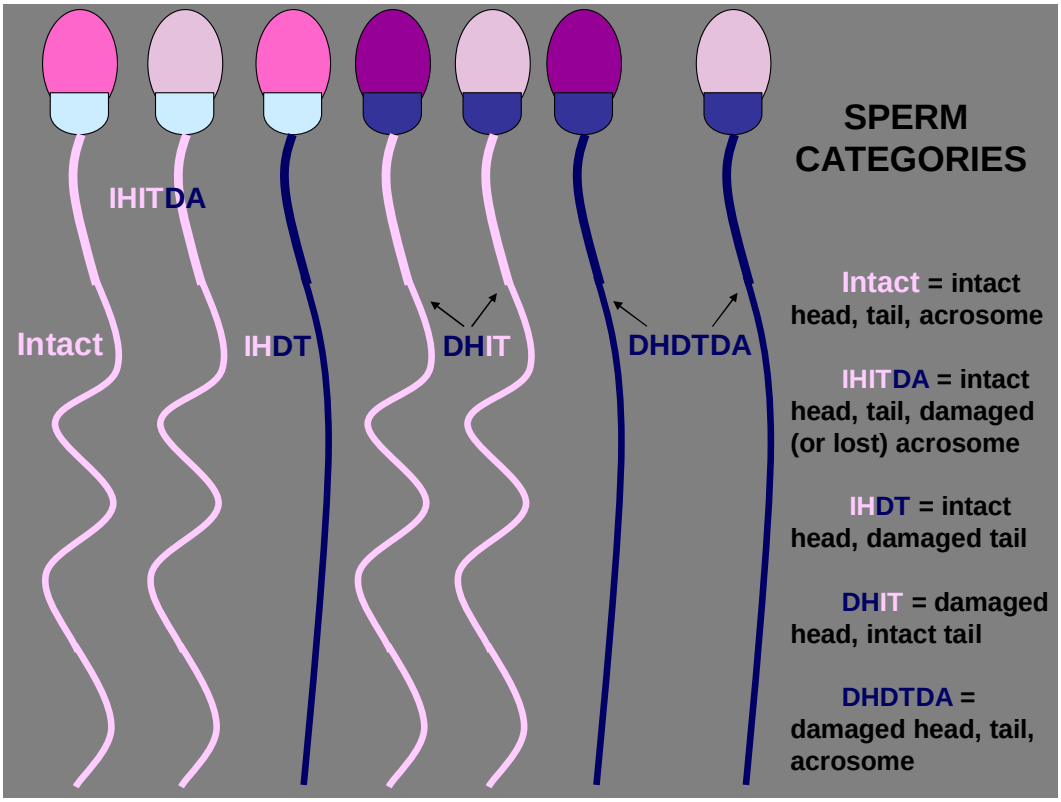


Figure 5. Different sperm categories classified for the viability evaluation in Experiment 1 and 3

- ***Eight sperm categories*** were classified ***based on membrane integrity combined morphology*** (Figures 6-7) for the light microscopy examination **in Experiment 2 and Experiment 4.** Two-three hundred cells were evaluated in each sample.

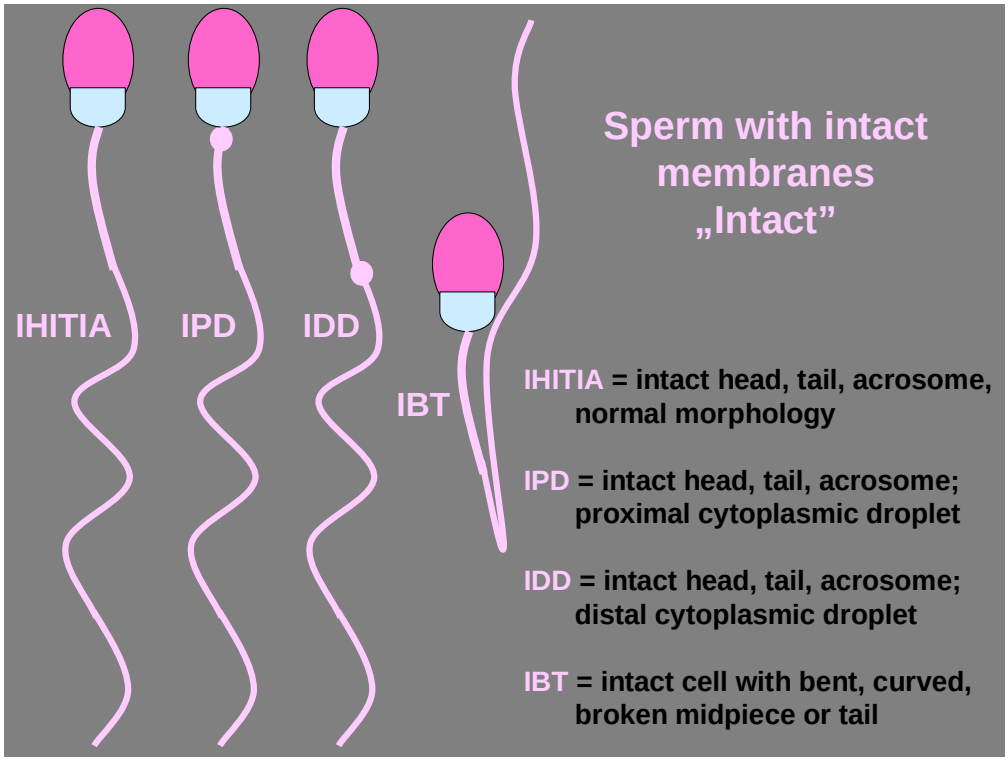


Figure 6. Spermatozoa categories with intact membranes

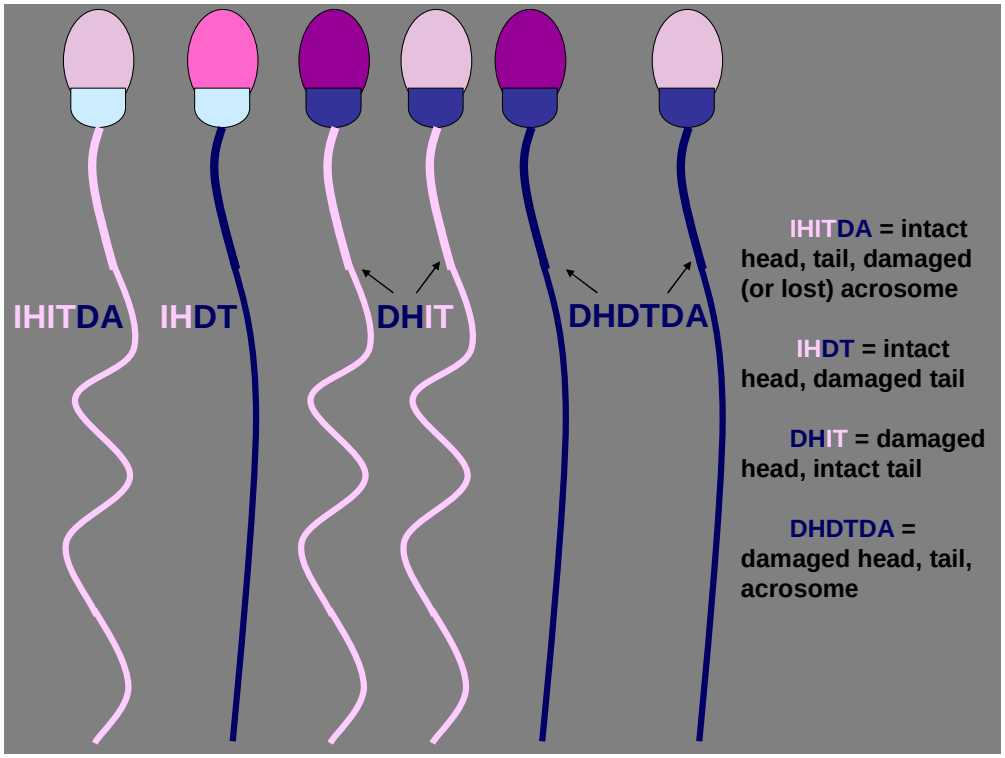


Figure 7. Sperm types with damaged membrane of any part of the cell

- In the statistical analysis of **Experiment 2** additional and combined categories were also evaluated:

1. Damaged spermatozoa with CD [**DCD**]
2. Damaged) sperm with bent, curved, broken midpiece or tail [**DBT**]
3. All cells with intact membranes [IHITA + IPD + IDD + IBT] **Intact**
4. Intact sperm with CD + Intact sperm with bent tail [IPD + IDD + IBT] **ICDBT**
5. All (intact and damaged) spermatozoa with CD [IPD + IDD + DCD] **IDCD**
6. All (intact and damaged) sperm with bent, curved, broken midpiece or tail [IBT + DBT] **IDBT**
7. All (intact and damaged) spermatozoa with CD or BT [IDCD + IDBT] **IDCDBT**

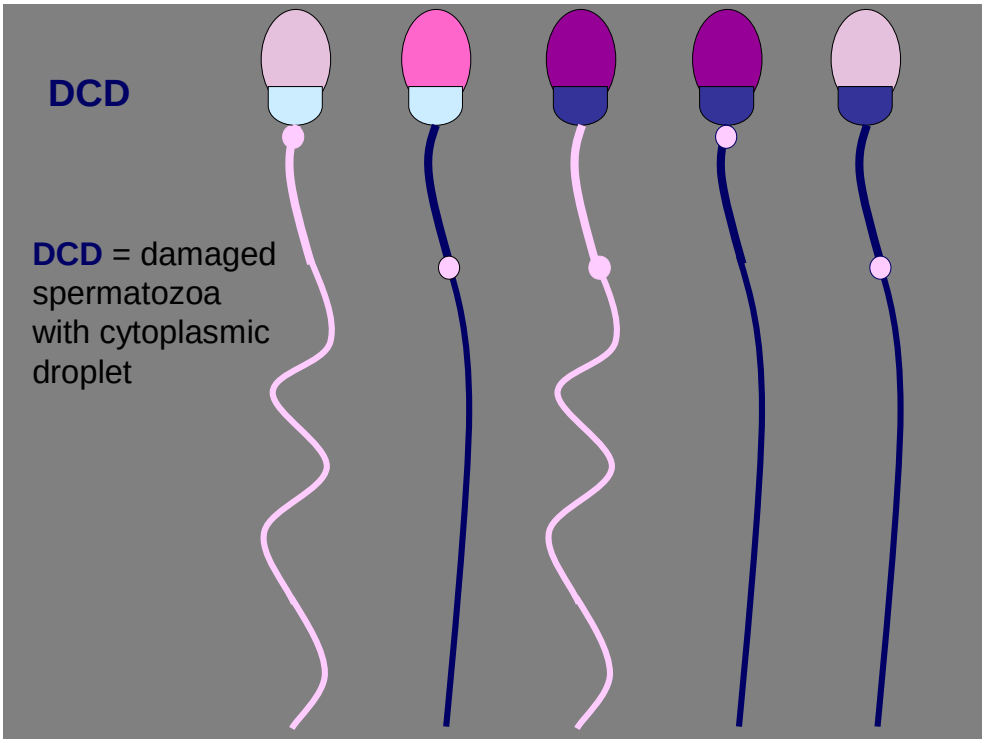


Figure 8. Damaged spermatozoa with cytoplasmic droplet

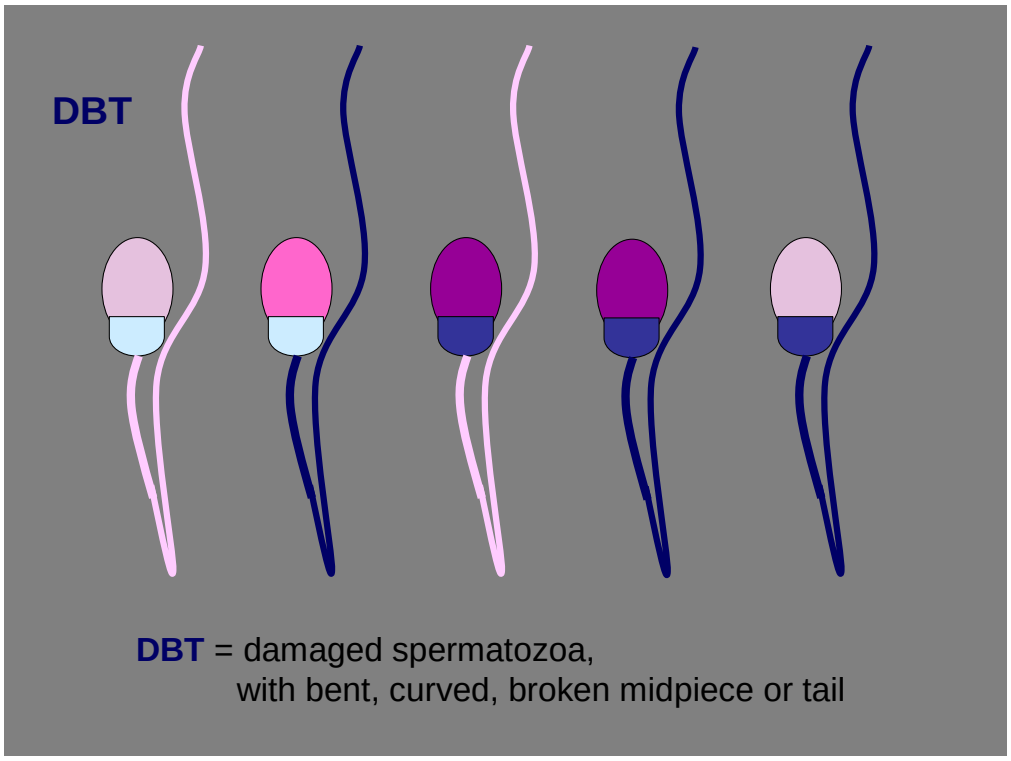


Figure 9. Damaged spermatozoa, with bent, curved, broken midpiece or tail

Different sperm types are shown in Fig. 10:

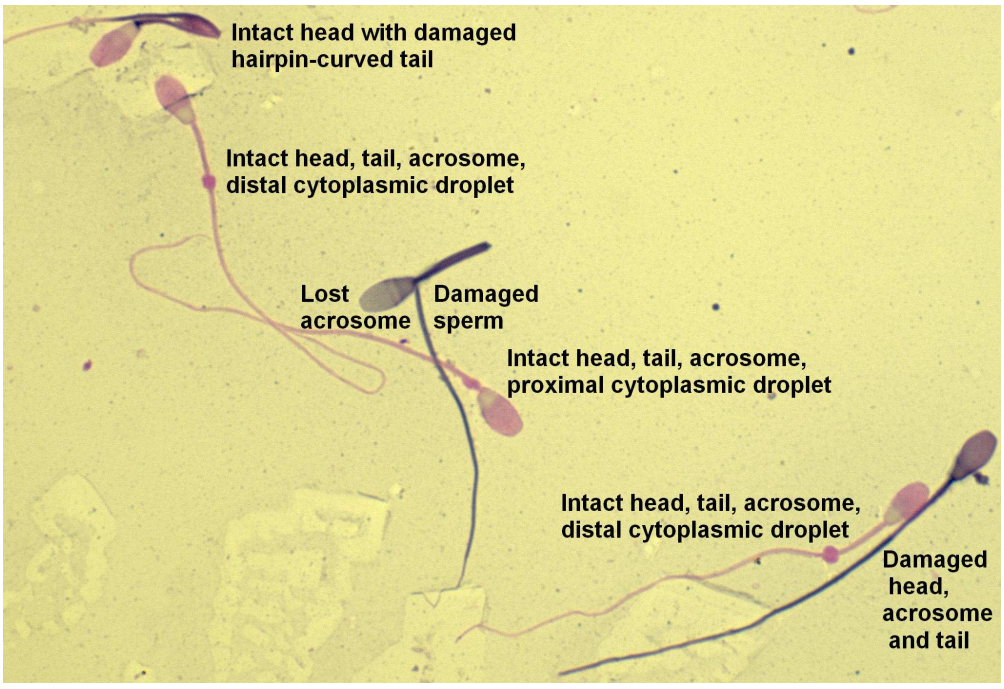


Figure 10. Microscopic picture of different sperm types (CSB/Giemsa staining)

Morphological evaluation

- Three hundred cells were classified in ***5 simple morphological categories*** in **Experiment 3**:

1. **normal**
2. **proximal cytoplasmic droplets (*PD*)**
3. **distal cytoplasmic droplets (*DD*)**
4. **midpiece and tail defect (*midp+tail*)**
5. **abnormal head (*head*)**

- Cells were classified in ***9 morphologic categories*** in **Experiment 4**:

1. **Normal morphology (*normal*)**
2. **Head abnormalities (*head*)** (microcephal, macrocephal, tapered, pyriform, nuclear vacuoles, acrosome defects)
3. **Midpiece defect (*midp*)** (swollen, bent, DMR, mitochondrial sheath defect, corkscrew, bowed)
4. **Tail abnormalities (*tail*)** (broken, bent, hairpin-curved, distal coiled tail)
5. **Coiled tail defect (*coiled*)** (tightly coiled tail, dag-like defect)
6. **Detached head (*detached*)**
7. **Proximal cytoplasmic droplet (*PD*)**
8. **Distal cytoplasmic droplet (*DD*)**
9. **Multiple forms (*multiple*)** (e.g. double midpiece, head, tail)

The standard counting and classifying system was used where a sperm cell was placed in one class of abnormality only (the most primary one) and identified the percentage of each of nine sperm categories. However increased proportion of sperm with multiple defects is also noted during the evaluation. Both live and dead spermatozoa were assessed for morphology during the count. Three hundred cells were evaluated in each sample to determine the percentage of different cell types. Classification of sperm abnormalities was based on previous publications and guidelines (Barth and Oko 1989, Kenney et al. 1990, Card 2005, Brito 2007, Varner 2008). Acrosome defects (knobbed, vacuoles, detached) were not enumerated separately from head defect here, however counted into distinct category among live cells (IHITDA) during viability evaluation. Distal midpiece reflex (DMR) defect was classified in midpiece defect according to Card (2005) and Brito (2007). Coiled tail defect (tightly coiled tail, dag-like defect) was enumerated in separated category from other tail defects because this can be caused by disturbance of spermatogenesis. Different sperm types are shown in the microscopic pictures in the Appendix.

4.3 Data analyses, statistical methods

Experiment 1. Repeatability and method agreement analyses

The Bland-Altman statistical method (Bland and Altman 1986, Nagy et al. 2003a,b) was used to assess agreement between the CSB and TB stains. To investigate the repeatability and agreement of CSB/Giemsa and TB/Giemsa staining, I used altogether 30 semen samples from 10 stallions: four raw ejaculates, 5 fresh-diluted with NFDSM-Glucose extender, 5 fresh-diluted with EY-SM extender, 16 semen samples were centrifuged and diluted with an egg yolk + glycerol extender (7 after dilution, 9 after frozen/thawed were evaluated). A smear was made using each viability stain and two measurements were obtained for each method. Three hundred cells were counted on each slide and classified into five categories: intact head, intact tail and acrosome membrane (Intact); intact head, tail, damaged acrosome (IHITDA); intact head, damaged tail (IHDT); damaged head, intact tail (DHIT); and damaged head, damaged tail, damaged acrosome (DHDTDA) (Fig. 5).

The analysis was calculated with the "intact" and the categories of the IHDT and DHDTDA merged, counting all damaged tails (DT). These two groups of cells clearly demonstrated the stained and unstained spermatozoa and were appropriate for comparing repeated measurements and the two different staining procedures. Statistical analyses of repeatability and agreement were accomplished using Microsoft Excel 2000 software (Microsoft, Redmond, WA). To assess the repeatability of a method, we calculated the differences between pairs of 30 repeated measurements and the mean of these differences (*d*). As a measure of repeatability, the British Standards Institution (BSI) repeatability coefficient (British Standards Institution 1975; Petrie and Watson 1999) was calculated as twice the standard deviation (2 SD) of the differences. The differences between the repeated measurements were plotted against their average (Figs.15-16).

The mean of the differences (*d)* and the BSI repeatability coefficient (± 2 SD) are presented on the diagrams. We expected 95% of differences to be < 2 SD. Method agreement analysis between the two staining protocols was carried out using the same statistical method. The mean of the differences between the paired measurements on the same samples (*d*) was calculated to estimate the average bias of one method compared to the other. The SD of the differences had to be corrected in this case (Bland and Altman 1986).

The corrected SD of differences (corrSD) was $\sqrt{s_D^2 + \frac{1}{4}s_1^2 + \frac{1}{4}s_2^2}$ where S_1 and S_2 were the standard deviation of differences between repeated measurements for each method separately, and S_D was the standard deviation of the differences between the paired means for each method, which was approximately $\sqrt{2s_D^2}$. We used this simplified formula for calculations. The 95% limits of agreement were calculated as *d* ± 2 corrSD (Bland and Altman 1986). The differences between the paired measurements were plotted against their average (Figs. 17-18). The mean of the differences (*d*) and the limits of agreement (± 2 SD) are presented on the diagrams.

Experiment 1. Densitometry (image analyses)

Twenty semen samples: five raw semen, five diluted with NFDSM extender, and 10 frozen using modified INRA82 extender (containing egg yolk and glycerol), from 15 different stallions were smeared and stained in parallel with TB and CSB. After viability staining, the entire procedure was the same according to the method described above. A Leica DM RA2 Microscope with a 100 x immersion objective, Leica Digital camera DC 500 and Leica IM50 1.20 image processing and archiving software were used to archive the photos. The settings were: 8 bit/channel color depth, 2600 x 2060 pixels image size, 16 shots, JPEG bitmap format. The digital images were stored for further analysis. Corel Draw 8.0; Corel Photo-Paint 8 software (Corel Corporation Ottawa, Ontario, Canada) was used for densitometry measurements. The parameters of the images were 440.3 x 348.8 mm (2600 x 2060 pixels), 24-bit RGB color, 150 dpi, JPEG bitmap format. Three digital images from each slide containing intact and “dead” cells stained with CSB or TB were saved to compare the color intensity of the stained vs. unstained tails and heads. The Magic Wand Mask tool of the Corel Photo-Paint 8 software was used to select the tail or head areas. The zoom level was 200%. For analysis of the individual digital images, RGB histograms were drawn for each different area of spermatozoa (Figs. 11 and 12).

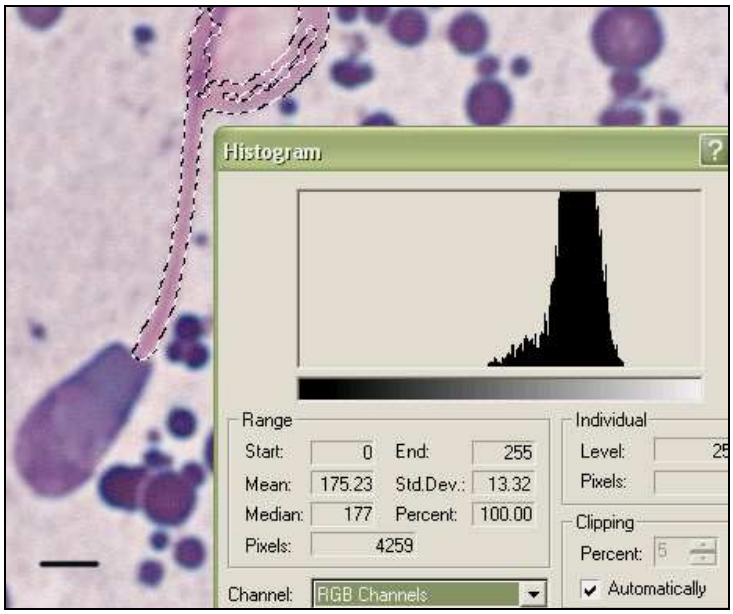


Figure 11. A spermatozoon with damaged head and intact tail membrane. CSB/Giemsa staining. The unstained tail is outlined with dotted lines. The histogram shows the RGB values of the selected area. Bar = 2 µm

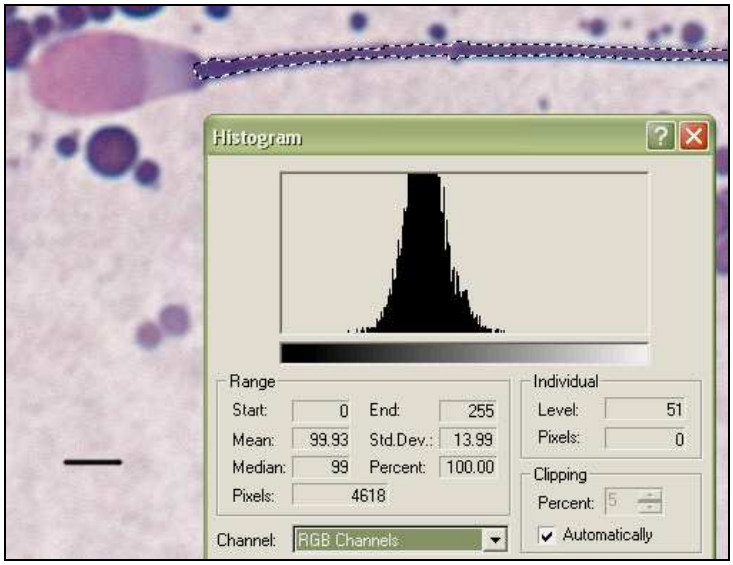


Figure 12. A Spermatozoon with intact head and damaged tail membrane. CSB/Giemsa staining. The stained tail is indicated with dotted lines. The histogram shows the RGB values of the selected area. Bar = 2 µm

The histogram plotted the brightness value of every pixel in the selected area of the image. Values ranged from zero to 255 (from darkest to brightest), and the histogram indicated how many pixels were at each brightness level. Means of the composite RGB values of the selected area on each of the intact or damaged tails (midpiece and principal piece, at least 4000 pixels per selected area measurement) and the stained and unstained heads (without acrosome, at least 1000 pixels/measurement) were registered from each picture. Differences between means of RGB values of live vs. dead tails and separately live vs. dead heads from each photo were used for comparing the two stains. Altogether, 120 photos were measured and 480 histograms of the total RGB value were made from the different areas. After evaluating the data for normality, the paired two-tailed T-test (SPSS 11.0. statistical analysis program, SPSS Inc. Chicago, IL) was used to compare the RGB differences between the stained and unstained tails or heads for CSB and TB staining.

In Experiment 2 paired T test statistical analysis was performed to compare the mean values of the percentages of different sperm categories in fresh, centrifuged and frozen samples using „R" software.

In Experiment 3 data (recovery rates and percentages of different cell types in the selected sperm after the 7 treatments) were arcsin transformed to achieve normality on the data and evaluated by GLM analysis of variance of SAS (SAS Inst. Inc., Cary, NC, USA). Differences among means were tested using Tukey's honestly significant difference (h. s.d.) procedure. In all cases, significance was set at $p < 0.05$ level. Data are presented as Least squares means and standard errors (LS means ± SE).

In Experiment 4 the evaluation of 10 subfertile stallion samples and the discussion with incorporation of previous data and observations from the stallions were interpreted in case reports. Mean values calculated from the results of the viability and morphology evaluations of stallions with good fertility (Fertile stallions), the average values, minimum requirements and the acceptable limits of the different sperm morphologic categories in fertile stallions according to the literature and to the guidelines of Hungarian standard for breeding stallion semen were considered as bases of comparison.

5. RESULTS

5.1 Experiment 1. Improvement of assessment of stallion sperm quality by Chicago sky blue and Giemsa viability and acrosome staining method

Subjective evaluation

In a preliminary study, we evaluated different fixation (2, 3, 4, 6 min) and Giemsa staining times (1-4 h, and overnight) following TB and CSB staining. Three different concentrations of CSB (0.26%, 0.16%, 0.13%) also were tested. Among these, the 0.16% CSB solution proved best, resulting in similar sperm heads, but better tail live/dead differentiation compared to 0.27% TB (Figs. 13-14).

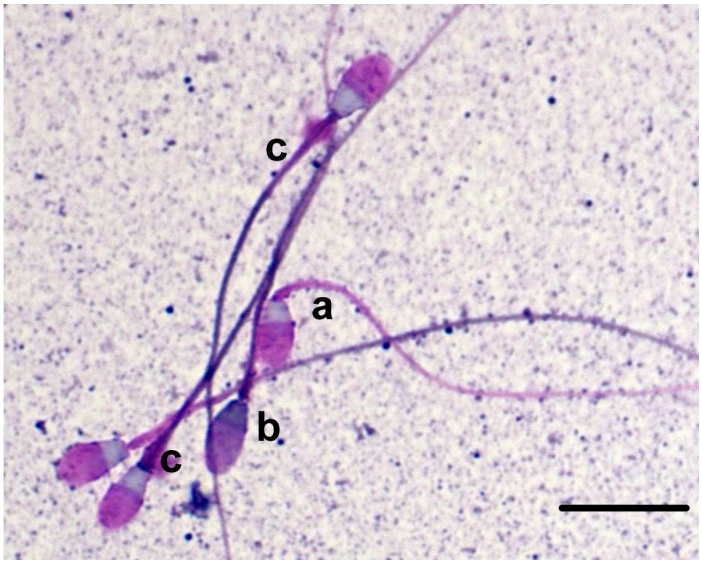


Figure 13. Frozen stallion spermatozoa stained with 0.16% CSB/Giemsa
a) Spermatozoon with intact head, tail and acrosome membrane
b) Spermatozoon with damaged head, tail and acrosome membrane
c) Spermatozoon with intact head and damaged tail membrane
Bar = 10 µm

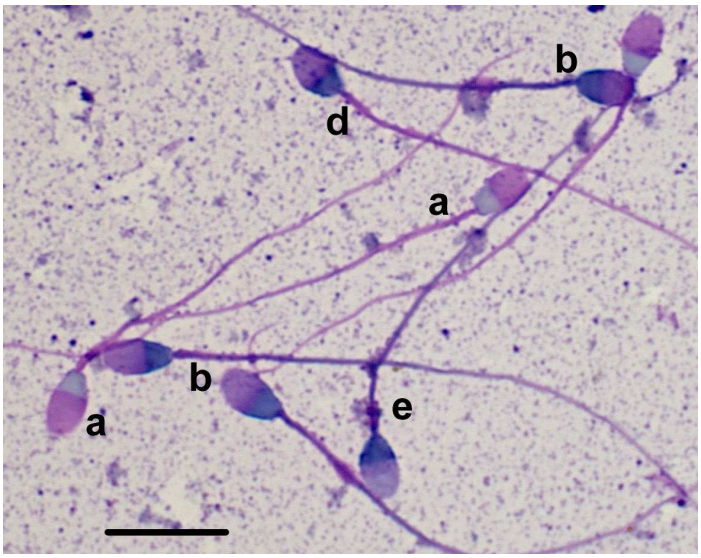


Figure 14. Frozen stallion spermatozoa stained with 0.27% TB/Giemsa
a) Spermatozoon with intact head, tail and acrosome membrane
b) Spermatozoa with damaged head, tail and acrosome membrane
d) Spermatozoon with damaged head and intact tail membrane
e) Spermatozoon with damaged head and tail membrane with no acrosome
Bar = 10 µm

Fixation for 4 min resulted in darker "dead" staining with acceptable background. Giemsa exposure for 2-4 h was sufficient for acrosome staining. The differences we found between CSB and TB viability staining are shown in Table 10.

Acrosome staining was the same for both procedures: intact acrosomes were purple or pink, loose and damaged acrosomes were lavender, and the anterior part of the sperm head with no acrosome was pale gray or pale grayish pink.

Table 10. Differences in CSB and TB staining

	Chicago sky blue	**Trypan blue**
Live heads	white, pale grayish blue	pale blue, pale grayish blue
Live tails	light pink	grayish pink
Dead heads	dark grayish-blue	stronger dark blue
Dead tails	black, stronger dark blue	dark blue

Repeatability and method-agreement analyses

The average difference between the repeated counts by microscopy of the intact cells on smears stained by TB was 0.28% (SD = 2.73%); the BSI repeatability coefficient (2 SD) was 5.47% (Fig. 15). The average difference between the repeated counts by microscopy of the intact cells in smears stained by CSB was -0.77% (SD = 2.83%); 2 SD was 5.67% indicating good repeatability (Fig. 16). The mean of the differences between the intact cells counted on the smears stained by the two viability stains was 0.03% (SD = 2.18%); (P = 0.78).

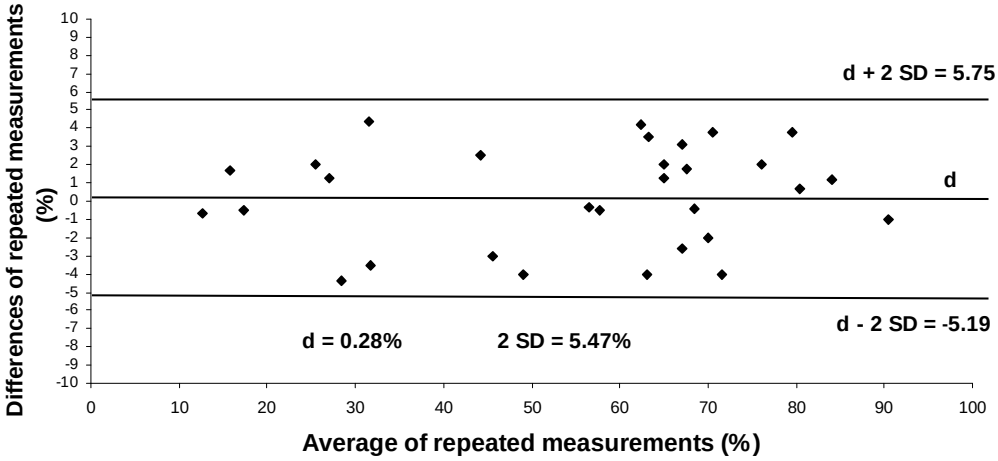


Figure 15. Repeatability of TB/Giemsa staining for counting intact cells on the smears. The differences between the repeated measurements are plotted against their average. The mean of the differences (d) and the British Standards Institution repeatability coefficient (2 SD) are presented (n =30)

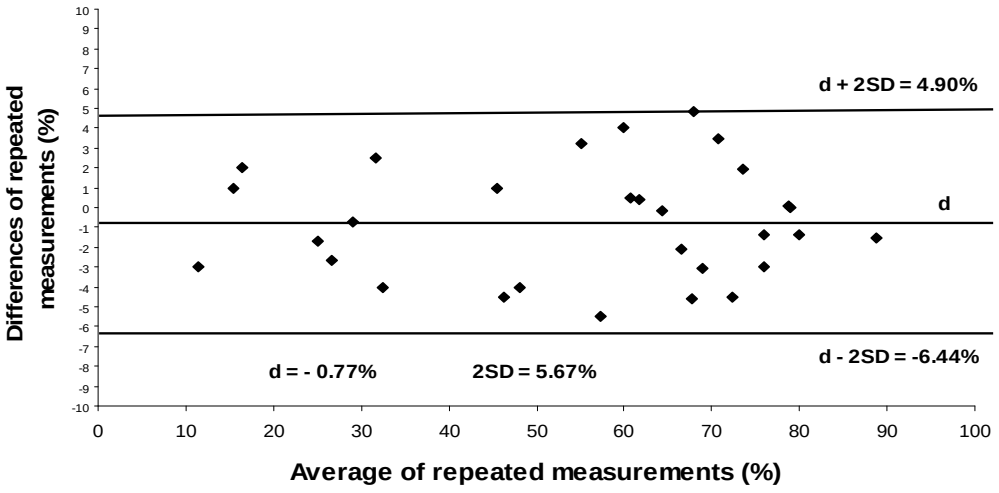


Fig. 16. Repeatability of CSB/Giemsa staining for counting intact cells on smears. The differences between the repeated measurements are plotted against their average. The mean of the differences (d) and the British Standards Institution repeatability coefficient (2 SD) are presented (n = 30)

The corrected SD = $\sqrt{2s_D^2}$ was 3.08% and the corrected repeatability coefficient (2 corrSD) was 6.18%. The 95% limits of agreement (d ± 2 corrSD) were -6.13 and 6.19% (Fig. 17). This interval was small and close to the d ± 2 SD of the repeated measurements of both methods (TB: -5.19, 5.75%; CSB: -6.44, 4.90%).

The average difference between the repeated counts by microscopy of cells with a damaged tail with TB/Giemsa staining was -0.19% (SD = 3.06%) and the BSI repeatability coefficient (2 SD) was 6.11%. The average difference between repeated measurements of the cells with a damaged tail with CSB/Giemsa staining was 0.64% (SD = 2.96%). The BSI repeatability coefficient (2SD) was 5.91%. The mean of the differences between the cells with a damaged tail counted on the smears stained by the two viability stains was -0.09% (SD = 2.40%). The corrected SD was 3.39% and the corrected repeatability coefficient (2 corrSD) was 6.79%. The limits of agreement (-6.88 and 6.70%) were small enough for us to be confident that the new method can be used instead of the old one (Fig. 18).

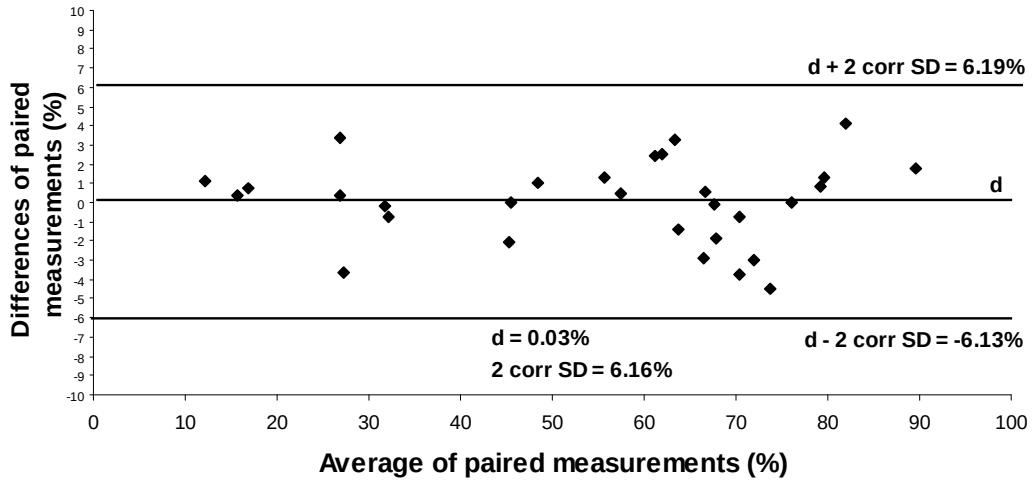


Figure 17. Agreement between the TB/Giemsa and CSB/Giemsa staining methods for counting intact cells on smears. The differences between the paired measurements are plotted against their average. The mean of the differences (d) and the limits of agreement (d ± 2corrSD) are presented (n =30)

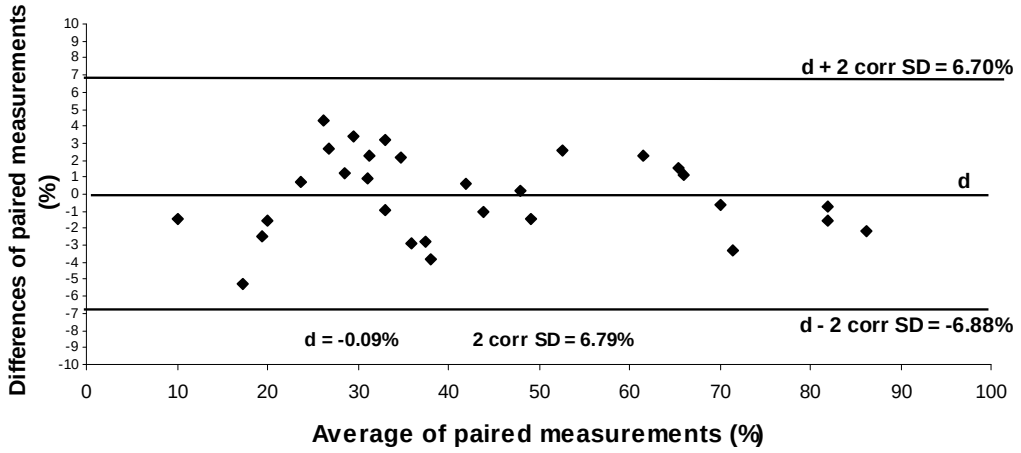


Figure 18. Agreement between the TB/Giemsa and CSB/Giemsa staining methods for counting cells with damaged tail on smears. The differences between the paired measurements are plotted against their average. The mean of the differences (d) and the limits of agreement (d ± 2corrSD) are presented (n =30)

Densitometry (image analysis)

Measuring the composite RGB value was the most objective and relevant method for comparing differences between the color intensity produced by the two stains (Figs. 11-12). The histograms showed the highest RGB values for intact tails unstained by CSB (171.2 ± 6.9) and the lowest RGB values for the stained tails by CSB (96.7 ± 10.6; Fig. 19). TB staining resulted in a small difference (~ 30 RGB) between the live and dead tails on half of the smears (Fig. 20).

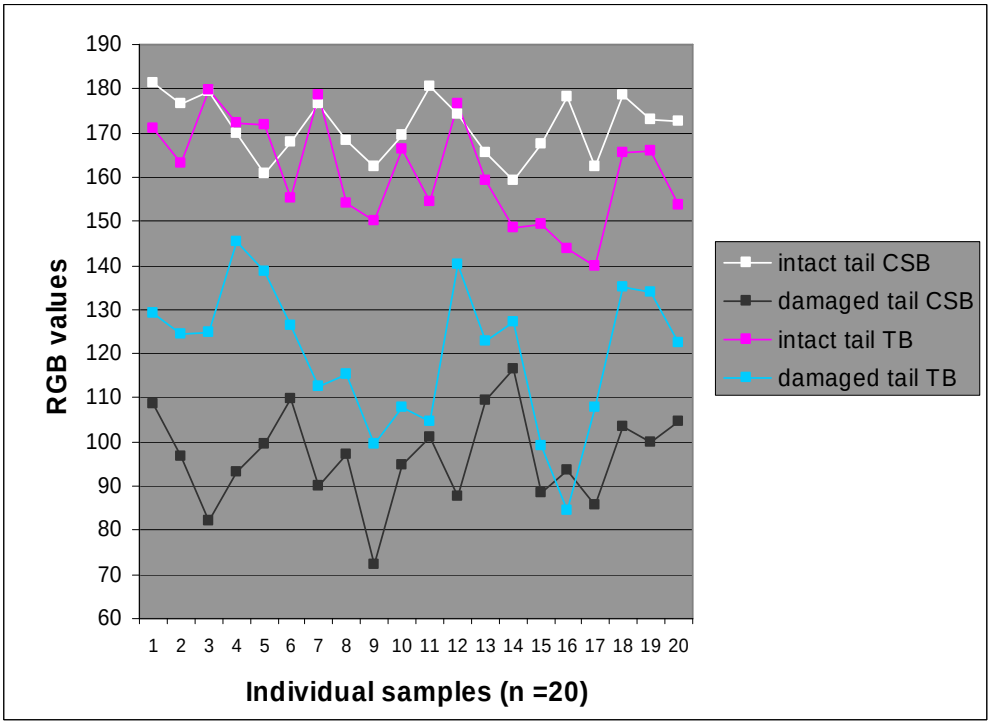


Figure 19. Means of RGB values of stained (damaged) and unstained (intact) spermatozoon tails in the individual samples

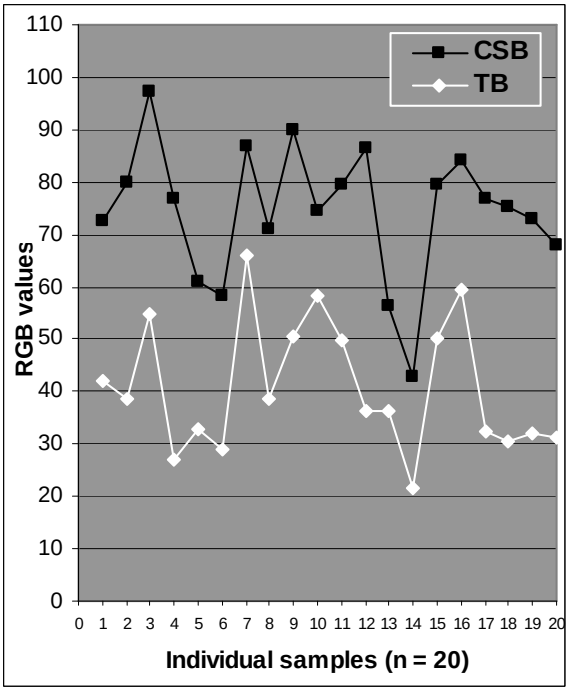


Figure 20. Comparison of RGB value differences between stained (damaged) and unstained (intact) spermatozoon tails using the two viability stains

The 20-30 RGB difference between live and dead sperm tails after TB staining was visible on the screen with higher magnification, but it was more difficult to distinguish the viability status by subjective evaluation by microscopy. We found an average of 80% higher measured values for differences in the brightness levels between the live and dead tails after CSB than after TB staining (mean ± S.D: 74.5 ± 12.7 vs. 40.9 ± 12.3; $P < 0.0001$; $n = 20$; Figs. 19-20). The difference between the two stains was also clearly seen by conventional subjective visual evaluation (Figs. 13-14). The measured values of the differences between live and dead heads obtained by TB staining were 14% higher than those obtained by CSB staining (mean ± S.D: 67.6 ± 16.4 vs. 59.3 ± 11; $P = 0.028$; $n = 20$). This difference was very small (average of 8.3 RGB) and not definitely visible by microscopy. There was no problem distinguishing live and dead heads in smears stained by either TB or CSB (Figs. 13, 14, 21).

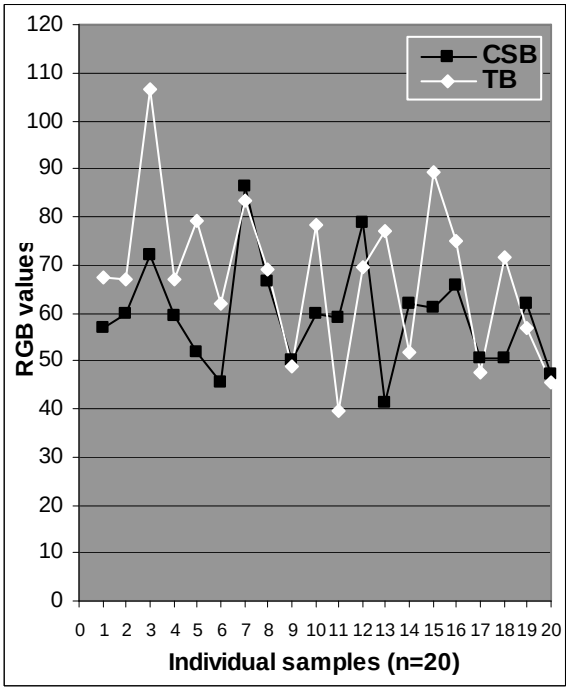


Figure 21. Comparison of RGB value differences between stained (damaged) and unstained (intact) spermatozoon heads using the two viability stains.

5.2 Experiment 2. Analysis of the injuries of stallion spermatozoa during the whole freezing procedure

Viability and cryodamages of various subdomaines

Proportion of eight main sperm categories based on membrane integrity combined morphology of the fresh, centrifuged and frozen semen is showed in Table 11. and Fig 22. Percentage of Intact spermatozoa wasn't changed after centrifugation (78±9 vs. 78±8%), but was lower in the frozen/thawed semen (38±11%, p<0.01). Tendency was the same in IHITIA category (58±16; 58±15; 26±9%; fresh, centrifuged and frozen respectively, p<0.01).

Table 11. Proportion of different cell types after technological steps of cryopreservation

%	Intact	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
fresh	78.1±9 ^{a}	58.5±17 ^{a}	6.5±4 ^{a}	5.7±5 ^{a}	7.5±6 ^{a}	0.2±0.3 ^{a}	3.9±3 ^{a}	0.8±1 ^{a}	17.0±8 ^{a}
centrifuged	78.3±8 ^{a}	58.0±15 ^{a}	6.2±4 ^{a}	4.7±4 ^{a}	9.4±8 ^{b}	0.1±0.2 ^{a}	4.0±3 ^{a}	0.6±1 ^{a}	17.2±7 ^{a}
frozen	37.7±11 ^{b}	25.6±9 ^{b}	3.4±3 ^{b}	2.1±2 ^{b}	6.7±6 ^{a}	0.7±1 ^{b}	19.1±7 ^{b}	4.1±2 ^{b}	38.5±10 ^{b}

10 stallions, n=33 freezing; a,b means within columns without common superscripts differ (p<0.05)

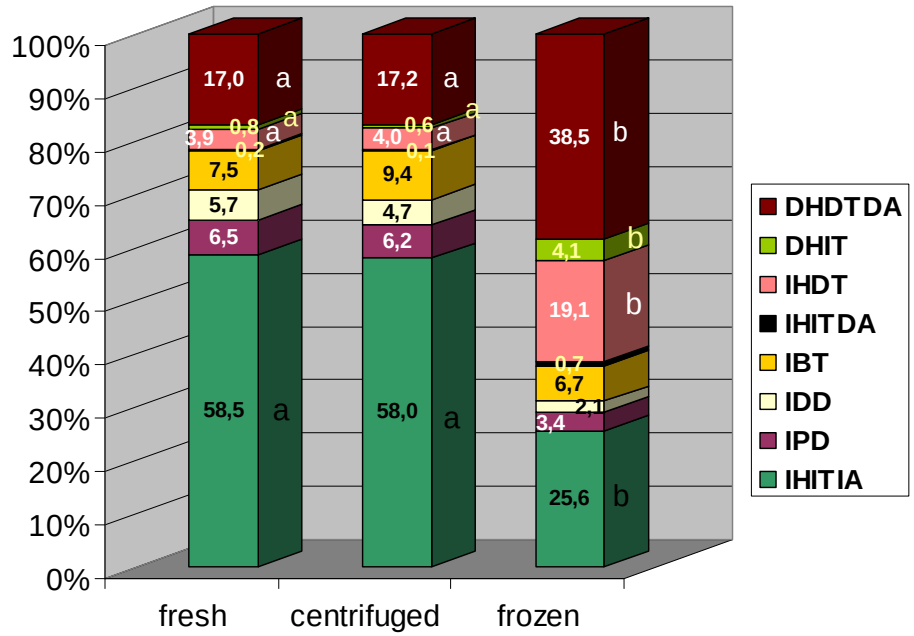


Figure 22. Distribution of different sperm categories during the freezing procedure. (10 stallions, 33 freezing). a, b means in the same cell category differ, p < 0.01

Proportion of IHDT increased considerably after freezing/thawing compared to fresh and centrifuged semen (19±7% vs. 4±3; 4±3; $p<0.01$) /Figs 22-25/. Rate of DHIT and DHDTDA are also significantly higher in the frozen semen (Table 11). Damages and depletion of acrosome of viable cells are uncharacteristic after freezing/thawing, because IHITDA was lower than 1% (0.7±1.1%).

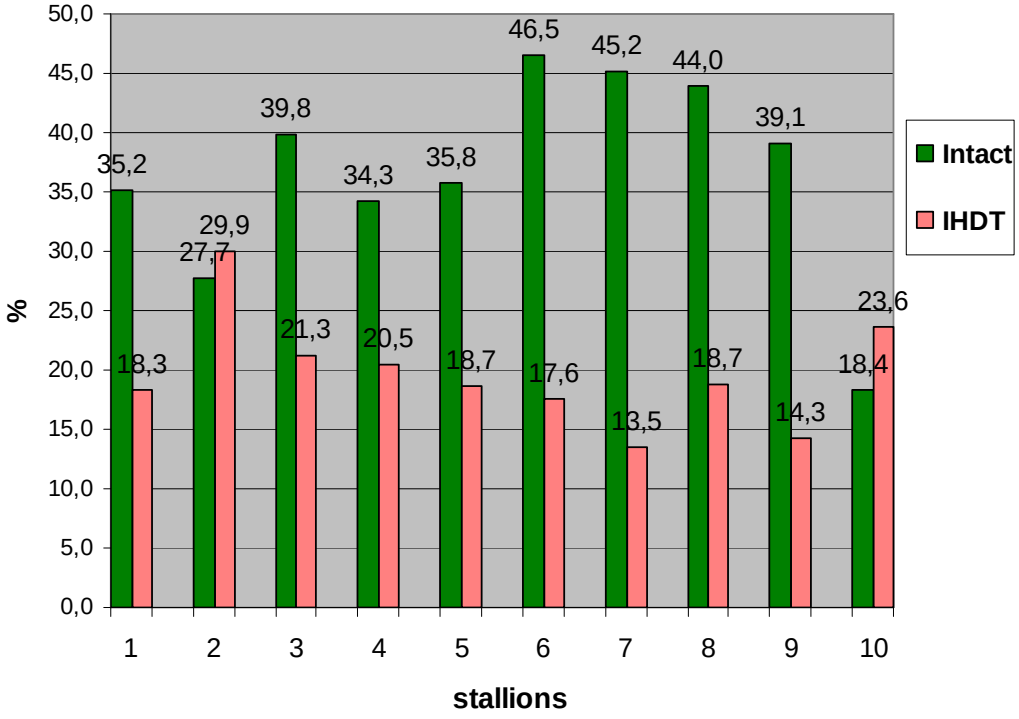


Fig. 23. Proportion of the cells with intact membranes (Intact) and IHDT after freezing/thawing in the semen of different stallions (mean values of 3-4 replicates)

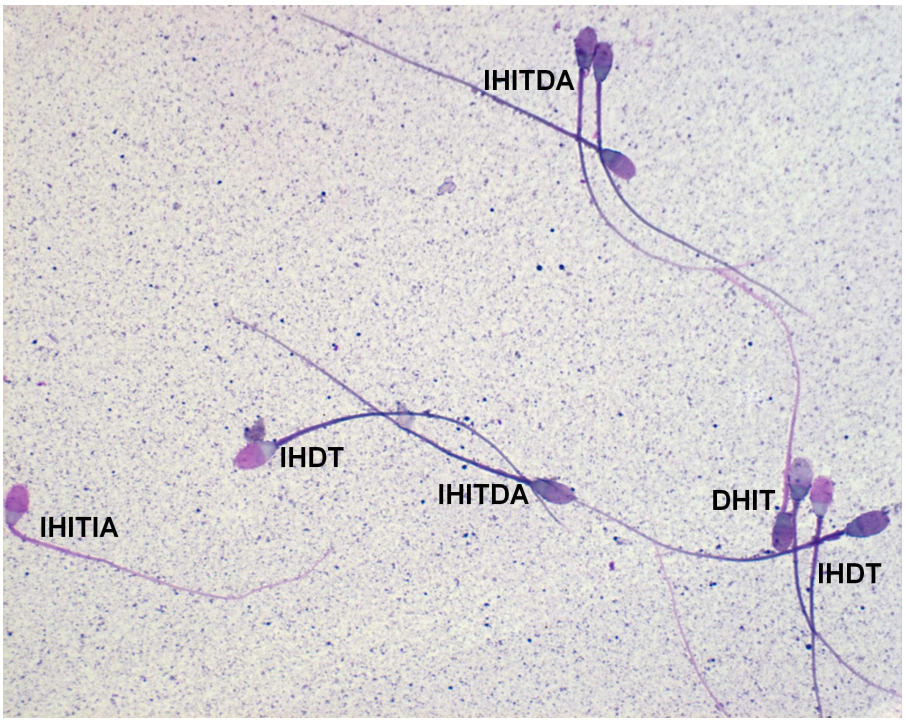


Figure 24. Microscopic picture of spermatozoa of "Stallion 4" after cryopreservation (CSB/Giemsa staining)

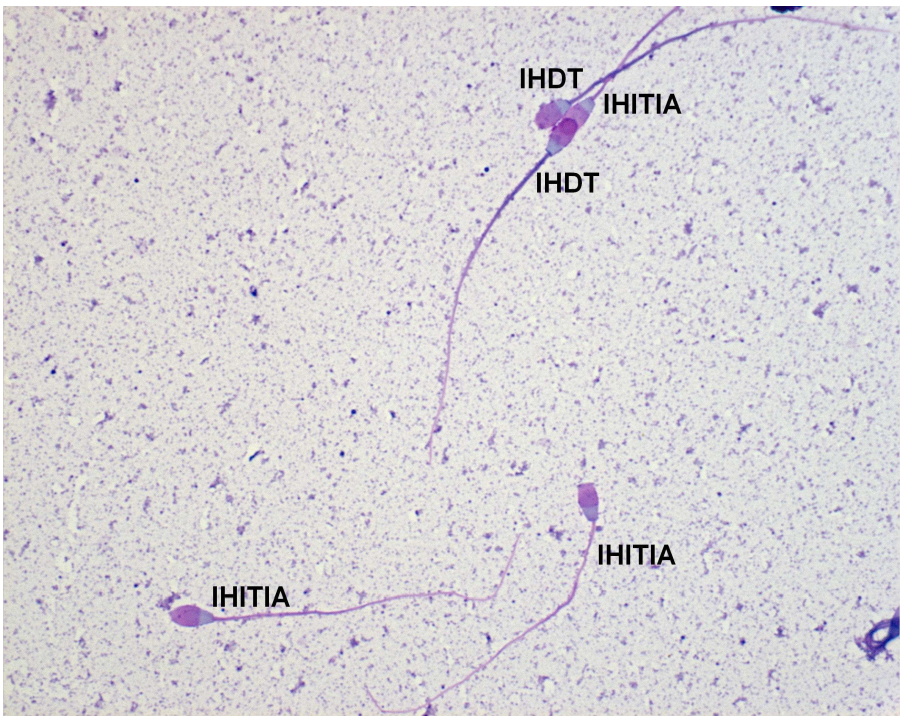


Figure 25. Microscopic picture of spermatozoa of "Stallion 3" after cryopreservation (TB/Giemsa staining)

Morphological changes, individual differences

Changes of proportion of sperm with midpiece and tail defect during the freezing process

We found individual susceptibility to centrifugation and differences among stallions in the proportion of sperm types (Fig. 26). After centrifugation (600 x g/10 min) proportion of viable sperm with tail abnormality (IBT) increased with 6-12% (9±2%) in 8 cases, in the other 25 ejaculates it changed by 0±5,5% (Fig. 27).
The eight cases related to 3 stallions (Stallion 6, Stallion 7, Stallion 9), which had fresh semen also with high percentage of this cell type (14±5%) unlike the other seven males (Group I.) which had fresh ejaculates presented 4±2% of this sperm type. In the centrifuged sperm of these 3 stallions (Group II.), IBT increased to 19±4% ($p<0,01$) and it was also high in the frozen/thawed semen (13±5%). It didn't change neither in the centrifuged (4±3%) nor in the frozen semen (3±1) in the other 7 stallions (Figs 28-29). Rate of all sperm with midpiece- and tail-defect (IDBT) increased significantly in centrifuged and frozen semen at Group II, but remained same proportion during the procedure in Group I. (Fig. 30).

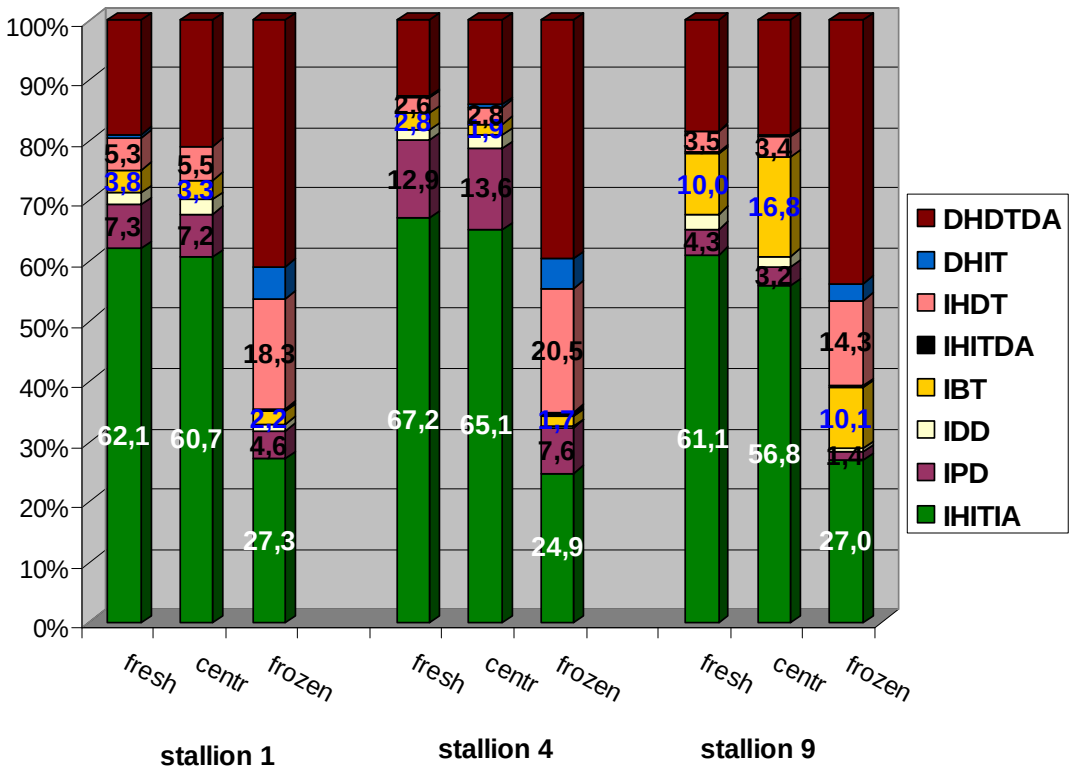


Figure 26. Distribution of different cell types of 3 stallion semen during the freezing procedure (mean values of 3-4 replicates)

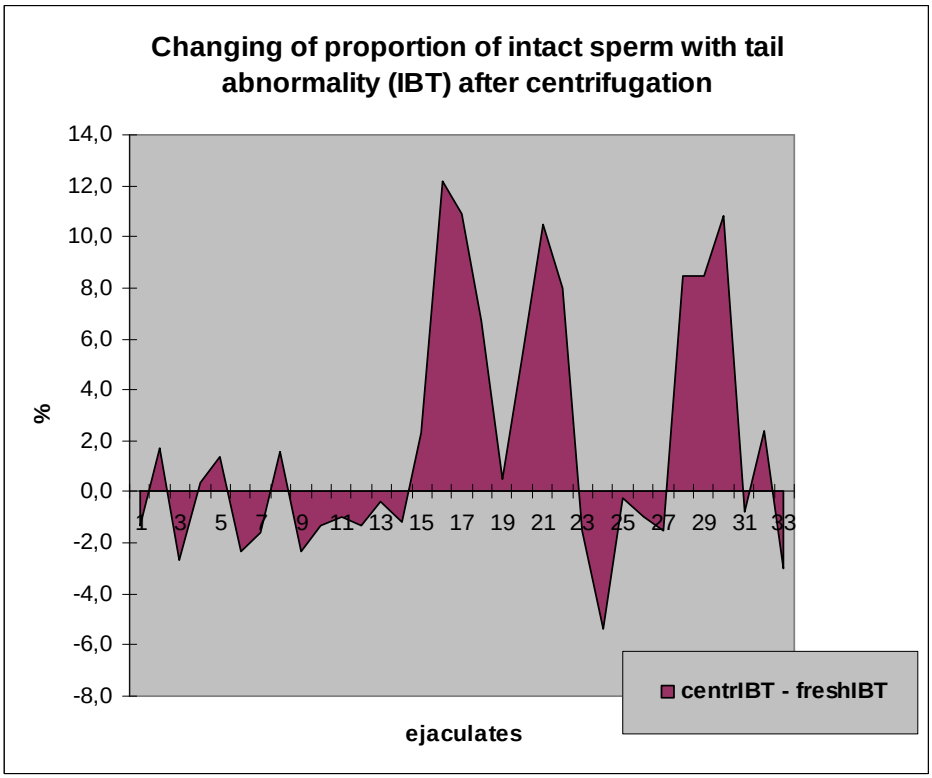


Figure 27. Differences of proportion of IBT between in centrifuged and fresh semen in each cases (n = 33)

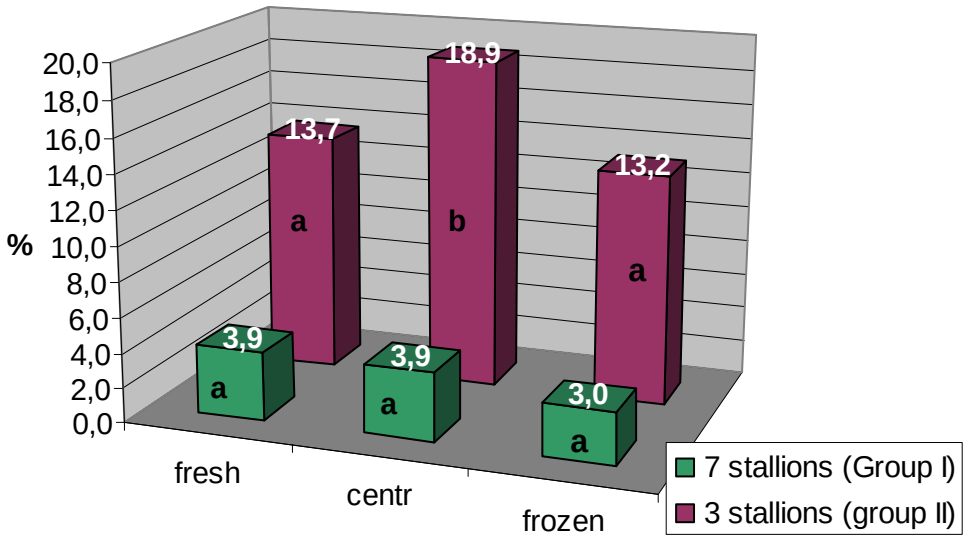


Figure 28. Proportion of IBT at different stages of the freezing process in the two groups of stallions (mean values of replicates, in Group I: n = 21 and in Group II: n = 12). a, b means in the same group differ, $p < 0.05$

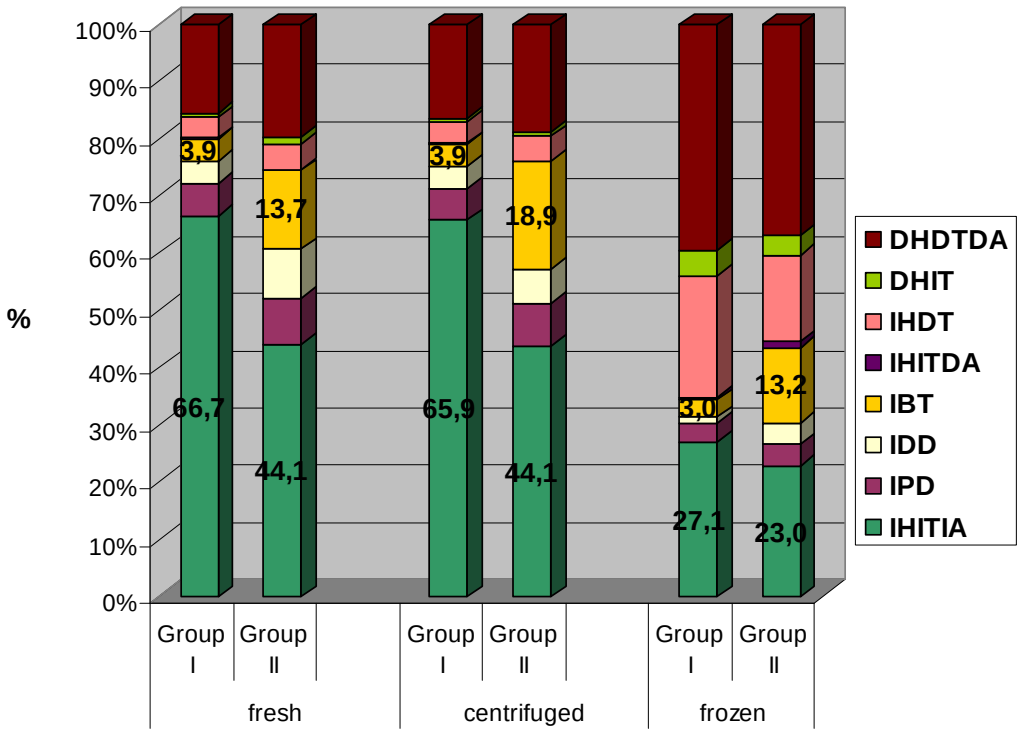


Figure 29. Distribution of different sperm categories during the freezing procedure in the two groups of stallions

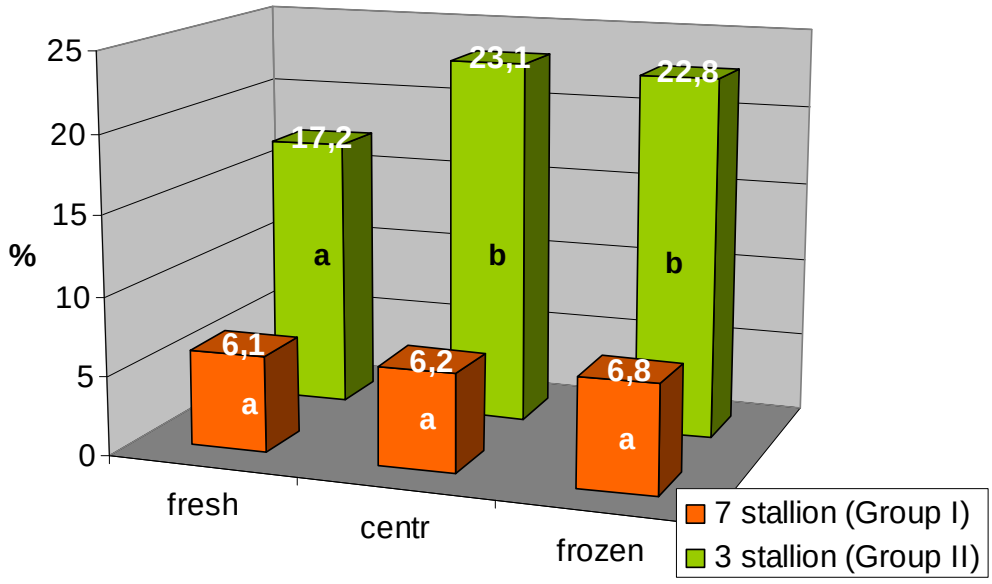


Figure 30. Proportion of IDBT at different stages of the freezing process in the two groups of stallion (mean values of replicates, in Group I: n = 21 and in Group II: n = 12). a, b means in the same group differ, $p < 0.05$)

In Group II. proportion of IBT within viable spermatozoa with intact membranes (Intact) was 30.3 % in the frozen/thawed semen while in Group I. this ration was 8.6%. IHITIA was nearly 80% of the intact sperm in Group I. while a little bit more than half of the viable cells (53%) in Group II. (Fig. 31).

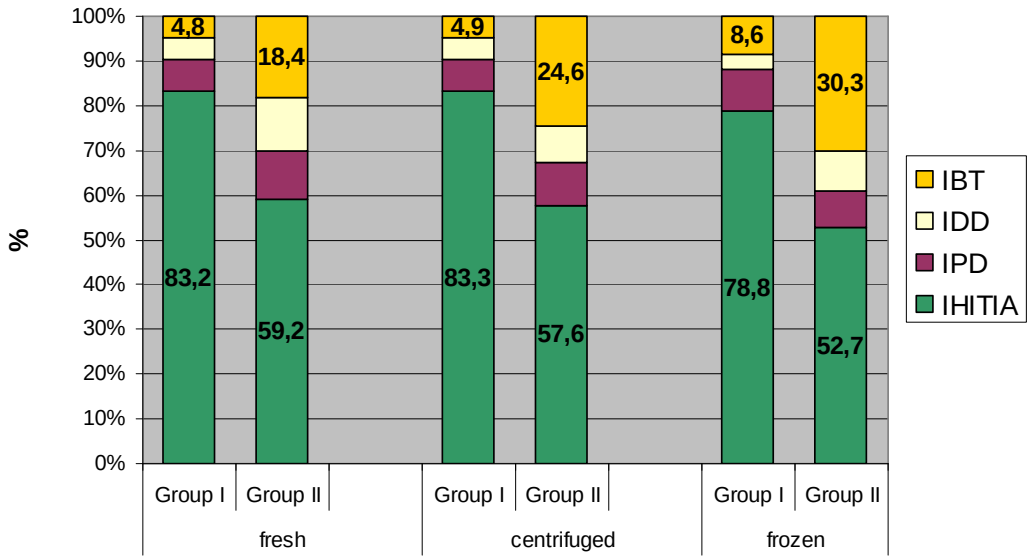


Figure 31. Proportion of different cell categories within intact viable spermatozoa during the freezing procedure in the two groups of stallions. (mean values of replicates, in Group I: n = 21 and in Group II: n = 12)

IBT ratios in fresh and centrifuged semen of Stallion 9 on each of the 17 collecting days are shown in Fig. 32. After centrifugation (600 x g/10min) a high percentage of IBT spermatozoa was observed (28 ± 13 %, n = 17). Motility evaluation by subjective estimation resulted in high percentage of motile sperm moving backwards. Frozen semen also contained high rate of IBT sperm (14 ± 6 %, n = 15). Mean percentage of all sperm with intact membranes (including IBT) was 36 ± 8% in the frozen semen. Within the total „live" Intact spermatozoa the rate of IBT cells was 38%. The proportion of different sperm categories with intact membranes in each of the 15 frozen semen samples of Stallion 9 are shown in Fig 33.

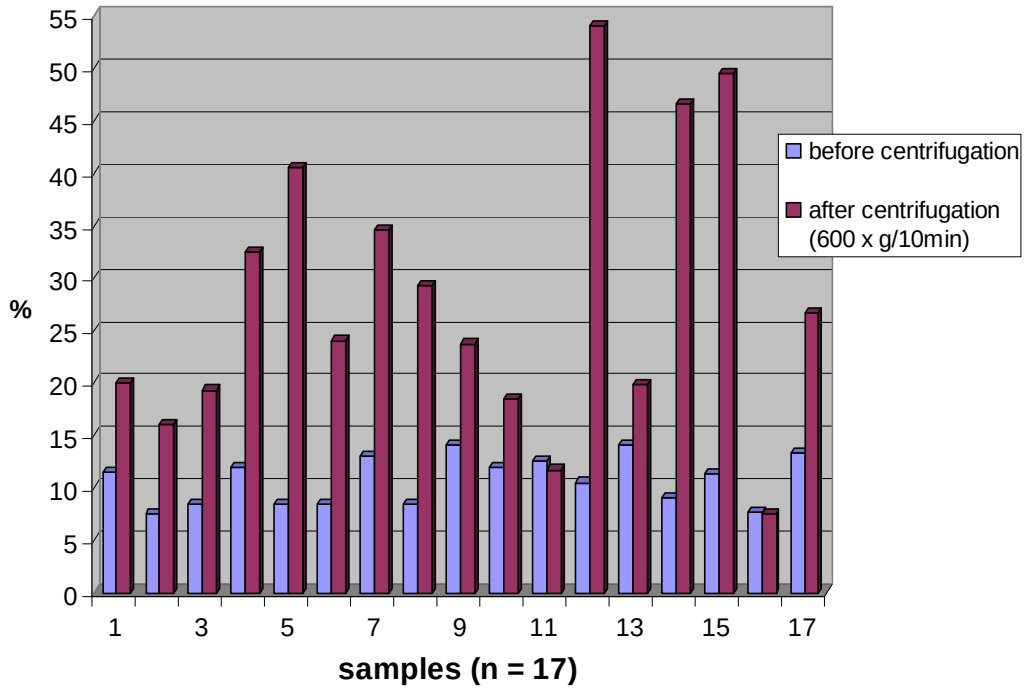


Figure 32. Proportion of IBT in fresh and centrifuged semen of Stallion 9 on different collecting days

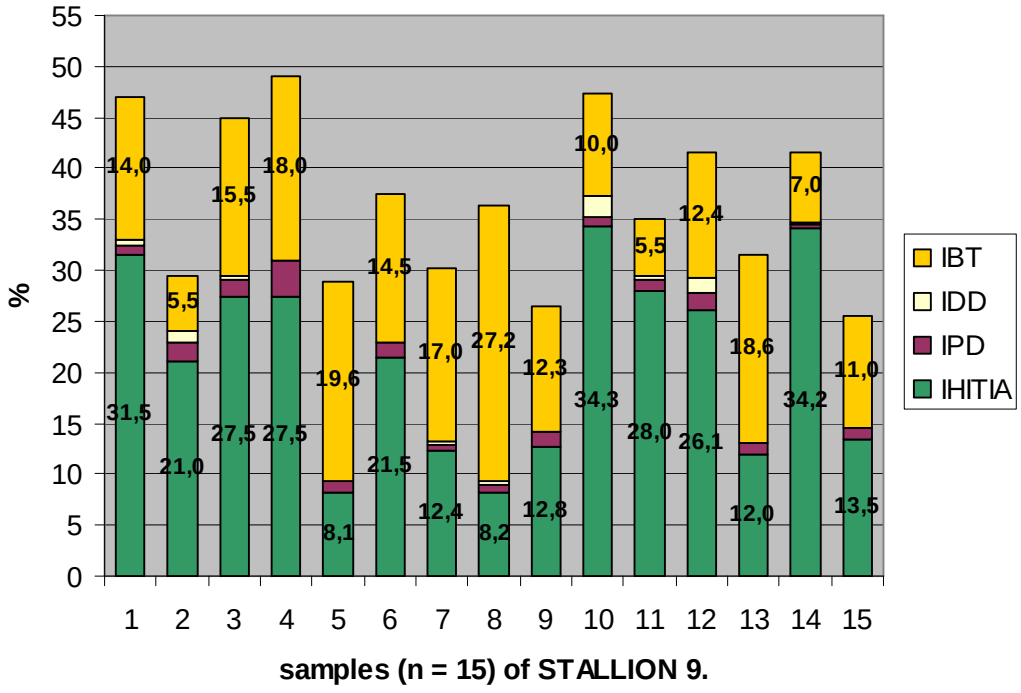


Figure 33. Proportion of different sperm categories with intact membranes in the frozen samples

Spermatozoa with midpiece and tail defect are shown in Fig. 34. Neither aberrations of axonemal filaments nor mitochondrial sheath disruptions were detected by scanning and transmission electronmicroscopy (Figs. 35-36).

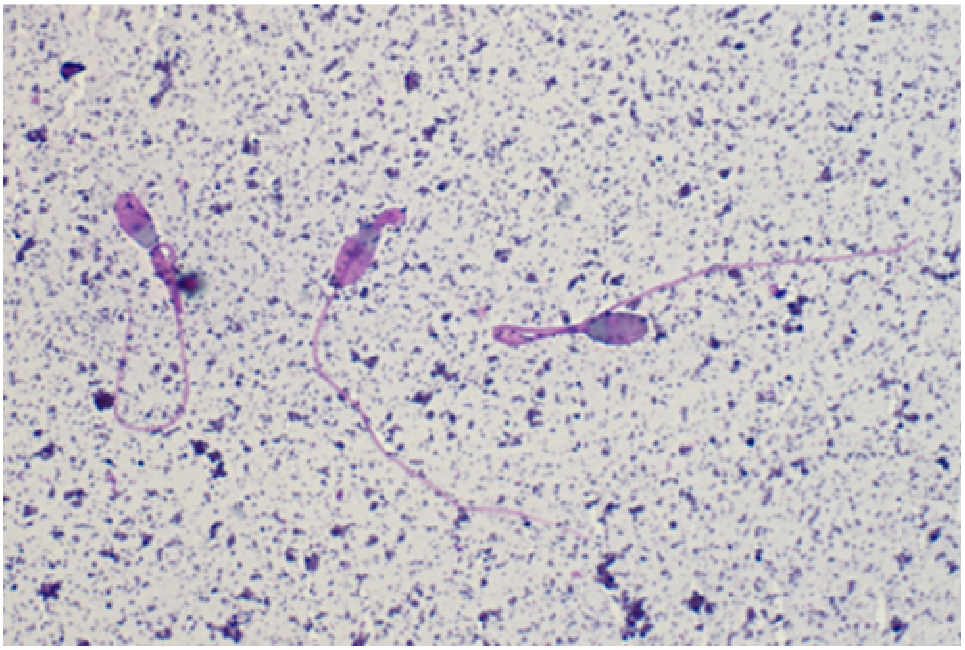


Figure 34. Microscopic picture of spermatozoa of "Stallion 9" after centrifugation (CSB/Giemsa staining)

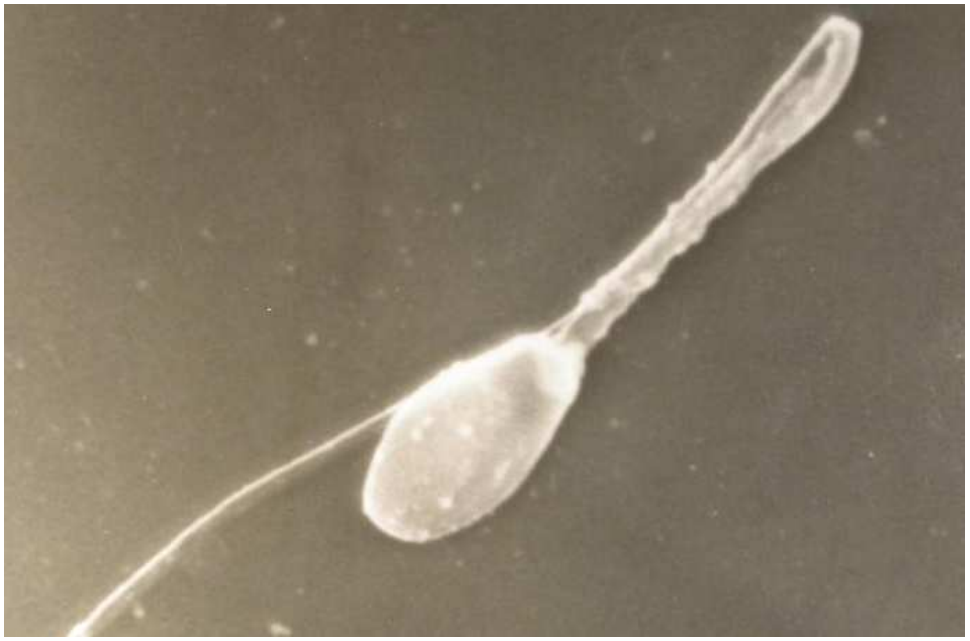


Figure 35. Tail defect (Scanning EM / 7000x magnification)

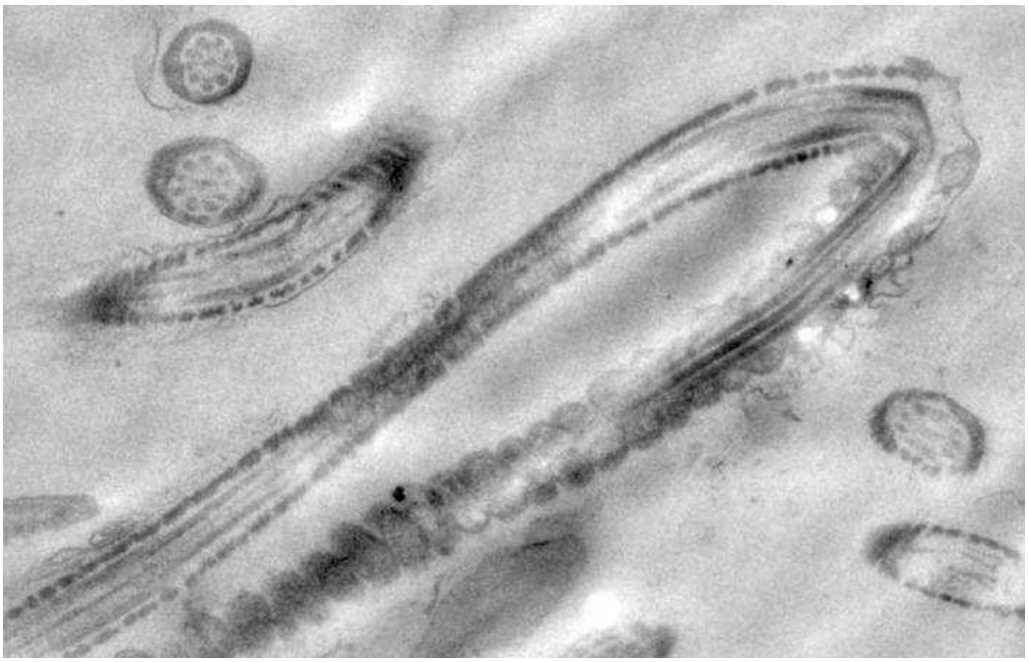


Figure 36. Tail defect (Transmission EM / 30000 x magnification)

Cytoplasmic droplets

Both of proportion of IPD and IDD were around 6 % in fresh and centrifuged semen and decreased in the frozen semen (3.4±3 and 2.1±2 %, $p<0.01$) /Table 11, Fig. 22/. Individual differences were found among stallions in the proportion of intact sperm with cytoplasmic droplets (CD) /Fig 26, 37/. IPD was more than 10 % in the fresh semen of Stallion 4 and Stallion 6, while IDD was higher than 10% in the fresh semen of Stallion 6 and Stallion 7. In the frozen semen both of these sperm categories decreased but they were presented in fairly high proportion related to IHITIA category at Stallion 7 (Fig. 37). Microscopic images of spermatozoa with CDs of Stallion 4 and Stallion 6 are shown in Figs. 38-39.

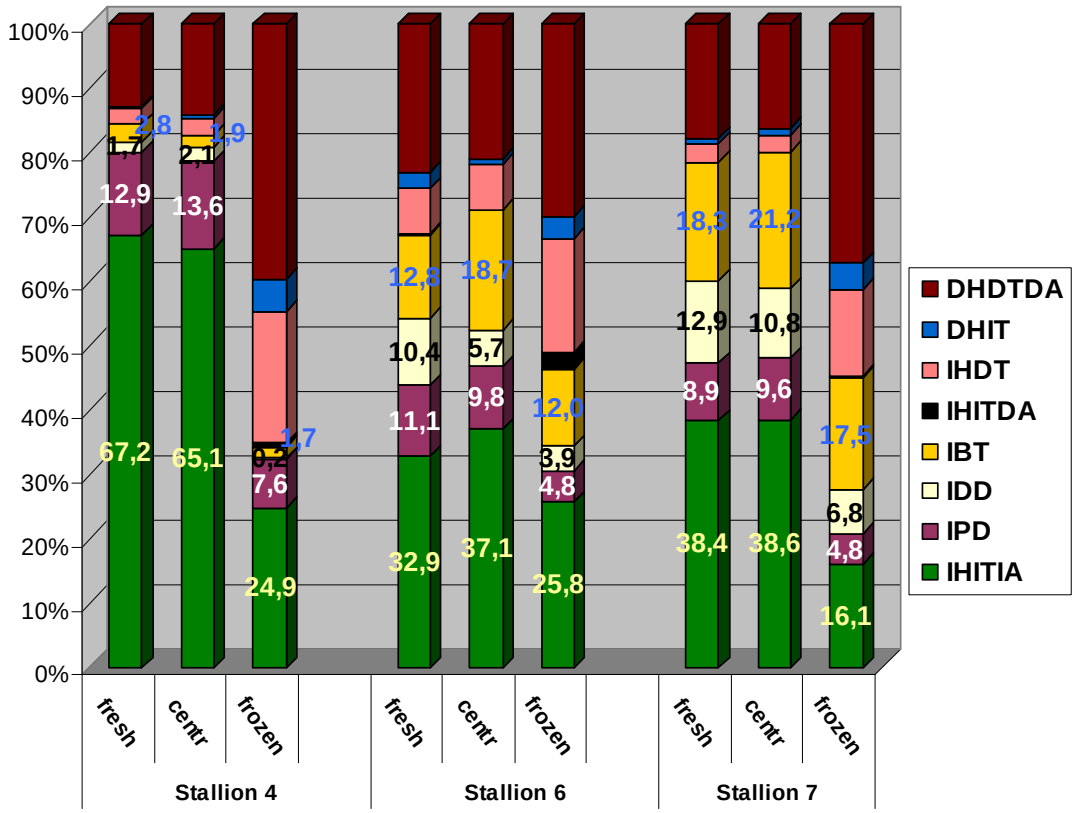


Figure 37. Distribution of different cell types of 3 stallion semen during the freezing procedure (mean values of 3-4 replicates)

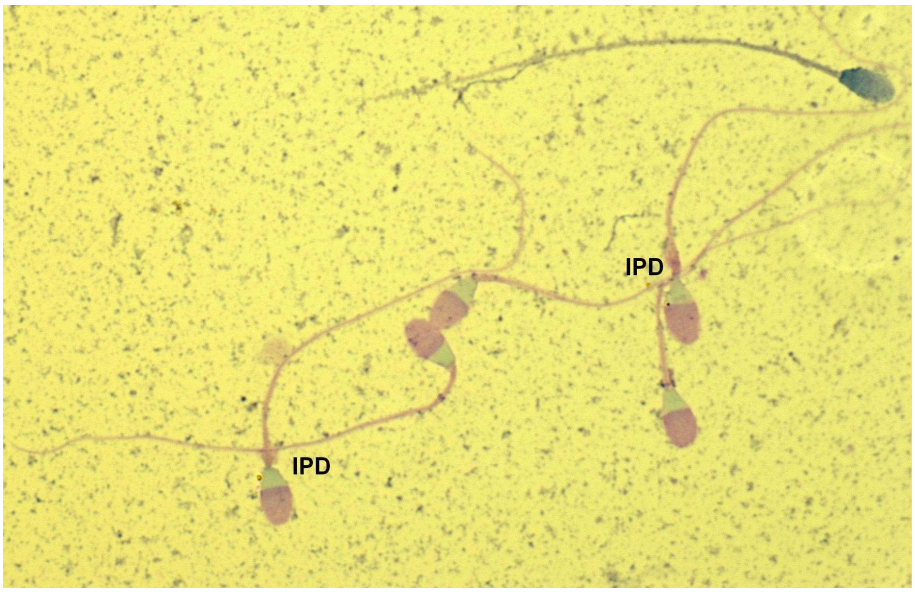


Figure 38. Spermatozoa in fresh semen of Stallion 4 (TB/Giemsa staining)

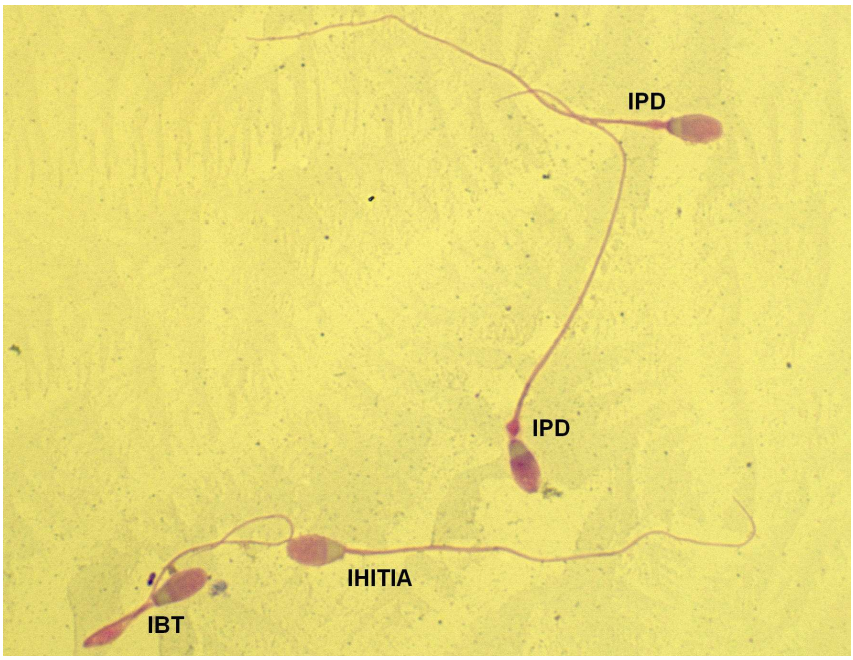


Figure 39. Spermatozoa in fresh semen of Stallion 6 (CSB/Giemsa staining)

In the combined categories all the sperm with CD-s (IDCD) slightly decreased during the process (15±9; 13±8; 12±8 %). All sperm with midpiece- and tail defect (IDBT) mildly increased after centrifugation (10±7; 12±10; 12±10 %). Proportion of IDCDBT didn't change during the freezing procedure (25±15; 26±15; 24±15%, fresh, centrifuged and frozen respectively) /Fig. 40/.

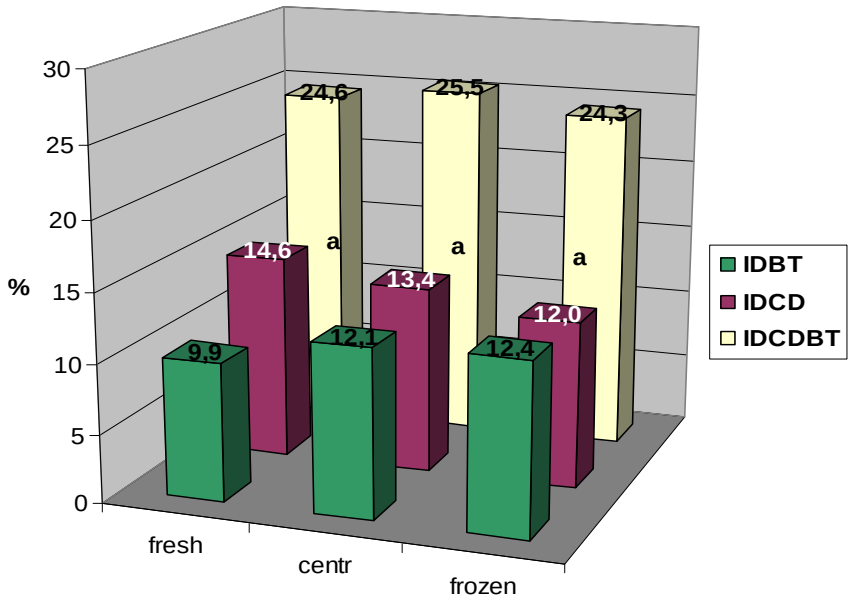


Figure 40. Incidence of sperm with CD and bent tail defect at different stages of the freezing process (mean values of 10 stallions)

5.3 Experiment 3. Use of pentoxifylline and hyaluronic acid for stallion sperm separation

Recovery

Better recovery rates were found in P-CON, P-PX, and SU-HA than P-NT, P-HA, SU-NT and SU-PX (Fig. 41).

Viability evaluation

P-CON and P-PX resulted in more intact sperm compared to all swim-ups (Table 12, Fig. 42). There were fewer DHDTDA sperm in Percoll® than in Swim-up treatments (Table 12, Fig. 42). IHITDA was higher in P-PX than P-NT ($P<0.01$) and P-CON ($P>0.05$) (17±1.6 %, 7±1.6% and 11±1.6 %, respectively) (Figs. 43, 44, 45, Table 12). There was a significant stallion x treatment effect in this sperm category. Samples of Stallion 2 and Stallion 3 caused this elevation of IHITDA after P-PX treatment (Fig. 46). After separations fairly high percentage of the sperm had damaged head but intact tail (DHIT) (Table 12, Fig 43, 44). This ratio was highest in P-CON (14±1.7 %), P-NT (13±1.7 %) and SU-PX (13±1.7 %) treatments. In the original frozen/thawed semen it was only 0.5 %. P-PX resulted in less DHIT cells compared to P-CON (8 vs 14 %) but it was not significant ($P>0.05$) (Fig. 43). IHDT was significantly lower after both Percoll® and swim up treatments compered to the beginning frozen/thawed sperm (2-6% vs. 17%, treated and thawed samples respectively).

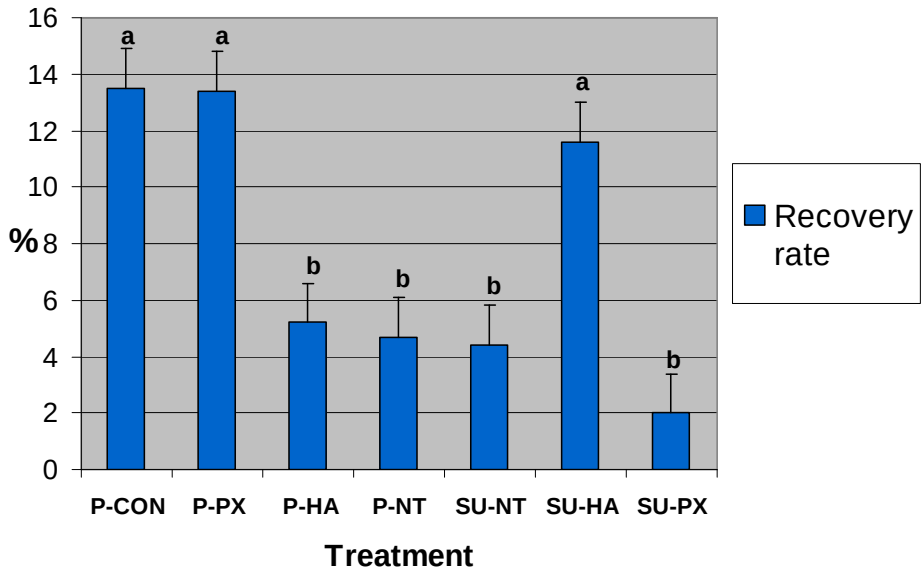
Morphologic evaluation

All Percoll® separations resulted in more „normal", and less sperm with droplets (proximal + distal droplets) and midp+tail defect compared to all swim-ups (91-92% vs.71-78%; 1% vs.4-7%; 6-7% vs.16-19% respectively, $p<0.01$) (Table 13, Fig. 47). There were no significant differences among Percoll® separations in any morphologic categories. „Normal" was lower in SU-HA than in SU-NT (71 vs.78%, $p<0.01$). „Midp+tail" was higher after swim-ups (Fig. 48) and lower after Percolls compered to the beginning thawed sperm (Table 13, Fig. 47). P-CON and P-PX resulted in the most intact, normal sperm. HA increased the recovery rate during swim-up, but not viability and proportion of normal cells in any of the treatments (Figs 41, 42, 47, 48).

Table 12. Percentages of different sperm in different categories and the recovery rates; LS means ± SE

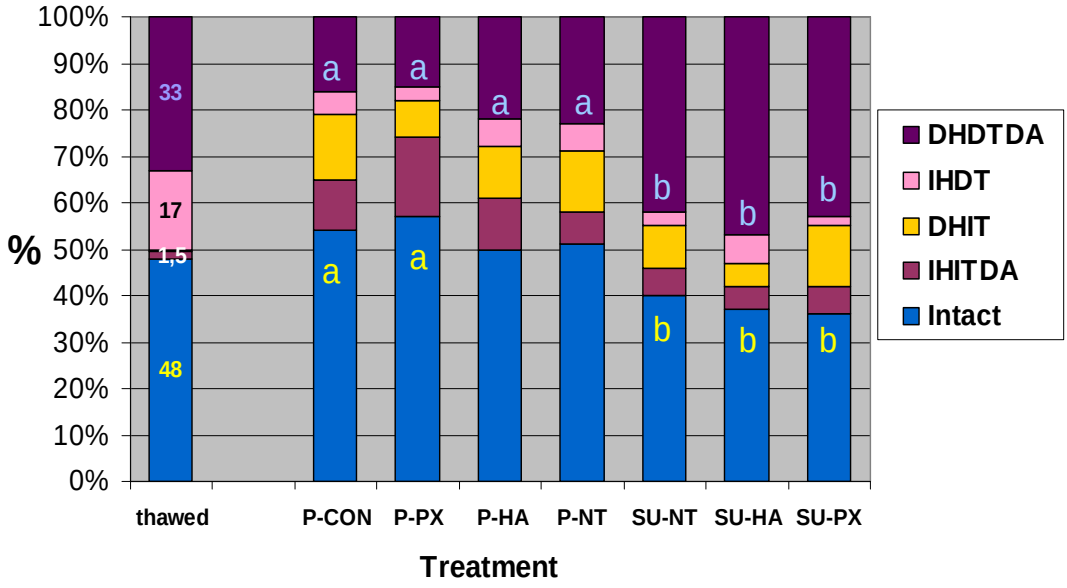
	Treatment						
Sperm categories	**P-CON**	**P-PX**	**P-HA**	**P-NT**	**SU-NT**	**SU-HA**	**SU-PX**
Intact (%)	54±3.4^{a}	57±3.4^{a}	50±3.4^{a,b,c}	51±3.4^{a,b}	40±3.4^{b,c,d}	37±3.4^{c,d}	36±3.4^{d}
IHITDA (%)	11±1.6^{a,b}	17±1.6^{a}	11±1.6^{a,b,c}	7±1.6^{b,c}	6±1.6^{b,c}	5±1.6^{c}	6±1.6^{b,c}
DHIT (%)	14±1.7^{a}	8±1.7^{a,b}	11±1.7^{a}	13±1.7^{a}	9±1.7^{a,b}	5±1.7^{b}	13±1.7^{a}
IHDT (%)	5±0.6^{a}	3±0.6^{a,b}	6±0.6^{a}	6±0.6^{a}	3±0.6^{a,b}	6±0.6^{a}	2±0.6^{b}
DHDTDA (%)	16±2.5^{b}	15±2.5^{b}	22±2.5^{b}	23±2.5^{b}	42±2.5^{a}	47±2.5^{a}	43±2.5^{a}
Recovery (%)	13±1.4^{a}	13±1.4^{a}	5±1.4^{b}	5±1.4^{b}	4±1.4^{b}	12±1.4^{a}	2±1.4^{b}

a,b,c,d means within rows without common superscripts differ (p<0.001) except DHIT (p=0.004). Samples are from 3 stallions and 3 replicates.



a,b in the same sperm category indicate significant differences between values (p<0.0001)

Figure 41. Recovery rate after different treatments



a,b in the same sperm category indicate significant differences between values ($p<0.01$)

Figure 42. Distribution of sperm viability categories after different treatments

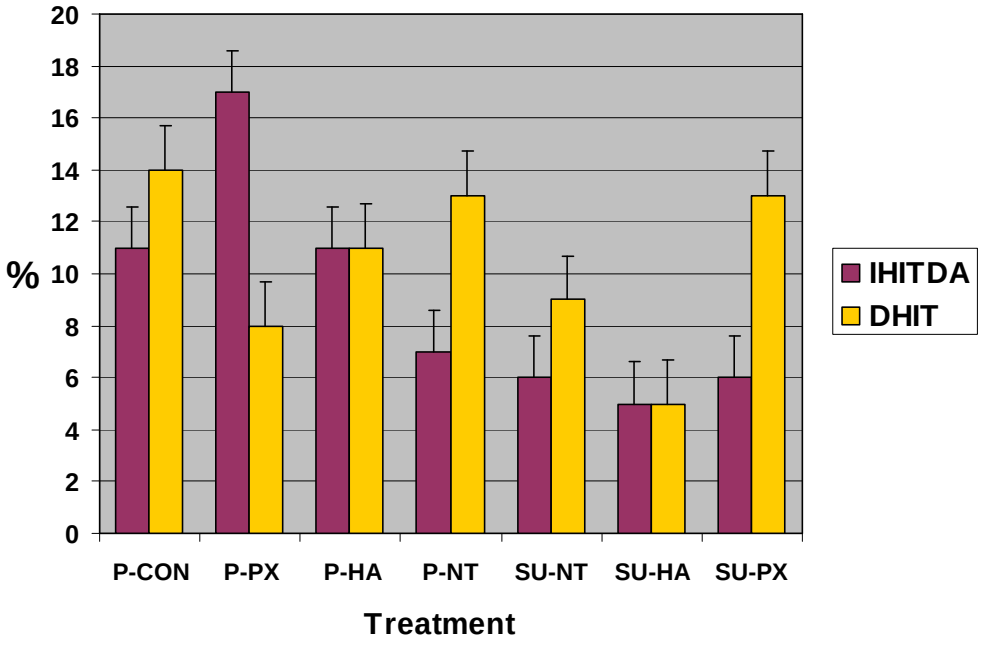


Figure 43. Proportion of IHITDA and DHIT after different treatments

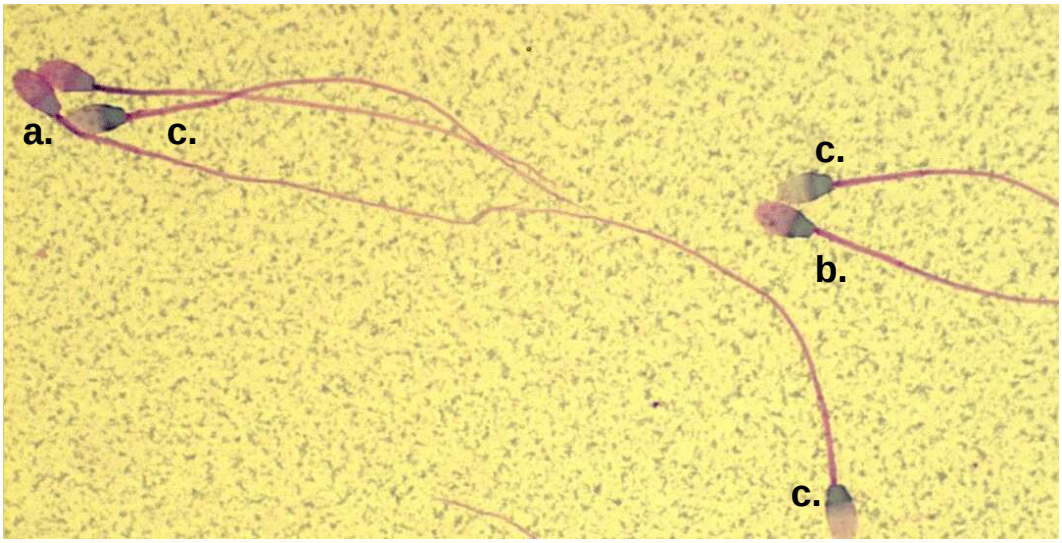


Figure 44. Viability and acrosome staining Percoll-Control (CSB/Giemsa staining)

a. Intact: intact head, tail and acrosome membrane
b. DHIT-1: damaged head, intact tail, loose acrosome
c. DHIT-2: damaged head, intact tail, lost acrosome

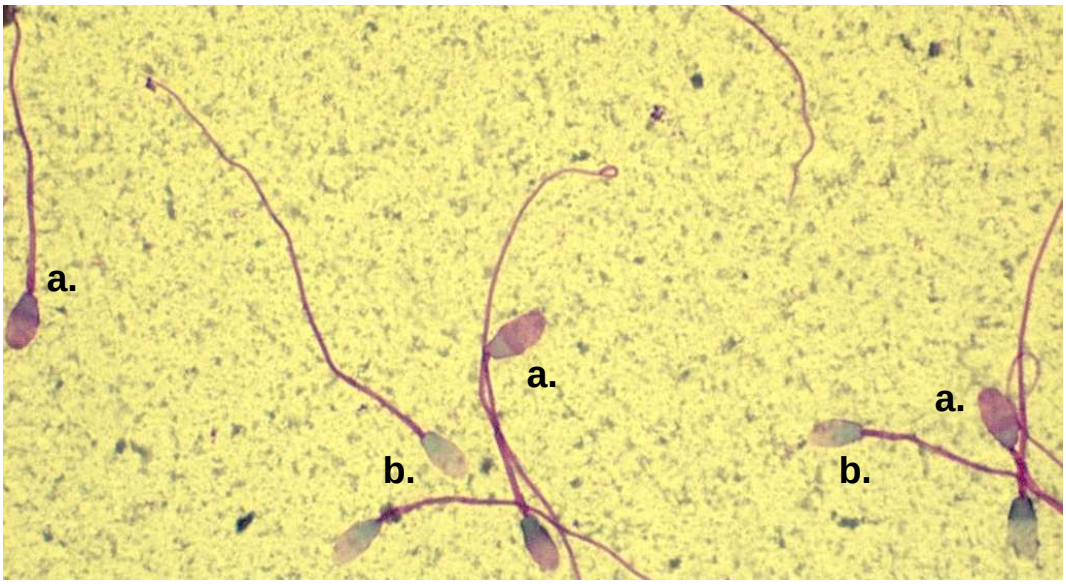


Figure 45. Viability and acrosome staining; Percoll-PX (CSB/Giemsa staining)

a. Intact: intact head, tail and acrosome membrane
b. IHITDA: intact head, tail, damaged (lost) acrosome
c. DHIT: damaged head, intact tail, lost acrosome

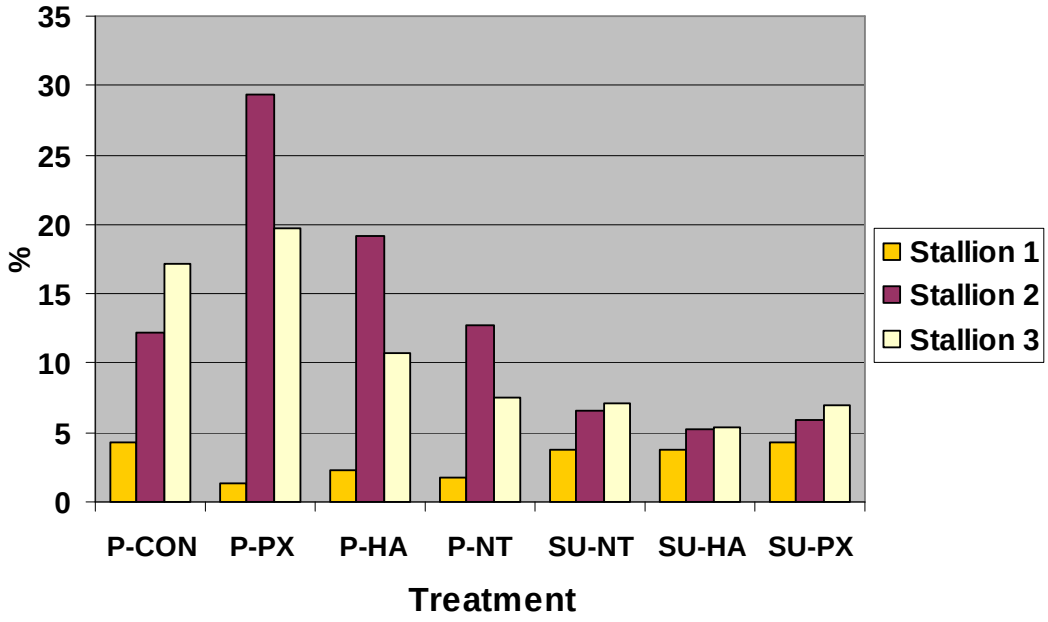


Figure 46. Proportion of IHITDA after different treatments in the individual stallions

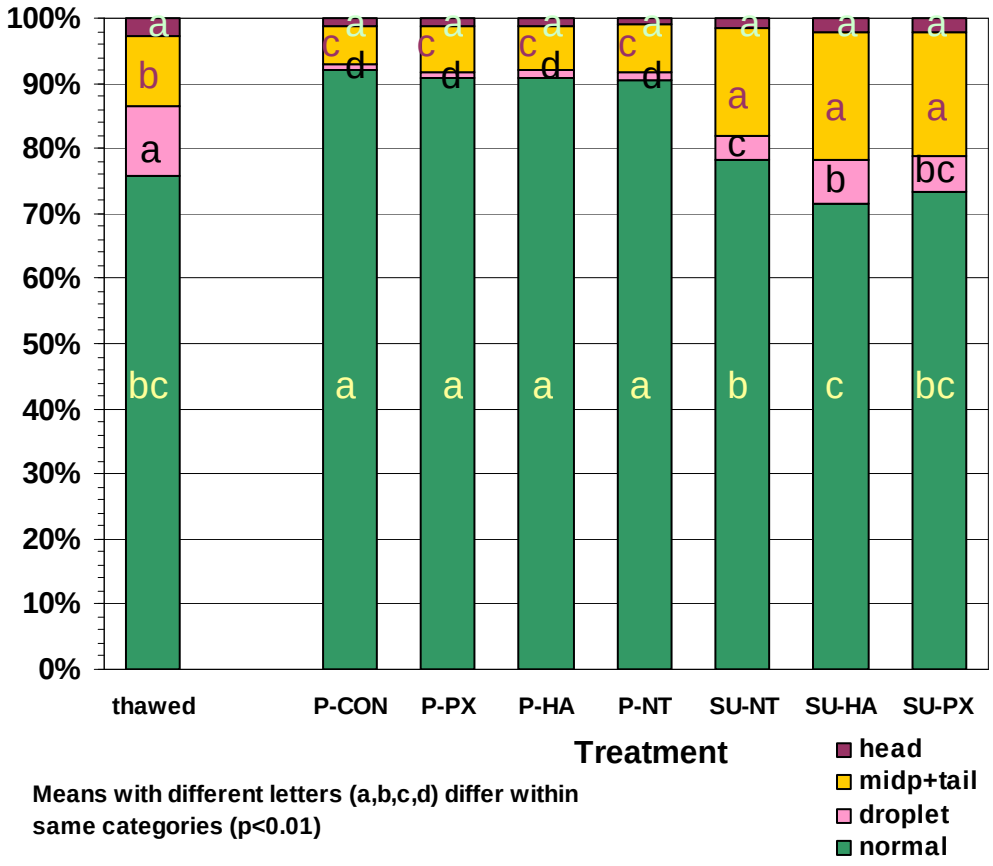


Figure 47. Distribution of morphologic categories of sperm after different treatments

Table 13. Percentages of sperm in different morphological categories and the recovery rates; LS means ± SE

		Treatment						
Sperm categories	Thawed	P-CON	P-PX	P-HA	P-NT	SU-NT	SU-HA	SU-PX
Normal (%)	76±0.9^{b,c}	92±0.9^{a}	91±0.9^{a}	91±0.9^{a}	90.5±0.9^{a}	78±0.9^{b}	71±0.9^{c}	73±0.9^{b,c}
Proximal droplet (%)	6.2±0.3^{a}	0.8±0.3^{c}	0.9±0.3^{c}	1±0.3^{c}	1.2±0.3^{c}	3±0.3^{b}	4.5±0.3^{a,b}	4±0.3^{b}
Distal droplet (%)	4.5±0.3^{a}	0.1+0.3^{e,d}	0.2+0.3^{e,d}	0+0.3^{e}	0.1+0.3^{e,d}	0.6±0.3^{c,d}	2.5±0.3^{b}	1.6±0.3^{c,b}
Droplets (all) (%)	10.7±0.5^{a}	1±0.5^{d}	1.1±0.5^{d}	1±0.5^{d}	1.3±0.5^{d}	4±0.5^{c}	7±0.5^{b}	5±0.5^{b,c}
Midpiece + tail defect (%)	10.7±0.7^{b}	5.6±0.7^{c}	7±0.7^{c}	7±0.7^{c}	7±0.7^{c}	16.6±0.7^{a}	19±0.7^{a}	19±0.7^{a}
Head defect (%)	2.9±0.3^{a}	1.3±0.3^{a}	1.2±0.3^{a}	1.1±0.3^{a}	1±0.3^{a}	1.5±0.3^{a}	2.2±0.3^{a}	2.3±0.3^{a}
Recovery (%)		13±1.4^{a}	13±1.4^{a}	5±1.4^{b}	5±1.4^{b}	4±1.4^{b}	12±1.4^{a}	2±1.4^{b}

a,b,c,d,e means within rows without common superscripts differ ($p<0.001$) except Head defect ($p=0.008$)
Samples are from 3 stallions and 3 replicates.

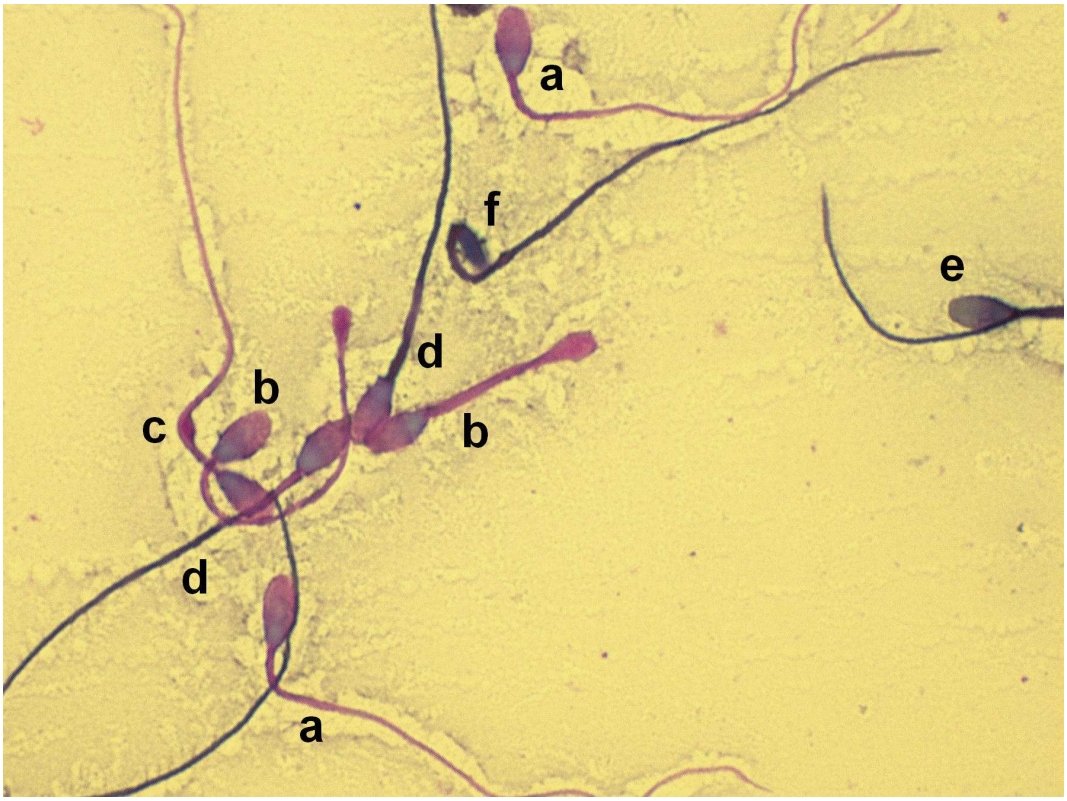


Figure 48. Viability, acrosome and morphology evaluation. Swim-up-HA (CSB/Giemsa staining)

a. Intact-1: intact head, tail and acrosome membrane; **normal morphology**
b. Intact-2: intact head, tail and acrosome membrane; **coiled or bent tail**
c. Intact-3: intact head, tail and acrosome membrane; **distal droplet**
d. IHDT: intact head, damaged midpiece and tail; **normal morphology**
e. DHDTDA-1: damaged head, tail, acrosome; **bent tail**
f. DHDTDA-2: damaged head, tail, acrosome; **microcephal head defect**

5.4 Experiment 4. Viability, acrosome integrity and morphology evaluation of sperm samples from subfertile stallions (Case reports and their interpretation)

Fertile stallions (Control group)

Quantitative and motility parameters of semen of fertile stallions are shown in the Table 14.

Table 14. Volume, concentration and motility parameters of the fresh semen of the individual fertile stallions

Stallion	gel free volume (ml)	concentration (x 10^6 sperm /ml)	motility (%)
Stallion 1	15-21	120-345	75-80
Stallion 2	25-58	50	70-80
Stallion 4	15	384	70
Stallion 5	25-110	83-110	70-80
Stallion 6/Stallion 12	10-40	85-155	70-75
Stallion 7	30-40	95-125	60-75
Stallion 9	30-100	65-100	60-90
Stallion 10	40	153	75-80
Stallion 11	45	235	70
Stallion 13	20-150	72-135	60-80
Stallion 14	20-40	68-140	80

Morphology assessment of fresh sperm of fertile stallions

Table 15. Percentages of sperm in different morphologic categories /fresh semen/

Stallion	normal	head	midp	tail	coiled	detached	PD	DD	multiple
1	73.5	1.7	3.1	2.9	1.0	1.8	14.1	2.1	0.0
2	76.5	0.8	1.9	2.5	1.8	0.5	4.5	11.7	0.0
3	71.7	0.9	3.6	7.3	2.7	0.4	5.0	8.4	0.3
4	78.5	1.5	6.9	2.9	1.4	0.4	3.9	4.8	0.0
5	81.3	5.2	3.2	2.8	1.3	0.7	3.7	1.8	0.0
6	73.4	4.0	2.5	4.3	1.9	1.5	9.5	2.9	0.0
7	76.6	3.6	3.3	5.1	1.2	0.9	5.3	3.1	0.9
8	89.2	0.0	1.6	4.9	0.9	1.3	1.2	1.2	0.0
9	80.1	1.5	5.3	3.1	1.0	0.4	6.3	2.4	0.0
10	83.4	0.7	7.0	1.9	0.5	1.8	2.5	2.3	0.2
MEAN	**78.4**	**2.0**	**3.8**	**3.7**	**1.4**	**1.0**	**5.6**	**4.1**	**0.1**
SD	**5.3**	**1.7**	**1.9**	**1.6**	**0.6**	**0.6**	**3.7**	**3.4**	**0.3**

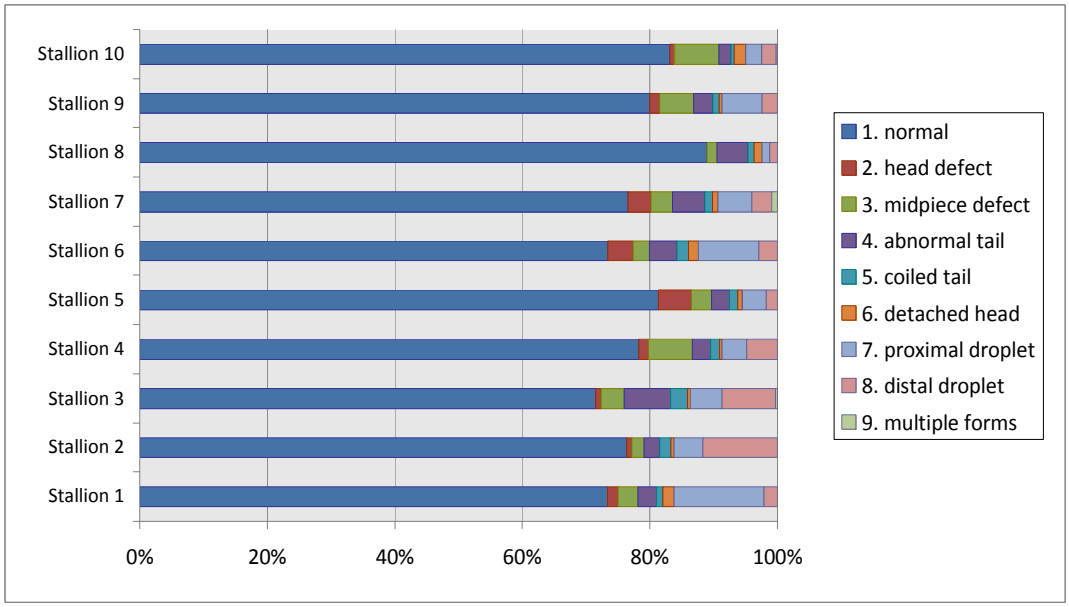


Figure 49. Distribution of morphologic categories in the semen of individual fertile stallions

Proportions of nine morphological sperm categories of fresh semen of the fertile stallions are shown in Table 15. and Fig 49. Data of different stallions are mean values of 2-3 ejaculates. Mean values and standard deviations (SD) of the 10 stallions' data are presented in Table 15 and Fig. 50. Data shows that proportion of morphologically normal sperm was higher than 70% and percentage of abnormal cells was less than 30% in each stallion. This result meets the Hungarian Standard for breeding stallion semen (7034/1999) which alows a maximum of 30% sperm with any morphologic aberrations, if less than half of these abnormal cells have primary defect. The avarege value of normal sperm in the 10 fertile stallions (78% ± 5.3%) was higher than mean percentages in the different publications shown in Table 2. The Proportion of various abnormal sperm categories was lower or corresponds to the value of the same category according to the different studies in Table 2. Average rates of the different sperm categories in fertile stallions described by Juhász and Nagy (2003): normal > 60%, head defect < 3-5%, neck abnormalities < 1-1.5%, midpiece defect < 4.5-6%, tail defects < 10-15%, proximal cytoplasmic droplets < 2.5-4%, distal cytoplasmic droplets < 2-5%. In the case of the studied stallions except two categories, the proportion of morphologic abnormalities were lower or not significantly higher than these values. In the semen of Stallion 2 and Stallion 3 the ratio of distal cytoplasmic droplet was elevated (11.7 and 8.4%. respectively), but these values are acceptable if the semen collection is frequent. Stallion 1 showed high proportion of proximal cytoplasmic droplet (14.1%), however percentage of morphologic normal cells was over than 70% and ratio of viable sperm with normal morphology (IHITIA) was

67.2% (Table 16), which is considered as high value and this compensates the presence of abnormal spermatozoa what is confirmed by excellent pregnancy results of the mares inseminated by “Stallion 1” semen.

Distribution of the nine morphologic categories of sperm in fertile stallions is shown in Fig. 50. Proportion of normal spermatozoa is nearly 80% and rate of abnormal cells is around 20% which implies good fertility of these semen.

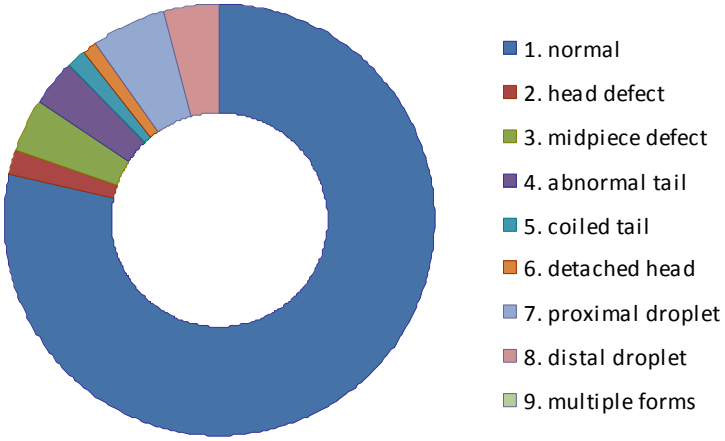


Figure 50. Distribution of morphologic sperm categories of fertile stallions (mean values of 10 stallions)

Membrane integrity assessment of spermatozoa of fertile stallions

Table 16. Percentages of sperm in different viability categories in fresh semen

Stallion	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
1	67.2	12.9	1.7	2.8	0.0	2.6	0.2	12.7
2	71.1	3.3	9.8	4.5	0.0	3.3	1.3	6.7
3	61.8	3.8	6.0	11.5	0.0	6.5	1.3	9.3
4	71.8	3.8	4.7	5.8	0.1	2.8	0.4	10.6
5	73.6	4.1	0.9	3.3	0.1	3.7	0.8	13.4
6	62.1	7.3	2.1	3.8	0.0	5.3	0.5	19.0
7	62.2	3.4	2.0	7.2	0.3	4.2	1.3	19.6
8	79.8	2.9	1.0	2.3	0.5	2.7	2.2	8.7
9	52.5	3.2	1.5	2.9	0.3	5.8	2.8	31.0
10	72.1	2.2	1.5	1.3	0.0	2.9	0.4	19.7
MEAN	**67.4**	**4.7**	**3.1**	**4.5**	**0.1**	**4.0**	**1.1**	**15.1**
SD	**7.9**	**3.2**	**2.9**	**3.0**	**0.2**	**1.4**	**0.8**	**7.3**

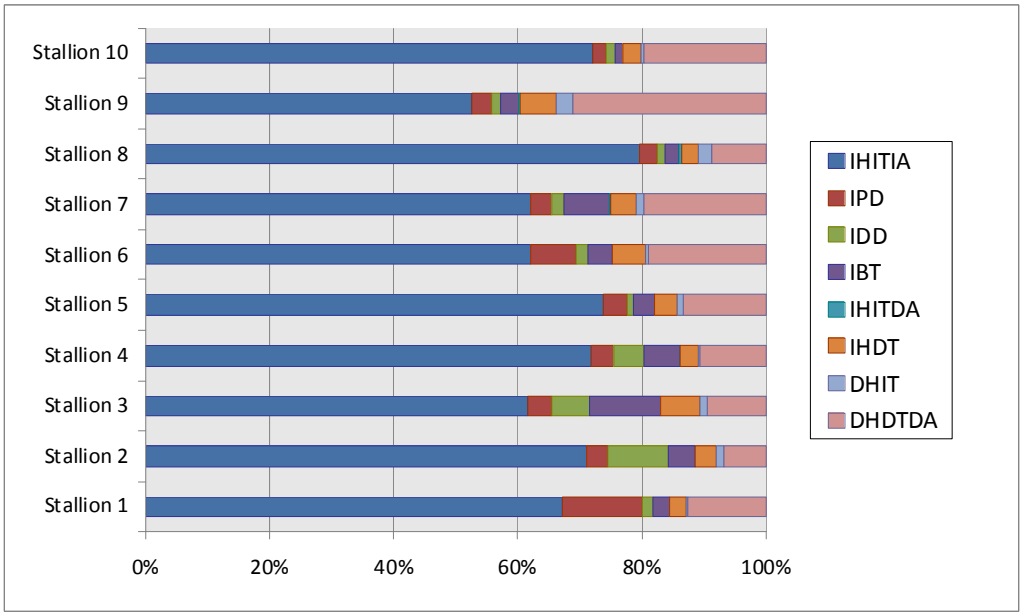


Figure 51. Distribution of viability categories in the semen of individual fertile stallions

Proportions of eight sperm categories based on membrane integrity combined morphology of the fresh semen of fertile stallions are shown in Table 16. and Fig 51. Data are mean values of 3 ejaculates of each stallion. IHITIA was higher than 60% in almost all stallion semen except at "Stallion 9" which had semen containing 52.5% of this cell type. This rate is feasible for fertilization if sufficient total sperm number is present in the insemination dose. Proportion of IPD is 12.9% in "Stallion 1" and rate of IDD is mildly increased in "Stallion 2" semen (9.8%). Presence of these spermatozoa can be compensated with the high ratio of IHITIA cells at both stallions. However in "Stallion 3" percentage of IBT sperm is higher (11.5%) compared to mean value of this sperm category of the 10 stallions. In morphologic evaluation - summarized midpiece and tail defects- the rate was 13.5% of "live" and "dead" sperm altogether and this ratio is below the allowable limit.

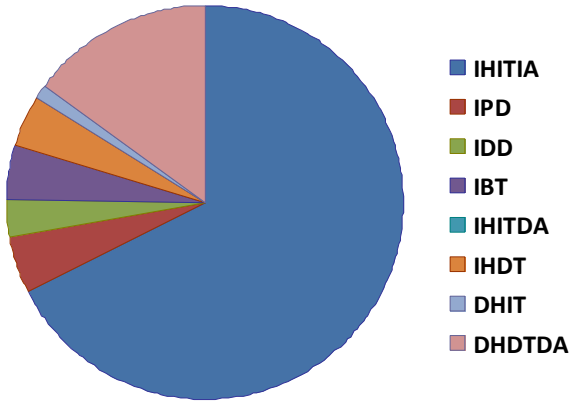


Figure 52. Distribution of viability sperm categories of fertile stallions (mean values of 10 stallions)

Mean values and standard deviations (SD) of the 10 stallions' data are presented in Table 16 and Fig. 52. Proportion of IHITIA is nearly 70% and percentage of DHDTDA sperm is only 15.1 ± 7.3 %. Rate of each sperm types which has damaged any part of the cell or has some morphologic defect is lower than 5%. The least cells are in IHITDA category (0.1 ± 0.2 %).

Viability evaluation of sperm of fertile stallions after 24 hours chilled storage

Table 17.Percentages of sperm in different viability categories in chilled-stored semen

Stallion	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
11	50.7	8.7	0.3	6.7	1.3	11.7	0.3	20.3
12	61.0	5.3	1.3	1.0	1.0	3.0	3.3	24.0
13	46.3	3.7	0.0	4.0	0.7	4.0	0.0	41.3
14	48.0	7.3	6.7	10.0	3.0	5.7	0.7	18.7
15	51.7	5.7	4.3	1.7	4.7	6.3	0.7	25.0
MEAN	**51.5**	**6.1**	**2.5**	**4.7**	**2.1**	**6.1**	**1.0**	**25.9**
SD	**5.7**	**1.9**	**2.9**	**3.7**	**1.7**	**3.4**	**1.3**	**9.0**

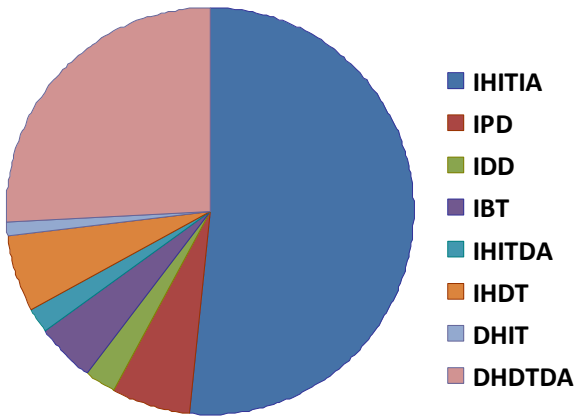


Figure 53. Distribution of viability sperm categories after chilled-storage (mean values of 5 stallions)

Results of evaluation of 5 fertile stallion sperm after 24 hours chilled storage based on viability combined morphology assesment are shown in Table 17 and Fig. 53. Results show that the proportion of IHITIA is lower (51.5 ± 5.7 %) than in the fresh semen of fertile stallions, however this rate is sufficient after 24 hours chilled-storage.

Subfertile stallions

Since the medical and breeding history of each stallion was not always complete and some important data (e.g. total number of sperm, or number of motile sperm in the insemination dose, the number of cycles of each mare inseminated, detailed data from the mares inseminated) were often missing, the evaluation of the given stallion samples and the discussion with incorporation of previous data and observations from the stallions will be interpreted in case reports.

Stallion „A” was an eleven-year-old Nonius breeding stallion. It had been housed under poor nutrition circumstances before it was stabled in the present artificial Insemination Station. The stallion was in poor condition. Gel-free sperm volume was 50-100 ml, sperm concentration was low: 40-50 million/ml and motility was 35-40% in the ejaculates. Per cycle pregnancy rate was 20% in the middle of the season and later increased to 30%. Viability and morphology evaluation of the semen was performed at the end of July.

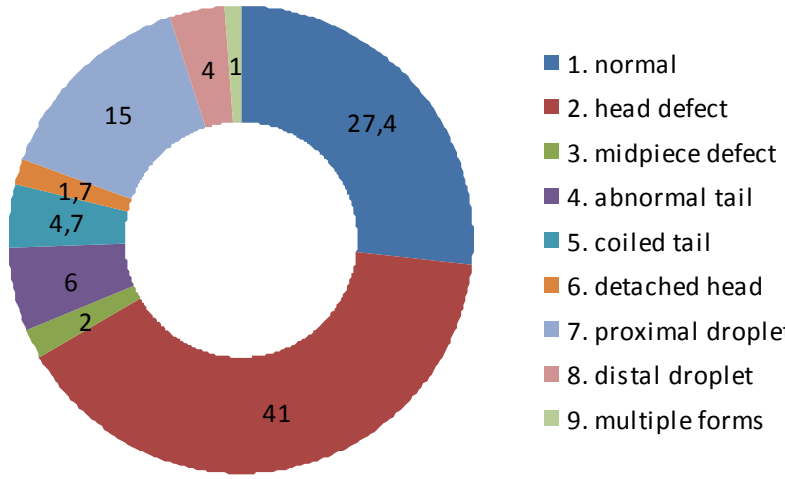


Figure 54. Percentages of sperm in different morphologic categories (fresh, diluted semen of Stallion „A”)

In Stallion „A” semen rate of head defect was extremely high (41%). Mainly microcephalic and tapered head were detected, often with proximal cytoplasmic droplets, or with tightly coiled tail together. Acrosome defects (often knobbed acrosomes) were also found frequently. Most of the spermatozoa with head defect were “dead”. Two percent of the sperm showed head defect in fertile stallions in our study. In the literature there are many different data in the range of 3-14% for proportion of abnormal head (Table 2.). Dowsett and Knott (1996) analysed the most different stallions and ejaculates (168 stallions from 9 breeds / 531 samples) and they found in average 5.4 ± 2 % head abnormality in the sperm. Percentage of head defects is ≤3-5% in stallions showing good fertility (Juhász and Nagy 2003). The proportion

of morphologically normal spermatozoa was 24.7%, which is much lower than this value in fertile stallions (78%). Percentage of abnormal sperm was more than 75% and within this around 60 % of primary defects. These rates are unsatisfactory and far above the strict guidelines of the Hungarian Standard for breeding stallion semen (7034/1999). Microcephalic sperm are probably the consequence of insults to primary and secondary spermatocytes that then have an uneven distribution of nuclear chromatin content after abnormal cell division (Brito 2007). Transport of sperm with tapered and pyriform heads is impaired and these sperm are selectively "filtered" throughout the female genital tract (Saacke et al. 1998) however some of them can reach the fretilization site. These cells have reduced ability to bind the zona pellucida but after binding can penetrate the zona and fertilize the oocytes (Kawarsky et al. 1995, Thundathil et al. 1999). Microcephalic spermatozoon is considered unable to bind and penetrate ZP. Because of selective filtration and reduced ability of zona binding these head abnormalities are considered compensable defects. The knobbed acrosome can be caused by environmental factors (eg. increased testicular temperature, stress, toxins) occurred more frequently in conjunction with other sperm abnormalities suggesting impaired spermatogenesis, but it could have a heritable basis in bulls which produce great percentages of affected sperm without significant changes in other sperm defects. Genetic origin has not been reported in stallions (Hurtgen and Johnson 1982, Barth and Oko 1989, Card 2005, Chenoweth 2005). In this case it appeared together with other head defects thus it most likely caused by impaired spermatogenesis. Occurrence of proximal cytoplasmic droplets is also considered primary defect in Stallion "A" because it accompanies with high rate of head defects in the ejaculates.

Viability evaluation of Stallion „A" semen

Table 18. Percentages of sperm in different viability categories (Stallion „A")

Status	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
Fresh, diluted	9.5	3.0	1.0	6.5	1.0	8.0	0.0	71.0
24 hours storage	7.0	0.0	0.0	2.0	0.0	5.0	1.0	85.0

The viability results of fresh and 24 hours chilled-stored semen at 4°C of Stallion "A" are shown in Table 18. In the fresh ejaculate there was only 9.5% intact, viable, morphologically normal sperm which decreased to 7% after 1 day storage. Proportion of DHDTDA sperm was very high (71%) already in the fresh semen. Probably the low rate of "live" sperm and the high proportion of primary defects caused the reduced fertility of Stallion "A". Primary defects are representing a failure of spermatogenesis

caused by pathological processes in the seminiferous epithelium. High proportion of "dead cells" is also implying to the disturbance of testicular function. High incidence of primary defects and damaged spermatozoa might be related to the poor conditition of Stallion „A" caused by unsatisfactory nutrition and probably some toxin-uptake from the forage. Most of the head defects are compensable abnormalities however the total sperm number was also reduced and together with the low viable cell rate, increasing insemination dose unlikely would have solved the problem. Appearance of high incidence of premature germ cells was not characteristic in the ejaculates thus the reason for subfertility seemed to be rather an intermittent stressor than testicular degeneration. After the collection of samples for sperm evaluation the condition of Stallion "A" was slowly improving and paralelly pregnancy results were also getting better. Sixty six percent of the mares (10 mares out of 15 mares) inseminated with semen of Stallion "A" became pregnant at the end of the breeding season (in October) which was still lower than generally observed in fertile stallions (Colenbrander et al. 2003, Juhász and Nagy 2003, Card 2010).

Stallion "B" was a nine-year-old Belgian coldblood breeding stallion. It had been housed under poor nutrition circumstances before it was stabled in the present artificial Insemination Station. Stallion "B" was in poor condition. Left testis was swollen, right testis had smaller than normal size. On this testicle later biopsy was taken but the histology result did not show pathological changes. Gel-free sperm volume was 50-100 ml, sperm concentration was 70-100 million/ml and motility was 30-40% in the ejaculates. Only some mares became pregnant after Stallion "B" (there is no precise pregnancy rate available). Viability and morphology evaluation of the semen was performed at the end of July.

In Stallion "B" semen, rates of head defects and proximal cytoplasmic droplets were increased (table 19, Fig. 55). Similar to Stallion "A", the proportion of sperm showed head abnormality was 41% (mainly microcephalic, tapered or degenerate head). In many cells head defect and proximal droplet occurred in parallel. Incidence of premature germ cells was slightly increased. Percentage of abnormal sperm was 72% and within this almost 60 % of primary defects (proximal cytoplasmic droplets are also considered primary defect in the case of Stallion "B" because these appeared with increased rate of head defects in the ejaculates).

Table 19. Percentages of sperm in different morphologic categories (fresh, diluted semen of Stallion "B")

Status	normal	head	midp	tail	coiled	detached	PD	DD	multiple
Fresh, diluted	28.0	41.0	4.7	4.0	5.7	3.7	10.0	3.0	0.0

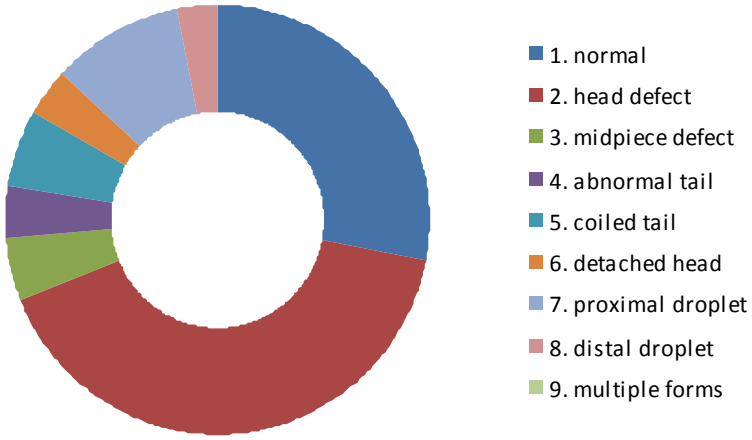


Figure 55. Distribution of morphologic sperm categories of Stallion "B"

Results of membrane integrity evaluation are shown in Table 20 and Fig. 56. In the fresh ejaculate there was only 14% intact, viable, morphologically normal sperm. After 1 day storage there wasn't seen any IHITIA sperm and percentage of each category in "membrane-intact spermatozoa with different morphologic defects" decreased to 1%.

Table 20. Viability evaluation of Stallion "B" spermatozoa. Percentages of different categories

Status	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
Fresh, diluted	14.0	6.0	0.0	5.0	0.0	4.0	5.0	66.0
24 hours storage	0.0	1.0	1.0	1.0	0.0	0.0	0.0	97.0

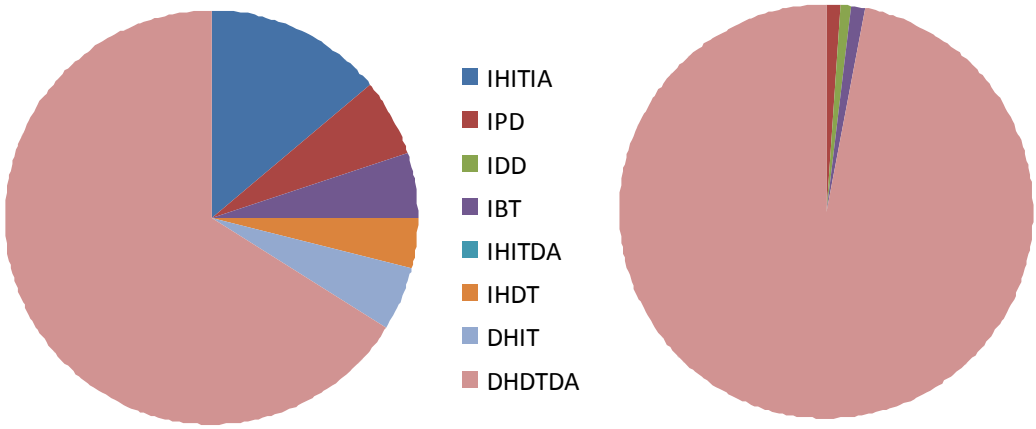


Figure 56. Distribution of viability sperm categories in the fresh semen and after 24 hours chilled-storage of Stallion "B" sperm

High proportion of primary morphologic defects was probably related to the observed testicular changes and the poor body condition of Stallion "B". Sixty six percent of "dead cells" also indicated the disturbance of testicular function. The cause of testicular disfunction in one hand might have been traumatic, in the other hand degenerative, but interestingly the result of histology evaluation was negative, consequently the stallion would have a chance for the possible recovery over time.

Stallion "C", a 22-year-old Shagya-Arabian breeding stallion had been suffering from RAO (Recurrent airway obstruction) disease for years. The sickness progressed and sperm quality was getting worse during the examined period. Therefore, only five mares were assigned to him in the studfarm, three became pregnant at the end of the season. Mares which were inseminated with transported semen did not get pregnant. Ejaculate's volume was 50-60 ml and contained much gel fraction. Gel-free sperm volume was 20-25 ml with low sperm concentration (50-70 million/ml). Viability and morphology evaluation of the semen was performed in June and also 2 months later in August.

Table 21. Percentages of sperm in different morphologic categories

Sample	normal	head	midp	tail	coiled	detached	PD	DD	multiple
June	32.5	16.0	9.5	1.0	2.5	4.0	31.0	3.5	0.0
August	30.0	19.3	11.3	2.3	5.5	4.0	24.3	3.3	0.0

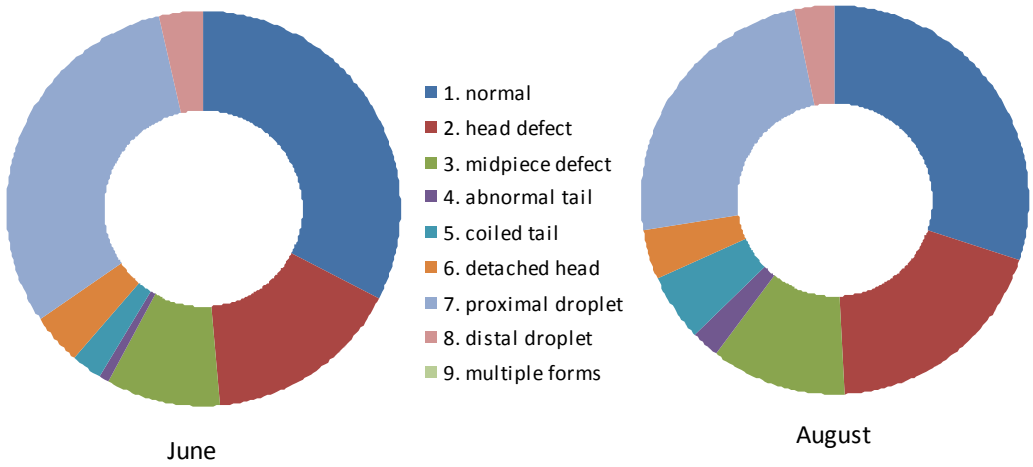


Figure 57. Distribution of morphologic categories of Stallion "C" spermatozoa in June and in August

In Stallion "C" ejaculates proportion of head defects (mainly microcephalic and tapered head), proximal cytoplasmic droplet and midpiece defect were increased (Table 21). Several cells showed more than one abnormality. A high percentage of

sperm with swollen, roughed midpiece or pseudodroplet and ‘‘corkscrew’’ defect was detected These rare defects might involve abnormalities of the mitochondrial sheet or accumulation of microtubular masses (Brito 2007) and are considered major morphologic problem with head defect and proximal droplet together. Major defects are mostly associated with a presumed disturbance of spermatogenesis. There was not found considerable changes in the percentages in different morphologic categories in the latter sample (in August) compared to the semen collected in June (Fig.57). Proportion of midpiece defect alone was 9.5% in June and 11.3% in August but in several cells the abnormality was also associated to head defect. The rate of this cell category is only 3.8 ± 1.9% in fertile stallions in our study and in most of the publications remains below 6% (Table 2). Percentage of abnormal sperm was around 70% and most of the defects were primary and also major abnormality.

Table 22. Percentages of sperm in different viability categories (fresh semen of Stallion “C”)

Sample	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
June	12.6	14.7	1.8	4.9	0.0	12.3	5.8	48.0
August	10.0	10.7	3.3	3.3	0.0	2.0	0.7	70.0

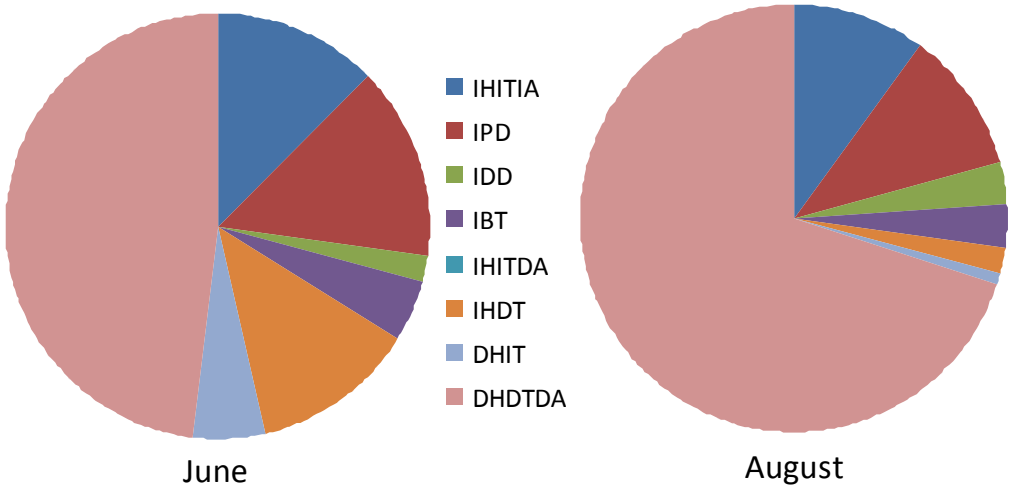


Figure 58. Distribution of viability categories of Stallion “C” spermatozoa in June and in August

The viability results heightened the morphologic problem, since the rate of intact, viable, morphological normal sperm (IHITIA) was fairly low at both collection time (12.6% in June and 10.0% in August). Not only the total value of proximal droplets irrespectively of live/dead status of spermatozoa was elevated (31% in June and 24.3% in August), but the proportion of sperm with proximal droplets within “live” cells (IPD) was also high (14.7% in June and 10.7% in August), this cell type

composed more than one-third of membrane-intact spermatozoa. Percentage of DHDTDA sperm increased considerable in semen collected in August compared to ejaculate taken in June (Fig. 58). Taking into account that Stallion "C" is an aged horse which has a progressive disease and sperm quality parameters has not improved over time the prognosis of fertility problem is bad. Changes in housing and environmental conditions, treatment of basic disease and using semen of the stallion only in the Stud, inseminating few mares with it may result in some pregnancies but regarding to the high proportion of primary sperm defects, chromatine analysis of spermatozoa is recommended preventing genetic abnormalities of the fetus.

Stallion "D" a 22-year-old Thoroughbred was used for natural service. The stallion was used as a sire on a commercial stud farm. There was no any foal born and any mare became pregnant after Stallion "D" in the year when the examination was performed. Gel-free volumes of ejaculates were ranged between 25-85 ml. Consistency was very thin, similar to watered milk contained 35-57 million/ml spermatozoa, motility and progressive motility was 65-80% and 33-42%, respectively. The motility results did not explained the infertility of the stallion so thus it came to perform the complex sperm staining method. Samples were collected in early September, 2 days apart and mean values were calculated.

Table 23. Percentages of sperm in different morphologic categories (fresh semen of stallion "D")

Sample	normal	head	midp	tail	coiled	detached	PD	DD	multiple
fresh	31.4	9.5	1.9	0.9	1.2	6.9	47.0	1.1	0.2

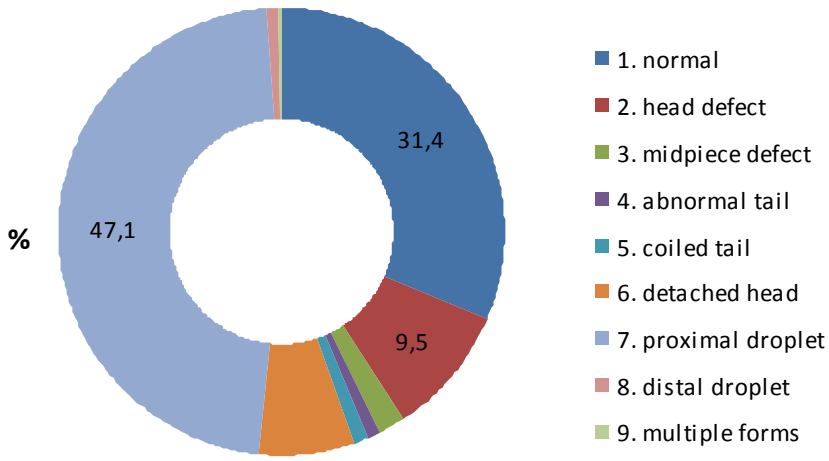


Figure 59. Distribution of morphologic sperm categories of Stallion "D"

Result of morphologic evaluation is shown in Table 23 and Fig. 59. It is clearly seen that proportion of spermatozoa with proximal cytoplasmic droplet was extremely high (47%) and the rate of these sperm within "live" cells (IPD) was also elevated (41.5%) composed about four-fifths of membrane-intact spermatozoa (Table 24, Fig. 60). Swollen, roughed midpiece also occurred together with proximal cytoplasmic droplet in some spermatozoa. Proportion of head abnormalities (mainly elongated, microcephalic and tapered head) was also higher (9.5%) than it was observed in sperm of fertile stallions (2%) in our study.

Proximal droplets may be either primary defect (result of a disturbance of spermatogenesis) or secondary defect (result of disturbance of epididymal function). Its occurrence together with increased proportion of head abnormality is considered primary defect. In the case of epididymal disfunction the number of sperm with distal droplets is also increased. In the ejaculates of Stallion "D" the proportion of sperm with retained distal droplet was only 1%, consequently elevated number of PD is due to testicular disfunction. Zona pellucida binding (ZPB) and also capacitation of PD spermatozoa are disturbed according to the literature (Peña et al. 2006). Moreover, other genetic defects in morphologically normal sperm which was capable of fertilizing oocytes probably contributed to the impaired embryonic development observed in vitro after the use of semen from bulls producing a large percentage of sperm with proximal droplets (Amann et al. 2000, Thundathil et al. 2001). High percentage of retained CDs in semen could negatively influence fertility directly by the function disorder (motility, capacitation binding affinity alteration) of affected spermatozoa or indirectly by releasing active enzymes and reactive oxygen species into seminal plasma and may be into the ovum. According to the theories - that sperm with CDs are partly filtered out during the sperm transport in the female genital tract but one portion of these cells reach the oocyte however supposed to fail to bind zona pellucida; otherwise enzymes of the droplets (ubiquitin, 15-LOX) effect on the non-defected normal spermatozoa - CDs are considered to be a non-compensable or semi-compensable defect. High proportion of sperm with PDs within intact spermatozoa in Stallion "D" has a negative effect on the fertility of the semen.

Table 24. Viability evaluation of Stallion "D" spermatozoa. Percentages of different categories (%)

Sample	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
fresh	12.0	41.5	0.5	1.0	1.5	9.0	11.0	23.5
24 hrs storage	11.7	40.0	0.5	3.7	0.0	13.7	3.6	26.9

*Fresh and 24 hours chilled stored samples were from semen collection on 2 different days

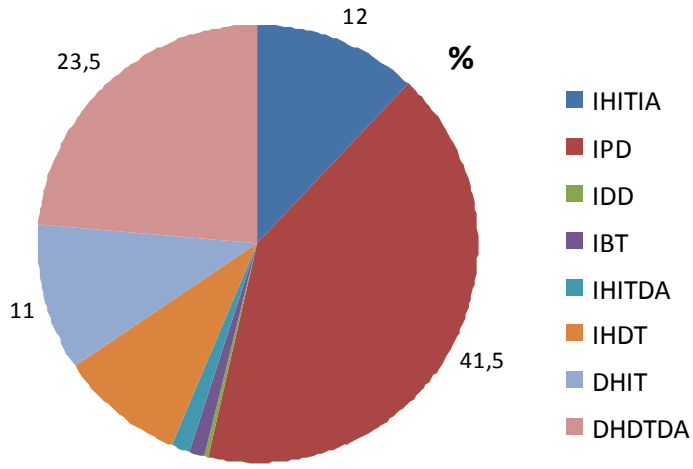


Figure 60. Distribution of viability categories of Stallion "D" spermatozoa in fresh ejaculate

The viability results showed increased rate of sperm with damaged head and intact tail membrane (DHIT: 11%). These spermatozoa could be visible as motile sperm in routine examination, however due to membrane lesions on the head and acrosome are not able to fertilize the ovum. Proportion of IHITIA was very low (12%). The low percentage of intact, viable, morphologically normal sperm and the absolute and relative (within membrane-intact sperm) high proportion of spermatozoa with proximal droplets could cause the infertility of Stallion "D".

Stallion "E" was a 9-year-old Shagya-Arabian breeding stallion which had low libido earlier in natural service and also in the artifitial insemination station when semen was collected from him. There had not been any foal born and any mare become pregnant after Stallion "E" in the previous two years. Routine examination of the semen showed very low motility. Viability and morphology evaluation of the semen was intended to be carried out in May before the final decision of withdrawal the stallion from breeding.

Table 25. Percentages of sperm in different morphologic categories (fresh semen of stallion "E")

Sample	normal	head	midp	tail	coiled	detached	PD	DD	multiple
fresh	40.5	6.7	11.3	13	2.5	1.3	22.7	2	0

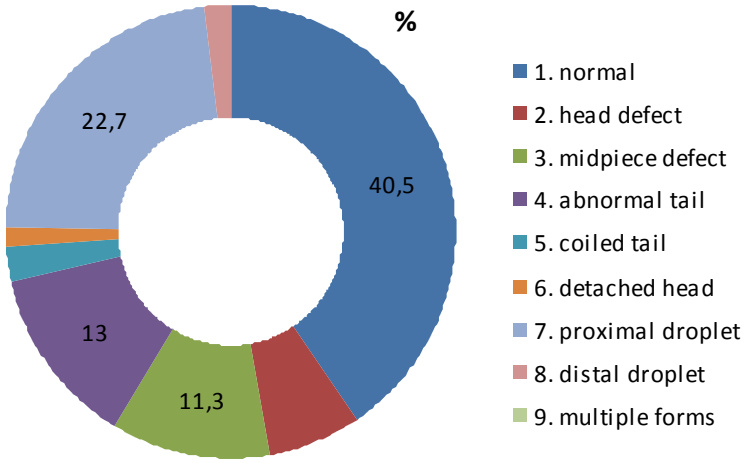


Figure 61. Distribution of morphologic sperm categories of Stallion "E"

Proportion of spermatozoa with normal morphology was 40.5% shown in Table 25. and Fig. 61. Rate of midpiece defects (mainly DMR and bent midpiece) and tail defects (broken, bent, hairpin curved, and coiled tails) was slightly elevated compared to the avarege values (see the references in Table 2). Percentage of spermatozoa with retained proximal cytoplasmic droplet was considerable increased (22.7%) without presentation of high rate of distal droplets. The cause of DMR, bending and coiling of the tail can be a response to abnormal secretion in the epidydimal tubules during sperm passage or of accessory glands during ejaculation (Swanson and Boyd 1962, Barth and Oko 1989, Barth 1994). Similar defects which involve fractures or double bends probably originate in the last steps of spermatogenesis and are usually found together with the epididymal forms (Barth 1994). Blom (1977) suggested that DMR and tail defects were minor defects. Due to the results of morphologic evaluation it may be hypothesized both testicular and epididymal origin of reduced fertility and together with poor libido, endocrine disfunction is also presumable.

Table 26. Percentages of sperm in different viability categories (fresh semen of Stallion "E")

Sample	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
fresh	1.5	4.5	0.5	5.0	1.5	28.0	0.0	59.0

Proportion of membrane-intact sperm and within this, viable, morphologically normal sperm (IHITIA) were very low (11.5% and 1.5%, respectively) in the fresh semen. Rate of spermatozoa with intact head but damaged tail membrane was 28%, DHDTDA sperm were presented in 59% in the ejaculate, which were in correspondence with the low motility results. Necrospermia (high proportion of dead

sperm) may be due to genetics, anabolic steroids, toxins, thermal injury, local infections, trauma etc. (Card 2010). Genetic examination, hormone analysis, testicular tissue histology and analytical examination of compound of seminal plasma are recommended to perform to clarify the cause of infertility. However due to the more years history of fertility problem the prognosis is not promising According to the very low viable sperm number, using the sperm for conventional AI, result of any pregnancy is almost impossible. After reassuring results of chromosome analysis a very few spermatozoa may be separated using adequate laboratory methods (Percoll, single layer centrifugation, glass wool centrifugation etc.) then these sperm might be applied for intracytoplasmic sperm injection (ICSI).

Stallion "F" was a 24-year-old Thoroughbred stallion which had had a low pregnancy rate for several breeding seasons. In the year of examination no pregnancies had been obtained from semen doses until July when the viability and morphology evaluation of the semen was performed. There was problem also with collecting semen from the stallion (time required for preparing and for semen collection was delayed, several jumping without ejaculation, and often no ejaculation at all). Gel-free sperm volume was 50-90 ml, consistency was white, milk-like or similar to watered milk. Sperm concentration was moderate or good and 60-70% motile sperm was observed.

Table 27. Percentages of sperm in different morphologic categories (fresh semen of stallion "F")

Sample	normal	head	midp	tail	coiled	detached	PD	DD	multiple
fresh	52.5	21.6	9.4	3.8	3.8	1.3	6.6	1.3	0.0

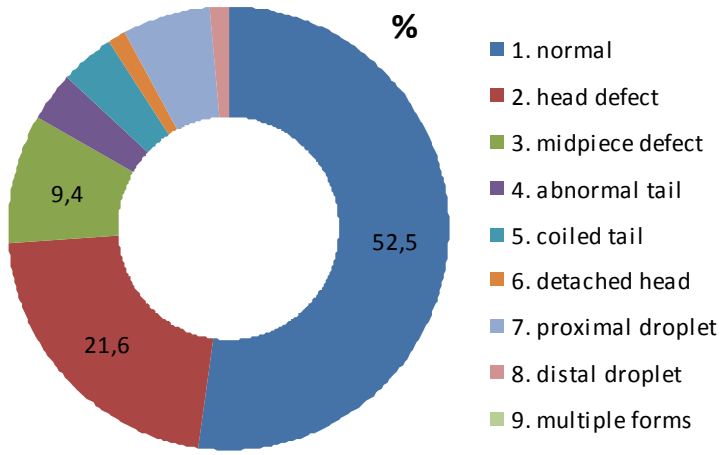


Figure 62. Distribution of morphologic sperm categories of Stallion "F"

Proportion of spermatozoa with normal morphology was 52.5%, which is lower than the average values according to the references in Table 2, but in special cases and when the morphologic abnormalities can be compensated with increased sperm number in the insemination dose, is acceptable. In our study the mean value of this category is much higher (78.4 ± 5.3%) in the fertile stallions. Number of sperm with head defect (21.6%) and midpiece defect (9.4%) was increased, several times these abnormalities occurred together in one sperm. Thus the proportions of the two categories are remarkable elevated in the ejaculate. Most of the spermatozoa with head defect were "dead". The head abnormalities were different, microcephal, tapered, elongated, pyriform and assimetric heads were detectable. Polymorphism of head shape and size were also observed. These findings indicated the disturbance of spermatogenesis and testicular origin of the problem. Rates of sperm in the other abnormal morphologic categories were not higher than in fertile stallions observed and correspond to the average values according to the references.

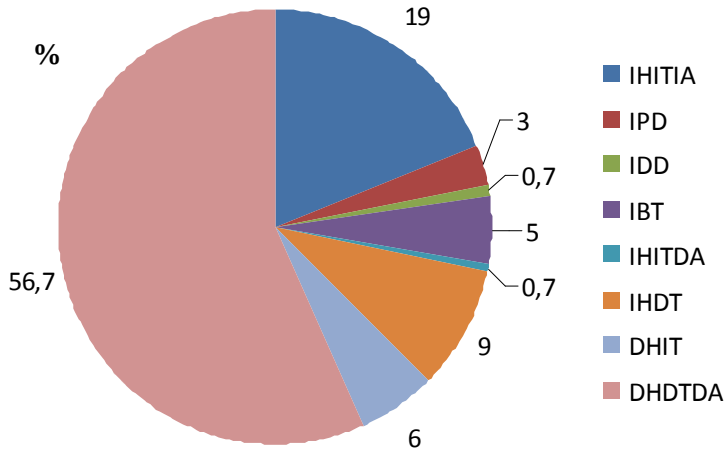


Figure 63. Distribution of viability categories of Stallion "F" spermatozoa in fresh ejaculate

Results of viability evaluation also reveal the cause of declined fertility of Stallion "F". Proportion of all membrane-intact sperm (IHITIA + IPD + IDD + IBT) was only 27%. The rate of intact, viable, morphologically normal sperm was 19%, which is much lower than the proportion of sperm in the same category in fertiles stallions (67.4 ± 7.9%). The low rate of intact, viable spermatozoa and the elevated proportion of primary defects can explaine the subfertility of Stallion "F". Declined fertility has a poor prognosis according to the old age of Stallion "F", a long history of subfertility and libido problem in anamnesis. Viable, intact, morphologically normal spermatozoa can be separated using adequate laboratory methods after semen collection. Selected sperm can be used for inseminating effectively some mares (Morrell et al. 2009b,c; Morrell and Rodriguez-Martinez 2010; Morrell et al. 2011). In this case this method

seems to be the last chance for resulting pregnancy. /It did not come to perform any separation method because at the end of the season the stallion had been withdrawn from breeding/.

Stallion "G" was a 10-year-old Trotter stallion. The gel-free volumes and sperm concentrations of the ejaculates were very low (the month average was in March: 53 ml, $70x10^6$/ml and in April when the examination was performed: 49 ml, $91x10^6$/ml). The low number of spermatozoa per ejaculates had limited the booking of mares outside of the Stud and pregnancy results of using transported semen of Stallion "G" had not been satisfactory therefore they decided to inseminate only the broodmares in the Studfarm. The stallion came from Germany in the beginning of the year and it had been the same problem with him in the previous breeding season hence they had not wanted to bother with him any longer. The gel-free volume of the semen was 60 ml, sperm concentration was $60x10^6$/ml and motility was 65-70% on the day of viability and morphology evaluation.

Table 28. Morphologic evaluation of Stallion "G" spermatozoa (%)

Status	normal	head	midp	tail	coiled	detached	PD	DD	multiple
fresh	50.0	1.0	13.2	3.6	2.9	2.1	14.7	11.5	1.0

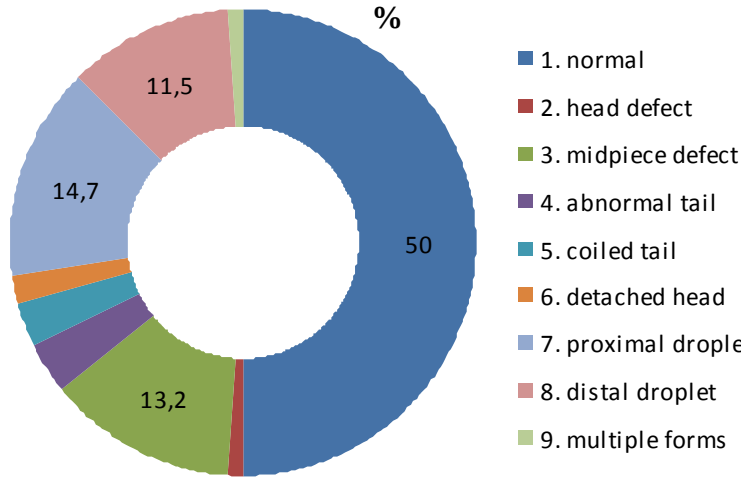


Figure 64. Distribution of morphologic sperm categories of Stallion "G"

Proportion of spermatozoa with normal morphology was 50%, which is lower than the average values according to the references in Table 2. Rate of midpiece defect was high (13.2%), mainly broken, bent and roughed midpiece was observed or fracture at the neck area. The percentage of this cell category is only 3.8 ± 1.9% in fertile stallions in our study and in most of the publications remains below 6% (Table 2).

Since both of the proportion of spermatozoa with proximal and distal cytoplasmic droplet was increased (14.7 és 11.5%), secondary origin of the sperm anomalies (epididymal disfunction and more frequent sperm collection) is presumable.

Table 29. Percentages of sperm in different viability categories of Stallion "G"

Sample	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
fresh	36.3	10.0	8.0	10.0	0.3	12.7	0.9	21.8
10 hrs storage	37.5	7.3	9.3	9.3	0.5	5.8	0.0	30.5
18 hrs storage	27.5	10.5	8.5	9.0	0.8	6.3	0.0	37.5
24 hrs storage	26.3	7.5	9.5	9.5	1.0	8.3	0.0	38.0

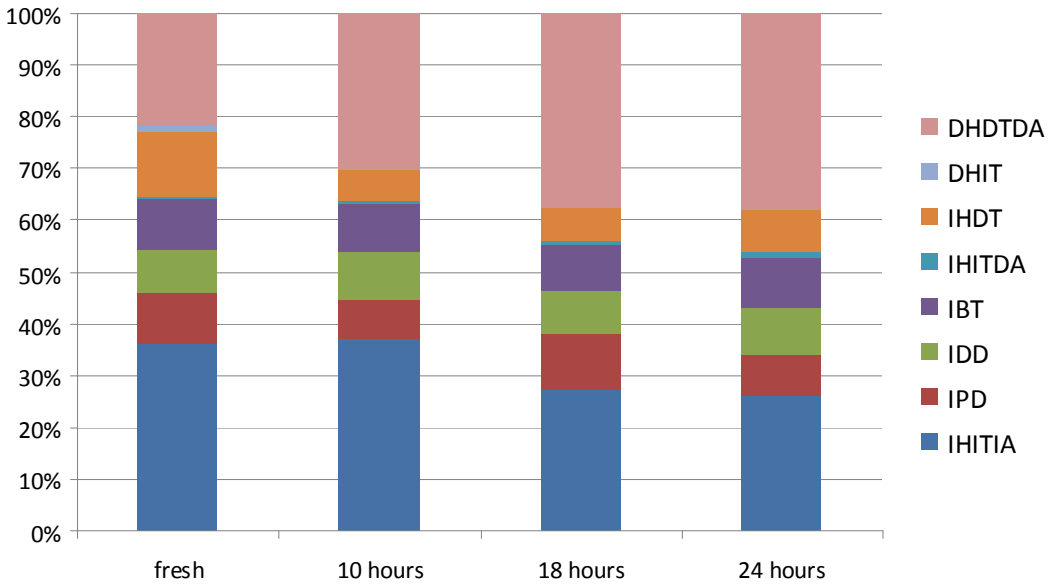


Figure 65. Distribution of viability categories of Stallion "G" spermatozoa in fresh and chilled-stored semen

Results of viability evaluation also contributed to the clarification of the problem. Analyses of the sample were performed after collection, 10, 18 and 24 hours chilled-storage. Proportion of all membrane-intact sperm (IHITIA + IPD + IDD + IBT) was 64.3% in the fresh semen. The percentage of these spermatozoa was 77% in fertile stallions in our study. The rate of intact, viable, morphologically normal sperm was low already after collection 36.3%, which decreased to 26.3% after 24 hours storage. These values were much lower than the proportion of sperm in the same category in fertiles stallions (67.4 ± 7.9% in the fresh and 51.5 ± 5.7% in the 1-day-stored semen).

Viable spermatozoa with retained proximal and distal cytoplasmic droplet and midpiece defect were represented in large proportion in the fresh semen. Percentage of spermatozoa in each category was 8-10 % and contained 44% of membrane-intact sperm. Their ratios were nearly not changed and were presented in 50% of all membrane-intact sperm in the 24-hour-stored sample. Since IBT spermatozoa have reverse motility it is unlikely that they would be able to penetrate the zona pellucida and initiate zona reaction. Therefore the presence of these sperm is compensable because the defected spermatozoa are not compiting with normal sperm in ovum fertilization. Proximal and distal droplet defects are semi-compensable problem. They are separated in the mare's reproductive tract and sperm with proximal droplet have also reduced capacity to bind ZP. However CDs affect on longevity of the sperm due to releasing active enzymes and reactive oxygen species into seminal plasma thus use of further applicability of the stored (transported) semen is limited.
These results have pointed out to the importance of defining of the ratio of membrane-intact and morphologically normal spermatozoa. Taking this into account in determining the sperm number in the insemination dose is recommended. The further distribution of the ejaculate and allocating the number of mares inseminated had been based on this way, hence it was possible to achieve good pregnancy outcome in the breeding season and 75% end of the season pregnancy rate of Stallion "G".

Stallion "H", a 5-year-old Arabian stallion came from England in the beginning of the season. This was the first season to start as a breeding stallion. Twelve mares had been inseminated with his sperm but no pregnancies had been obtained from semen doses prepared by conventional semen processing until the date of examination. The stallion had good libido, and there were not any abnormality found during physical examination. Volume of the ejaculates was 15-20 ml, gel-free volume was 12-15 ml and sperm concentration was medium or good. Motility values were varied, but marked anomaly was not observed in native microscopic examination. Generally non-fat dry skim milk (NFDSM) extender was used for dilution of each stallion semen routinely in the AI station. In this case the veterinarian intended to try also an egg-yolk-skim-milk-based (EY-SM) extender. Viability combined morphology evaluation of the semen was performed at the end of June. Aliquots of gel-free ejaculate were diluted with NFDSM or EY-SM extender. Analyses of the samples were performed after collection and after cooled-storage for 24 and 48 hours. Results are shown in Table 30 and Fig. 66.

Table 30. Percentages of spermatozoa in different viability categories of Stallion "H" (fresh and chilled-stored samples)

Sample	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
Fresh (EY-SM)	35.8	8.3	0.8	11.3	1.0	3.5	1.3	38.3
Fresh (NFDSM)	12.0	2.5	1.5	18.0	0.0	5.0	0.0	61.0
24h (EY-SM)	29.0	3.5	0.5	11.5	7.5	3.5	0.0	44.5
24h (NFDSM)	1.0	1.0	0.0	9.0	0.0	6.0	1.0	82.0
48h (EY-SM)	22.5	2.5	0.0	9.0	6.0	6.5	1.0	52.5

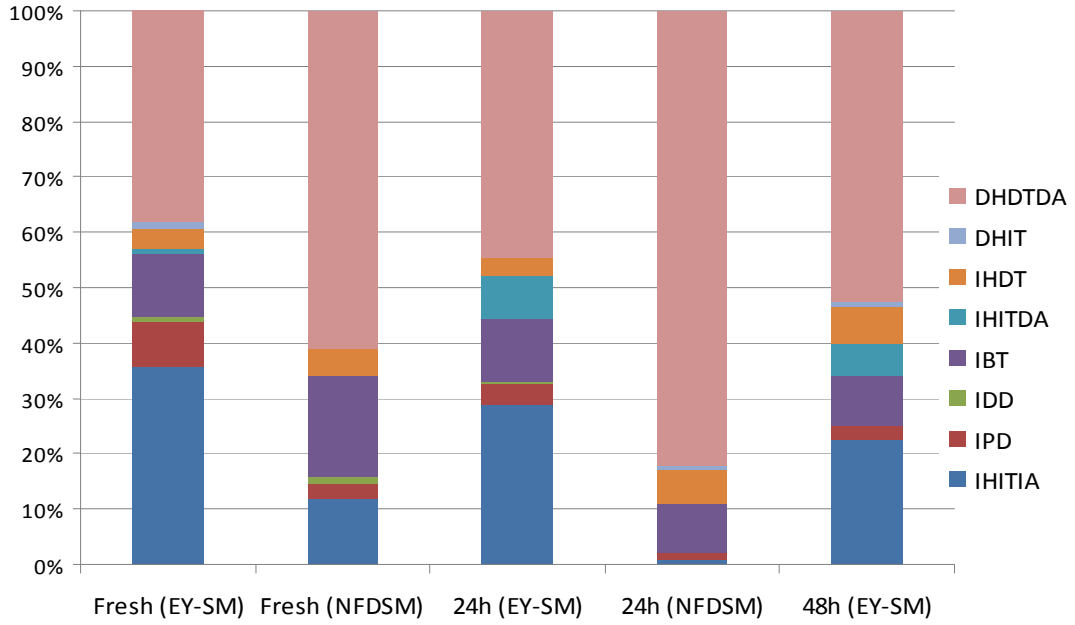


Figure 66. Distribution of viability categories of Stallion "H" spermatozoa in fresh and chilled-stored semen

The percentage of spermatozoa with intact head-, tail- and acrosome membrane and normal morphology in the fresh semen diluted with egg-yolk based extender was 35.8%, which is lower than can be seen at the avarage and good quality semen of fertile stallions. Proportion of all membrane-intact sperm (IHITIA + IPD + IDD + IBT) was 56 % in the EY-SM diluted fresh semen and decreased to 44.5% and 34% after 24 and 48 hours storage, respectively. Reduction of number of membrane-intact and IHITIA cells during storage is considered normal and acceptable. Remarkable differences were found in the results of viability evaluation between EY-SM and NFDSM diluted samples (Fig. 66). Spermatozoa showed poor sperm survival in NFDSM diluted-cooled semen. Sperm couldn't tolerate NFDSM extender for any reason, what can be observed in both of fresh and cooled-stored semen: proportion of DHDTDA sperm was 61% in the fresh and 81% in the 24 hours-stored semen. IHITIA

spermatozoa were presented in 12% in the fresh semen and only 1 % in the 1 day-stored sample. Despite this, interestingly the percentage of IBT sperm was much higher in NFDSM diluted semen (18%) than in EY-SM diluted semen (11.3%) which shows an effect of some stress factor caused by NFDSM extender on Stallion "H" spermatozoa. Spermatozoa response to sublethal effects characteristically. Cold-, warm and hypoosmotic shock induce bent-looped, coiled tail of sperm due to changes in water-permeability of cell membrane (Devireddy et al. 2002). The stallion produced low volume of ejaculates which suggested that maybe non-physiological ratios of components of seminal plasma caused by one or more accesory sex gland disfunction contributed to elevated sensitivity of spermatozoa to environmental stress factors. Probable the spermatozoa of Stallion "H" were highly sensitive for the compounds of extenders and it may be possible also for the chemical milliou of the genital tract of the mare. The latter presumption had been not confirmed afterwards: The breeding utilization of the stallion was changed for natural service at the end of the season, two mares were mated and both of them became pregnant.

Stallion "I" was a 13-year-old Trotter breeding stallion, came from Germany in April for only one season. The Stud veterinarian was not satisfied with the quality of the semen, which had been declined during the season (sperm concentration decreased, many cells were moving in circle and backwards) and pregnancy results were also getting worse. Viability and morphology evaluation of the semen was performed in early June and also at the end of July. Fresh (in the Stud in June and July) or cooled-transported /TRP/ (in 6 hours after collection in July) sperm was analysed. The transported semen collected at the end of July was also evaluated after 24 hours chilled-storage. Results of morphologic examination are shown in Table 31.

Table 31. Morphologic evaluation of Stallion "I" spermatozoa (%)

Sample	1 normal	2 head	3 midp	4 tail	5 coiled	6 detached	7 PD	8 DD	9 multiple	3+4+5
06.09. Fresh	52.7	2.3	22.7	3.7	2.3	7.0	4.7	4.7	0.0	28.7
07.20. Fresh	42.0	2.0	21.3	10.8	5.0	7.5	2.1	9.3	0.0	37.1
07.28. TRP 6 hrs	44.5	2.5	13.6	13.1	8.9	11.0	2.0	4.4	0.0	35.6
07.28. 24 hrs	46.5	4.0	14.5	14.0	7.5	7.5	1.5	4.0	0.5	36.0

Rate of morphologically normal sperm was decreased with 8-10% from June (52.7%) to July (42 and 44.5%). Proportions of midpiece abnormality (21-23% in the fresh semen) and also tail defects were high, mainly included DMR, bent midpiece and tails, coiled or broken tails. Cytoplasmic droplet was very often entrapped in the bend. Proportion of midpiece and tail defects altogether (categories 3 + 4 + 5; Table 31) was 28.7% in the ejaculate collected in June and increased to 36-37% in the samples taken in July. Rate of midpiece defect was only 3.8 ± 1.9% in fertile stallions in our study and in most of the publications is less than 6% (Table 2). Percentage of tail defects was in average around 5% in the case of fertile stallions in our study (Table 15). Much more tail defects were found in ratios of 16-22% in Stallion "I" sperm collected in July (category 4 + 5; Table 31).

Viability evaluation of Stallion "I" semen

Table 32. Percentages of spermatozoa in different viability categories (fresh, transported and 24 hours cooled-stored samples)

Sample	IHITIA	IPD	IDD	IBT	IHITDA	IHDT	DHIT	DHDTDA
06.09. Fresh	37.7	2.3	4.3	13.3	0.0	2.7	4.0	35.7
07.20. Fresh	30.0	0.0	11.0	17.5	0.5	5.0	2.5	33.5
07.28. TRP 6 hrs	33.3	0.5	6.0	20.3	0.0	4.0	0.3	35.8
07.28. 24 hrs	19.3	0.7	0.7	15.3	2.0	4.0	0.7	57.3

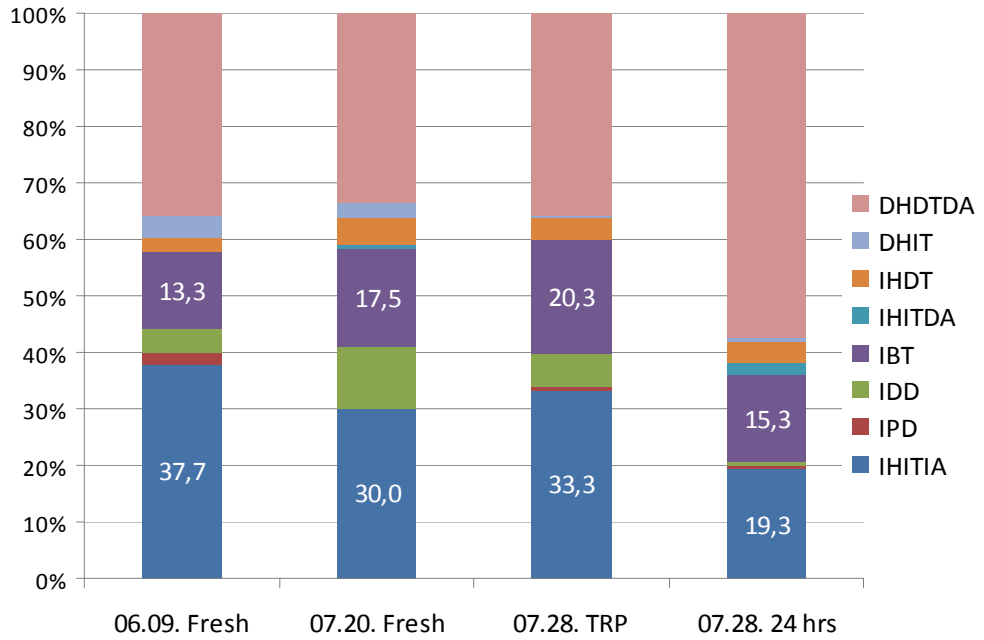


Figure 67. Distribution of viability categories of Stallion "I" spermatozoa in fresh and chilled-stored semen

Percentage of IHITIA sperm was 38% in June and 30-33% in July in the fresh or transported semen. This ratio decreased to 19.3% after 24 hours cooled storage. Semen contained high proportion of "live" cells with midpiece and tail defect (mainly bent, broken, curved, coild midpiece and tail) /13-20% in the fresh and transported semen/. The rate of this cell category was only 4.5 ± 3 % in fertile stallions in our study. Proportion of all membrane-intact sperm (IHITIA + IPD + IDD + IBT) was 58-60% in the fresh and transported semen, and half of these spermatozoa had normal morphology and one-third of membrane-intact sperm showed midpiece or tail anomaly in the samples collected in July. These types of sperm generally show abnormal motion (circular and reverse motion characteristics) which observation was in correspondence with the motility results in the case of Stallion "I". After 24 hours storage the ratio of IBT sperm was increased to 42.5% within membrane-intact cells. The low rate of IHITIA sperm presented in the ejaculates may have caused the reduced fertility of the stallion. The sperm morphologic problem of the Stallion "I" is considered due to epididymal disfunction and maybe stress factors caused by transportation, arriving to the new stable and environmental changes. Alteration of accessory sex glands' secretums may also influenced the last steps of sperm maturation hence release of droplets and formation of loop, bend in the midpiece and tail with enclosed CD. These sperm are either selectively filtered in the female genital tract or unable to penetrate the zona pellucida at the fertilization place (Barth 1994, Saacke et al. 2000), and can be compensated by increasing sperm number in the insemination dose. Management also plays a critical part in reproductive performance and good, intensive management can substantially improve the 'apparent' fertility of many poorly performing stallions, primarily by ensuring insemination closer to the time of ovulation (Colenbrander et al. 2003). In conclusion measuring of total number of sperm in the ejaculate and determining and increasing of the insemination dose based on the previous viability and morphology results is advised. In this way fewer mares can be inseminated from one ejaculate and more intensive mare management and/or induction of ovulation may be necessary but efficiency would be enhanced.

Stallion "J" was a 16-year-old Trotter breeding stallion, came from Germany in the beginning of the season. Some mares inseminated had become pregnant in early of the season, later the fertility results declined. The ejaculate contained large amount of gel fraction, consistency of gel-free semen was very thin, water-like, with a low sperm concentration which caused a problem in obtaining sufficient spermatozoa for a standard sperm dose (500 million PMS) in a manageable volume (20-30 ml of extended semen). Motility of the low numbers of spermatozoa was acceptable. Viability and morphology evaluation of the semen was performed at the end of July.

Table 33. Percentages of sperm in different morphologic categories (fresh semen of stallion "J")

Sample	normal	head	midp	tail	coiled	detached	PD	DD	multiple
fresh	50.5	3.3	8.8	2.0	1.5	1.7	5.0	26.8	0.3

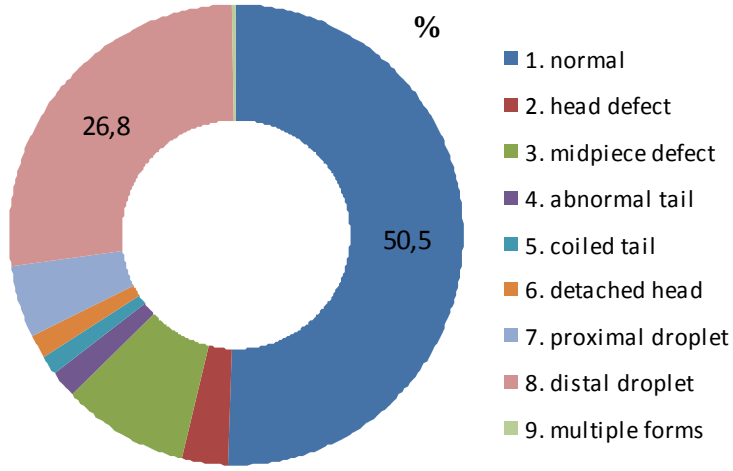


Figure 68. Distribution of morphologic sperm categories of Stallion "J"

In Stallion "J" semen the proportion of spermatozoa with normal morphology was 50.5%, which is lower than the average values according to the references in Table 2. Among the values of different morphologic categories the rate of spermatozoa with distal cytoplasmic droplet (DD) was emerged (26.8%). Percentage of this sperm abnormality was 2-6% in the stallions studied in different publications (Table 2) and in average 4.1 ± 3.4% in fertile stallions in our study (Table 15). Nine percent of the cells showed midpiece defect, mainly DMR, some bowed, thick or roughed midpiece was observed. The effect of a retained DD on fertility is less well defined, although there are more and more results suggesting a negative impact for such semen used in artificial insemination (Kuster et al. 2004, Pesch and Bergmann 2006) due to the active enzymes of retained CDs and elevated ROS levels in the seminal plasma. In boars, the proportion of spermatozoa with distal CDs in stored semen had a negative correlation with pregnancy rates and litter size (Waberski et al. 1994).

Percentage of all membrane-intact spermatozoa (IHITIA + IPD + IDD + IBT) was 83% in Stallion "J" semen which is rather high rate. The proportion of intact, viable, morphologically normal sperm was 47% and the rate of "live" cells showing distal droplet (IDD) was elevated (21.3%) /Fig. 69/. IBT sperm was also presented in slightly increased percentage (11.3%). Ratio of IDD spermatozoa is 3.1 ± 2.9% in the

ejaculates of fertile stallions (Table 15). Incidence of sperm with proximal droplet was not prominent. High proportion of cells with distal droplet can be the result of epididymal malfunction but may be caused by a lack of a haemolytic factor in seminal fluid which is one of the products of seminal vesicle that enhances cytoplasmic droplet release (Barth and Oko 1989).

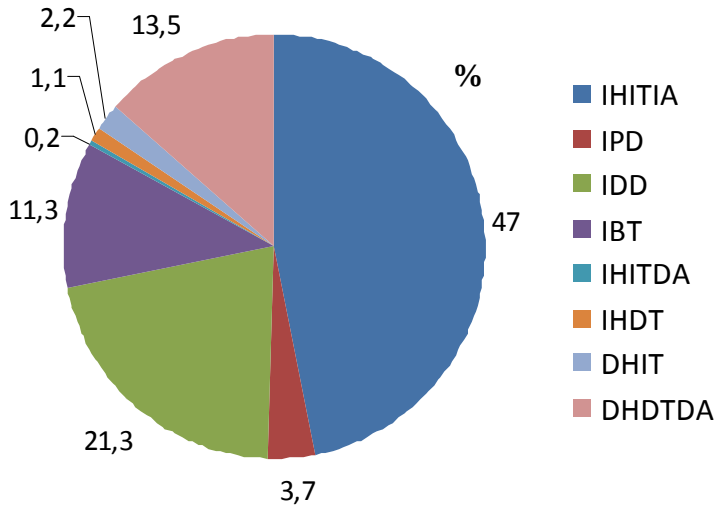


Figure 69. Distribution of viability sperm categories in the fresh semen of Stallion "J"

Due to the anamnesis, sperm concentration of the semen was low. And although the all membrane-intact spermatozoa were presented in high proportion and the motility of the sperm was also good, 43.5% of these cells showed morphologic abnormality. Therefore, the low fertility observed in this case was likely because of insufficient total number of fertile, normal, intact spermatozoa. It is important to allocate the ration of intact, normal sperm and to take into account this in determining cell number in the insemination dose. Recently a new method, single layer centrifugation (SLC) through a species-specific colloid (Androcoll) was developed which uses only one layer of colloid. Thus, time is saved during preparation and the method can be scaled-up successfully to allow large volumes of semen (e.g. the whole stallion ejaculate) to be centrifuged to produce sufficient numbers of spermatozoa for stallion AI doses. SLC consistently improves the quality of stallion sperm samples which can be used for inseminating some mares resulting in successful pregnancy (Morrell et al. 2009c, Morrell and Rodriguez-Martinez 2010, Morrell et al. 2011). The viability and morphology evaluation was performed at the end of the season, so thus further sperm preparations had been not carried out, and the stallion went back to Germany.

6. DISCUSSION AND CONCLUSIONS

6.1 Experiment 1. Improvement of assessment of stallion sperm quality by Chicago sky blue and Giemsa viability and acrosome staining method

Chicago sky blue 6B stain has been applied widely in the biological sciences including studies in vivo. For example, increased uterine vascular permeability can be detected by CSB staining. This "blue spotting" method is used as a marker of the implantation site at early stages of pregnancy in rats (Lundkvist and Ljungkvist 1977), rabbits (Garside et al. 1996) and mice (Cheng et al. 2004).

After freezing and thawing, a high proportion of spermatozoa with unstained heads and stained tails are observed. These cells are immotile (Nagy et al. 1999, Domes and Stolla 2001). Therefore, unambiguous differentiation of intact and damaged sperm tail membrane is important for evaluating semen quality. Using the TB/Giemsa method for staining stallion sperm, differentiation of intact or damaged sperm tails was problematic, mainly with frozen and thawed samples. Stallion spermatozoa are small and the larger number of seminal plasma and extender proteins binding to TB make color differentiation of live and dead tails less clear. Dyes such as TB and CSB are used simply as anionic dyes in histology, but they also have the capacity to bind directly, presumably by hydrogen binding, to different proteins including those with linear structures (Lillie 1977). CSB has a stronger affinity for the proteins of the sperm tail than does TB. This probably is due to the structure of the molecules, because CSB has two more groups that are capable of hydrogen binding than TB (Fig. 3). CSB resulted in similar live/dead sperm head differentiation, but a better tail differentiation than TB. This was verified by densitometry (Figs. 19-21) and with subjective evaluation by light microscopy (Figs. 13 and 14). By microscopy, the cells are much smaller than on the screen; therefore, the greater difference in the color intensities of the live and dead cells is important. The 80% greater differences in the brightness levels between the live and dead tails after CSB compared to TB staining (Figs. 19 and 20) allows easier differentiation. The background after CSB staining is more uniform compared to TB.

Repeatability of staining methods is critical. The CSB/Giemsa staining method gave good repeatability and agreed with the standard TB/Giemsa method (Fig 16-18). Therefore, we conclude that the TB viability stain can be replaced by CSB for staining stallion sperm, thereby providing more reliable evaluation.

In our preliminary study, fixation for more than one day after viability staining caused pale discoloration of the head. Therefore, fixing smears as soon as possible after the viability staining, is advised. Fixation for 4 min resulted in darker dead staining with acceptable background. Acrosomes of equine spermatozoa are stained more rapidly than those of other domestic mammals. Giemsa staining below 20° C does not work; it is more effective at 25-40° C. Contact with air is also important, especially with short dye exposure. The dilution rate of stallion semen with PBS is only 1:4-1:9 owing to its low cell concentration. Therefore, the proteins of the seminal plasma and the extender (milk and/or egg yolk) cause denser background staining. Overnight staining can disturb the differentiation of the live and dead tails mainly in the case of frozen semen. Background caused by seminal plasma and extender proteins was greatly reduced on slides stained for only 2-4 h with Giemsa. In conclusion, after staining with 0.16% CSB and 4 min fixation, 2 - 4 h Giemsa staining at 25-40° C is recommended for stallion semen.

The acrosome of dead spermatozoa usually is damaged or missing, and rarely intact (≤ 1%) in stallion semen. For evaluating membrane integrity based on staining characteristics of the sperm cell subdomains, we generally classified the cells into five practical categories: intact head, tail and acrosome membrane; intact head, tail and damaged or lost acrosome; intact head with damaged tail; damaged head with intact tail; damaged head, tail and acrosome. This viability evaluation also can be performed in combination with morphological assessment. Consequently, more informative classifications of sperm among the live, intact cells can be made. Intact sperm with no morphological abnormalities and those with different morphologic aberrations (categorizing the most common defects such as proximal cytoplasmic droplets; distal cytoplasmic droplets; midpiece or tail defects) can be identified and together with the four cell types with damaged membranes in any part of the sperm, a useful complex classification system with 8 combined categories would be applied. Sperm based on morphology are also can be classified into five simple or nine more differentiated categories (details are found in the Materials and Methods).

For light microscopic techniques, usually the minimum number of spermatozoa analysed is one hundred cells. Using our staining method for the quick analysis of the slide, 200 cells are counted, which is far satisfactory. However, the accuracy of the evaluation can be higher by increasing the number of cells analyzed.The counted sperm number depends on the density of cells on the smears. In the case of fresh semen, -especially when we examine raw ejaculate-, the particules of extender do not interfere with the assessment because of background staining. In this case 5-6-fold dilution is sufficient and in most cases cell-rich smear can be obtained. For the correct

assesment, covering the entire slides, higher cell counts must be implemented. In the semen diluted with freezing extender, egg-yolk can disturb the evaluation. In this case, at least ten-fold dilution is necessery which ensures the proper quality smear, however the cell concentration on the slide would be lower; e.g. when we use sperm with 100 million / ml concentration. In these cases, it is not possible to find large numbers of spermatozoa on the slides therefore 200 cells counted per sample are considered feasible. In general 200-300 sperm per sample need to define the percentages of different cell types accurately.

6.2 Experiment 2. Analysis of the injuries of stallion spermatozoa during the whole freezing procedure

An increased proportion of spermatozoa with damaged membranes appear to be an important component associated with reduced fertility of frozen–thawed spermatozoa (Zhang et al. 1990). In the present study TB/CSB - Giemsa complex staining was used to evaluate mebrane integrity of head, tail and acrosomal status, and morphology of stallion spermatozoa during the whole freezing process. Freezing procedure followed the advised protocol of Vidament et al. (2000) using modified INRA 82 extenders (Vidament et al. 2000, 2001, Table 7) was utilized in our study because this was one of the most standardised and proven method which is applied and routinely used in one of the worldwide most organized system, in the French National Stud at the time of experiment. Three-four ejaculates were frozen from 10 stallions (n=33), the collection dates performed randomly throughout between the years 2001-2004 (Table 5-6) in order to exclude the seasonal effect on semen quality and freezability (Janett et al. 2003). Viability evaluation in combination with morphological assessment was utilized in order to define the proportion of intact sperm with no morphological abnormalities and those with the most common morphologic aberrations (with cytoplasmic droplets; with midpiece or tail defects) after the technological steps of cryopreservation (Table 11, Fig 22). Neither of “Intact” and IHITIA cells proportion was changed after centrifugation but both were decreased significantly in the frozen/thawed semen. After freezing/thawing not only the proportion of DHDTDA sperm was higher but IHDT also increased considerably compared to fresh and centrifuged semen (19±7% vs. 4±3; 4±3; $p<0,01$) /Figs 22-25/. Our result is in accord with the study of Domes and Stolla (2001) in which after freezing and thawing, a high proportion of spermatozoa with unstained heads and stained tails were observed. These cells are considered as immotile (Nagy et al. 1999). At most of the techniques for viability evaluation - including light microscopic or fluorescent combined stainings -, examination of the midpiece and tail membrane separately is not feasible.

Recently measuring mitochondrial membrane potential has started to be involved in the viability estimations which could correlate with the tail membrane integrity evaluation. These tests are currently not available in routine examinations only for some laboratories. The results clearly demonstrate that the most sensitive subdomain for the freezing/thawing stress is the flagellum. 57.6% of the spermatozoa had damaged tail membrane after cryopreservation. Although it seems the sperm head area and shape influence sperm freezability (Esteso et al. 2006, Leibo 2006) the damage of the midpiece and tail membrane is also of great significance during the freezing process. Damages and depletion of acrosome of viable cells were uncharacteristic after freezing/thawing since the proportion of IHITDA was less than 1 %. The rate of this cell type is lower (1.8 % of the Intact "live" cells) than in other studies detected by combined fluorescence staining methods where the proportion of spermatozoa with intact acrosomes was found in 79.3-84.5% of the viable cells after Percoll separation (Kavak et al. 2003a) or 87–88% acrosome intact and 12 % acrosome damaged sperm of live spermatozoa according to Wilhelm et al. (1996). Differences can be explained by the discrepant staining methodologies because fluorescens stainings normally included 1-2 washing steps and a short period incubation while using TB/CSB-Giemsa staining the smears are made after a quick dilution of frozen/thawed sperm. Another reason may be the different classification of the cell types. In the studies referred spermatozoa with only intact head membrane was classified as viable sperm while in our experiment sperm with intact head and tail membrane was identified as viable cell and furthermore sperm with damaged tail and damaged membranes of any part of the head (head or acrosome membrane) was considered functionally damaged sperm (DHDTDA).

Fresh ejaculates and frozen/thawed semen samples of stallions show individual characteristics in point of viability and morphology. The stallion sperm showed different susceptibilities to stress of dilution, freezing and thawing, independently from initial quality (Loomis and Graham 2008). We found individual susceptibility also to centrifugation. Current protocols for equine sperm cryopreservation require the centrifugation of semen in order to separate sperm cells from the seminal plasma and to concentrate the sperm population so that they can be rediluted with freezing extenders. It has been shown that for several species, for example human (Ng et al. 1990), and mouse (Katkov and Mazur, 1998) centrifugation is a potentially sperm-damaging step during semen processing. There is still no complete or accepted explanation of how centrifugation induces sperm damage. However, it has been hypothesized that the damage to the spermatozoa is due to a direct mechanical effect on the sperm membranes (Alvarez et al 1993) as well as to an indirect adverse effect

caused by excessive ROS formation (Aitken and Clarkson, 1988). Centrifugation somehow weakens the membrane or compromises the permeability barriers. There is at present no defined mechanism for this effect, but it may involve stresses on the sperm plasma membrane similar to the stresses induced during cryopreservation by osmotic forces and phase transitions (Hammerstedt et al. 1990, Alvarez et al, 1993). Impact of centrifugation can be influenced by the duration and force of centrifugation. Studies on the effect of different centrifugation adjustments on human sperm (Shekarriz et al. 1995) have concluded that the time of centrifugation is more critical than the *g*-force for inducing sperm damage; thus, the use of short-term centrifugation is recommended in the preparation of sperm for assisted reproductive techniques. Carvajal et al. (2004) evaluated the influence of different centrifugation regimes (400, 800, 1600, and 2400 x g for 3 or 5 minutes, using the standard protocol - 800 x g for 10 min - as a control) on both boar sperm recovery and yield, after centrifugation and on sperm cryosurvival. The highest recovery and yield values were achieved using 2400 x g for 5 or 3 minutes and 1600 x g for 5 min. After thawing the samples which were centrifuged with 2400 x g for 3 min and 1600 x g for 5 min before freezing showed significantly higher postthaw sperm motility, viability, and percentage of uncapacitated sperm than control samples. They recommended using short-term centrifugation with a relatively high g-force (2400 x g for 3 minutes) in boar sperm cryopreservation protocol.

There is not much literature about the impact that centrifugation before freezing has on the quality of frozen-thawed stallion spermatozoa. Only a few experiments were achieved in which the effect of centrifugation on stallion sperm quality was studied and none of them used morphology evaluation to analyse the affect of centrifugation: Moore et al. (2005a) demonstrated the deleterious effect of seminal plasma on stallion spermatozoa during cryopreservation. However, retention of 5–20% seminal plasma in the suspension after centrifugation has been considered to be essential for cryosurvival (Sieme et al. 2008). Membrane plasticity could also play a role in survival during centrifugation. Membranes are still in liquid phase at 22 °C and could therefore undergo less damage during centrifugation at 22 °C than at 4 °C (Vidament et al. 2000). Cetrifugation/glycerol-addition at 22 °C followed by cooling to 4 °C results in an improvement of post-thaw motility, spermatozoa recovery rate and per cycle fertility compered to the procedure carried out at 4 °C. In an experiment, 9 centrifugation methods (600-1000 x g for 3-5 min) were tested. 800xg/10min and 1000xg/10min caused significant reduction of motility and membrane integrity as compared to the other 7 treatments. The best recovery rates with better motility and viability were observed after 600 xg/10 min and 1000 xg/5 min centrifugation

(Dell'Aqua et al. 2001). In a recent study a standard centrifugation protocol (600 x g for 10 min) was compared to four protocols with increasing g-force and decreased time period (600 x g, 1200 x g, 1800 x g and 2400 x g for 5 min). The authors didn't find reduction of stallion sperm quality after centrifugation or remarkable differences in sperm quality between the different centrifugation protocols. However the results showed that the loss of sperm cells in the supernatant after centrifugation could be substantially reduced by increasing the g-force up to 1800 x g or 2400 x g for a shorter period of time (5 min) compared to the standard protocol without apparent changes in semen quality, resulting in a considerable increase in the number of insemination doses per ejaculate (Hoogewijs et al. 2010).
Weiss et al. (2004) compared three centrifugation regimes (600 x g /10 min /I./, 1000 x g /2 min /II./and 2000 x g /2 min /III./) to investigate the influence of various centrifugation methods on sperm loss and quality of frozen-thawed semen. Mean sperm loss (I, 1.9%; II, 8.7%; III, 3.7%) was significantly different between the three centrifugation regimes. There were no significant differences among treatments in percentage of HOS positive cells and motility after centrifugation and in motility and viability after freezing/thawing, only the proportion of HOS positive cells in method III (52.1%) was significantly lower than in methods I (55.5%) and II (55.3%) after thawing. They concluded that stallion semen should be centrifuged at 600 x g during 10 minutes before freezing in order to obtain low sperm loss and a good quality of frozen-thawed semen. According to our results that after centrifugation more IBT cells were observed in some cases it is suggested to consider that in the study of Weiss et al. (2004) there is no information about morphology before and after centrifugation or after freezing/thawing. HOS-test shows the proportion of sperm with functionally intact tail membrane, but couldn't make a difference between the reacted sperm and the originally abnormal morphologic sperm.

Spermatozoa response to sublethal effects characteristically. Cold-, warm and hypoosmotic shock induce bent-looped, coiled tail of sperm due to changes in water-permeability of cell membrane. (Devireddy et al. 2002). The motion characteristics of the motile spermatozoa are altered. High percentage of motile sperm is moving in circle or backwards. We found that centrifugation also may cause similar morphologic alterations and this occurred intensively in some stallions (Figs. 28, 32, 34). Increasing IBT cells were observed related to 3 stallions (Stallion 6, Stallion 7, Stallion 9), which had in their fresh semen also high percentage of this cell type (14±5%).
Early observations showed that bulls produce quite frequently ejaculates containing high proportion of spermatozoa with bent, looped or coiled tails. The loop in the midpiece appears to be permanent so that sperm which are once bent or coiled in this

fashion cannot regain straight normal condition. If the affected sperm are motile, the side or reverse position of the tail causes them to move in circles or backwards at a lower speed than normal sperm. Another part of the sperm with coiled tails are only weakly motile or are immotile. Swanson and Boyd (1962) tried to find the causes of incidence of coiled tails. These tail defects are considered a secondary abnormality which means their epididymal or postejaculation origin (Campbell et al. 1960). Distal midpiece reflex (DMR), bent and coiled tail defect usually develop in response to environmental insults as sperm migrate to the distal half of the epididymal tail, probably in association with altered ion concentration (Barth and Oko 1998; Brito 2007). It has been not clear that it affects or not on fertility, but most of the authors indicated that high percentages of abnormal tails and midpieces caused reduced fertility (Campbell et al. 1960). In the centrifuged sperm of the Group II. stallions, IBT increased to 19±4% ($p<0.01$) and it was also high in the frozen/thawed semen (13±5%) (Fig 28-29.), While the rate of IHITIA sperm decreased considerable from 44% to 23%. Elevation of this defect can be more important in the frozen semen, because it could show equal or higher proportion among the viable sperm than the cells with normal morphology (Fig. 33, 37) which could affect on the fertility of these semen doses. In Group II. proportion of IBT within viable sperm with intact membranes was 30.3 % in the frozen/thawed semen while in Group I. this ration was only 8.6%. IHITIA was nearly 80% of the intact sperm in Group I. while a little bit more than half of the viable cells (53%) in Group II. (Fig. 31). DMR, bent and coiled tail defect (Fig. 34) are compensable defects. These sperm are either selectively filtered throughout the female genital tract or unable to penetrate the zona pellucida at the fertilization place (Barth 1994, Saacke et al. 2000). In this aspect fertility of the sperm can be improved with higher number of spermatozoa in the insemination dose.

The formation of coiled, bent tails can be produced in most sperm samples by rapid chilling (cold shock) or by hypoosmotic shock. It seems spermatozoa flushed from epydidymis are more sensitive to cooling than ejaculated sperm probably due to the lack of accessory sex glands secretums; however the flushing medium is also important (egg-yolk-citrate solution prevented the sperm from cold shock and development of coiled tails). The cause of this abnormality can be also an abnormal secretion in the genital tract. The normal amount and contents of seminal plasma can prevent sperm damages (Swanson and Boyd 1962). Two of our three stallions with increased level of tail defect produced low volume (gel-free volume: ~10-20 ml) of the ejaculates with high sperm concentration 280-450 x 10^6/ ml, it is suggested that maybe a low portion or non-physiological components of seminal plasma caused by accesory gland function failure resulted in the elevated sensitivity to stress effects, in this case to the centrifugation.

We also tried to find answer whether the ratio of cells with CD-s changed and how they survived during the process. Both of proportion of IPD and IDD were around 5-6 % in fresh and centrifuged semen and decreased in the frozen semen (3.4±3 and 2.1±2 %, $p<0.01$) /Table 11, Fig. 22/. Individual differences were found among stallions in the proportion of intact sperm with cytoplasmic droplets (Figs. 26, 37). Regarding to the literature spermatozoa with retained cytoplasmic droplets are fairly common in the equine semen. They represent a failure of maturation, because normally the residual cytoplasm is released down the tail during spermiogenesis. Proximal droplets are thought to have a great impact on fertility and therefore are classified as major defects (Jasko et al. 1990, Amann et al 2000, Thundathil et al. 2001, Peña et al. 2006), however today, retained distal droplets are also considered to be more detrimental to fertility than previously presumed (Kuster et al. 2004, Pesch and Bergmann 2006), because of releasing active enzymes (ubiquitin, 15-LOX) and reactive oxygen species which may influence also normal spermatozoa without defect, fertilization and further embryo development. (Sutovsky et al. 2001, Thundathil et al. 2001, Kuster et al. 2004, Fischer et al. 2005). High proportion of sperm with CD-s among intact spermatozoa may have a negative effect on the fertility of frozen stallion semen.

In the combined categories all the sperm with CD-s (IDCD) were slightly decreased during the process (15±9; 13±8; 12±8 %) and sperm with midpiece- and tail defect (IDBT) were mildly increased after centrifugation (10±7; 12±10; 12±10 %) /Fig. 40/. Our results in contrast with the study of Blottner et al. (2001) in which a percentage of spermatozoa with CD was 10–23% in May and 12–15% in December (tested on 4 stallions) and greatly decreased to percentages lower than 2 and 4% respectively after cryopreservation. Proportion of IDCDBT didn't change during the freezing procedure (25±15; 26±15; 24±15% fresh, centrifuged and frozen respectively) /Fig. 40/. The relative ratios of IDBT, IDCD and IDCDBT during the process might be explained in some cases with the effect of centrifugation which results in curve of the midpiece containing CD which is very often entrapped in the bend (Figs 34, 70). Double bends of the midpiece usually accompany coiling of the principal piece with retention of cytoplasmic material (Brito 2007).

The ratio of intact, viable spermatozoa is the most important parameter of the quality of frozen semen. However, for the further development of cryopreservation technologies or determination of freezability of individual stallion and usability of frozen semen, it is also important to define accurately the localization of cell injury during the cryopreservation process for which each of the part of the sperm need to be assessed. Our staining method is well-applicable for subdomain-specific examination of spermatozoa.

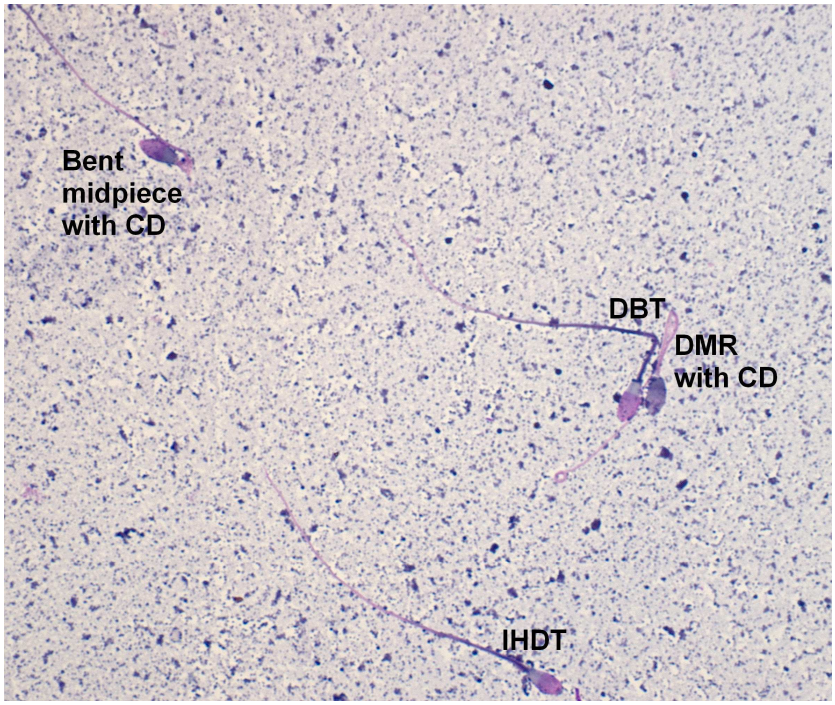


Figure 70. Microscopic picture of spermatozoa of "Stallion 9" after cryopreservation (CSB/Giemsa staining)

6.3 Experiment 3. Use of pentoxifylline and hyaluronic acid for stallion sperm separation

Both Percoll® gradients and swim-up procedures are generally used for separating equine spermatozoa for ICSI (Landim-Alvarenga et al. 2008). Therefore Percoll® and swim-up methods were modified for the separation of low volume stallion spermatozoa in our experiment. Standard sperm separation methods are not always effective with low numbers of total and viable sperm. In humans for oligozoospermic and asthenozoospermic men the regular Percoll® gradient centrifugation yielded low rates of sperm recovery. Therefore, a discontinuous mini-Percoll gradient (0.3 ml of each of 95%, 70% and 50% Percoll®) was developed and resulted in better recovery of clean motile spermatozoa (Ord et al. 1990, Smith et al. 1995). In human sperm preparation washing procedures are usually very time-consuming processes. Stallion spermatoza (mainly frozen semen) are very sensitive to protracted procedures like the ones used in humans; however they are not sensitive to higher centrifugation speed (Dell'Aqua et al. 2001, Hoogewijs et al. 2010 and personal experiences). The proposed approach was to reduce the volumes of separating media (Mini-Percoll in 1.5 ml microcentrifuge tube and mini-swim up method), the time of centrifugation and

swim up duration and use higher g-force for mini-Percoll to increase the yield of viable sperm for ICSI when few sperm are available. Another factor was the number of layers of Percoll® gradients. Since the 3 layer-Percoll® density gradient centrifugation (90%–70%–40%) was not found more effective than the 2 layer-method (90%–45%) (Chen and Bongso 1999), the 2 layer method was chosen for this experiment. In human studies 5-15 ml tubes were used for mini-Percoll separation. In our experiment we used small 1.5 ml microcentrifuge tubes because it was easier to layer the two gradients and aspirate the pellet from the bottom of the tube.

The most effective Mini-Percoll method, based on a preliminary study, was used for this experiment. In a preliminary study different combinations of forces and times of centrifugation from 300 to 1200 x g for 5-10 min were compared. Frozen sperm was centrifuged through two layer gradients (0.5 ml of each of 90% and 45% Percoll®). The pellet was washed in H-CDM-1 at 300 x g for 5 min. Recovery of the sperm and percentage of intact sperm with normal morphology were analyzed and compared after different centrifugations. Using low force (300 x g) there was very low recovery rate while high force (1200 x g) or longer time resulted in more dead sperm and debris in the pellet. At the combination of 600 x g for 5 minutes, considerable good recovery and yield was observed. Therefore I chose these centrifugation parameters in the subsequent experiment.

Commonly there are two different main approaches to increase the effectiveness of sperm separation. One is modifying and developing separation methods and the other is adding chemical stimulators to the media. Pentoxifylline and hyaluronic acid are successfully used for initiating and inducing motility and viability of spermatozoa. In the present study we tried to find answer weather PX or HA treatments enhance the effectiveness of PG and SU separation when small volume and low numbers of stallion sperm is available.

The incubation period is typically between 30 and 60 minutes at 37-39 °C in waterbath or 5% CO_2 in air incubator for swim up procedures in the IVF laboratories. I placed the semen into the medium for the shortest time - 30 minutes -, in CO_2 incubator at 38 °C in all swim up treatments, also because of similar duration as spending for Percoll separation treatments was required.
Pentoxifylline as a non-specific inhibitor of phosphodiesterase would result in both, stimulation of motility and acrosome reaction depending on the conditions, the time of stimulation and concentration of pentoxifylline in the medium (Tesarik et al. 1992, Jayaprakash et al. 1997, Calogero et al. 1998, Fisch et al. 1998, Henkel and Schill 2003). PX improved the ability of thawed spermatozoa to undergo the acrosome

reaction in response to calcium ionophore (Esteves et al. 2007). In our study IHITDA was higher in P-PX than P-NT ($P<0.01$) and P-CON ($P>0.05$) (17±1.6 %, 7±1.6 % and 11±1.6 %, respectively) (Table 12, Figs. 43, 45). PX seems to promote acrosome reaction, maybe mainly on sperm with destabilized membranes. There were individual differences among stallions in the reactivity of acrosome-membranes. There was a significant stallion x treatment effect in this sperm category. Samples of Stallion 2 and Stallion 3 caused this elevation of IHITDA after P-PX treatment (Fig. 46). These spermatozoa are loosing or already have lost acrosomal material what can be advantage in ICSI fertilization of oocyte but also disadvantage because sperm theoretically may loose "sperm-factor". The spermatozoon carries an oocyte-activating factor associated to the acrosome-membranes or post-acrosomal region that stimulates Ca^{2+} release and Ca^{2+} oscillations important for the initiation of development (Malcuit et al. 2006). The acrosome and its contents never enter the oocyte under natural conditions. The removal of sperm membranes may make the sperm-borne oocyte-activating factor more easily available to the ovum's cytoplasm. The studies indicate that acrosomal enzymes have potentially harmful effects on embryo development and when sperm with intact membranes are microinjected, changes underlying oocyte activation may still occur, although at a slower pace (Morozumi and Yanagimachi 2005, Morozumi et al. 2006). Therefore the removal of acrosomes before ICSI is preferable (Morozumi and Yanagimachi 2005) in human and necessary to facilitate sperm head decondensation and fertilization in bovine (Goto et al. 1990) and in sheep (Gómez et al. 1997). In contrast none of the studied parameters (oocyte activation, pronuclear formation, and embryo development) was affected by the acrosomal or the live or dead status of the spermatozoa injected in porcine (García-Roselló et al. 2006). In pig sperm, phospholipase Cζ (PLCζ), which is thought to be the oocyte-activating factor in mammalian sperm was localized to both the post-acrosomal region and the tail area. Thus raising the possibility that injection of whole sperm may be required to attain successful activation in pigs. (Nakai et al. 2011a, 2011b). In equine species the activation process seems to be more complicated. Oocyte activation rates after ICSI in equine species have been largely inconsistent and generally low among laboratories (Choi et al. 2002). There are few publications in equine species and these are contradictory about affectivity of using chemicals for acrosome reaction and plasma disruption or activation of fertilized oocytes (Matsukawa et al. 2002, 2007, Bedford et al. 2004). Therefore a simple mechanical membrane disruption within the pipette is currently performed during sperm injection in equine species (Hinrichs 2010).

HA supplementation prior to freezing appeared to preserve post-thaw boar spermatozoa viability and maintained membrane stability after cryopreservation (Peña et al. 2004). In equine sperm HA did not prevent cryodamages. (Mari et al. 2005, Ottier and Curtis 2005). Hyaluronic acid was used successfully in combination with swim-up for separating motile spermatozoa from frozen bovine semen. Better motility, increased proportions of spermatozoa with intact plasma membrane and acrosome were observed after HA-swim-up method compared to control samples (Shamsuddin et al. 1993, Shamsuddin and Rodriguez-Martinez 1994). In our experiment HA - in the same concentration as the authors used in the previous studies for bull sperm separation - increased the recovery rate during swim-up, but not viability and proportion of normal cells in any of the treatments (Figs 41, 42, 47, 48). Equine spermatozoa are more sensitive to environmental stress factors and handling processes than bovine sperm. The reason for good sperm concentration (recovery rate) but poor viability (survival rate) and morphology of the SU-HA selected sperm may be attributed to the detrimental effect of the final washing procedure after SU as it was concluded in the studies of Shamsuddin et al. (1993) and Shamsuddin and Rodriguez-Martinez (1994).

Numerous studies have previously been carried out to compare swim-up and Percoll® separation of spermatozoa with very varied results. Our results agree with the findings of Menkveld et al. (1990), van der Zwalmen et al. (1991) and Somfai et al. (2002b) in superiority of Percoll separation compared to swim-up. P-CON and P-PX were the most effective separation procedures when beginning with low numbers of sperm. In the study of Somfai et al. (2002b) the same staining method was used as in our experiment however they evaluated different sperm categories based on membrane integrity of subdomains. They did not counted separately sperm with damaged head and intact tail but they classified different types of acrosome damages (damaged-, loose-, lost acrosome) within “live” and “dead” cells. Head of stallion spermatozoa is much smaller than bovine’s and to distinguish the grade of acrosome damage of the “dead” sperm is not always possible. IHITDA cells have generally loose or lost acrosome. In the respect of sperm separation, to categorize spermatozoa with intact head or tail plasma membranes to subcategories is more important than classify “dead” cells. Therefore we focused on to define the ratio of functionally different sperm types in the separated sperm suspension such as DHIT or IHITIA. After separations fairly high percentage of the sperm had damaged head but intact tail (DHIT) (Table 12, Fig 43). This ratio was highest in P-CON (14±1.7 %), P-NT (13±1.7 %) and SU-PX (13±1.7 %) treatments. In the original frozen/thawed semen it was only 0.5 %. P-PX resulted in less DHIT cells compared to P-CON (8 vs. 14 %) but it was not significant ($P>0.05$) (Figs. 43, 44). Twenty-five to 35% of the sperm

with intact midpiece and tail membrane, which possibly are motile (Nagy et al. 1999), have damaged head or acrosome membranes after separations (Fig 71).

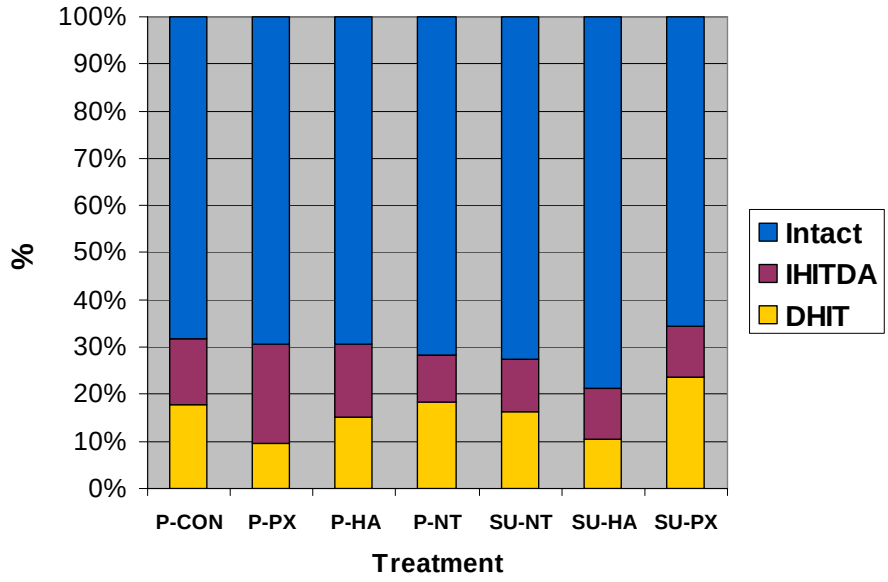


Figure 71. Distribution of different sperm categories with intact midpiece and tail membrane after different treatments. Diagram shows the relative rates of the 3 sperm types to each other

This could affect the success and results of ICSI procedures, in which final selection of sperm is based on motility. The results point out a weakness of this method since there is a quite high proportion of the sperm having intact tail but damaged head and acrosome consequently these cells could be also functionally damaged. All Percoll® separations produced high proportion of normal cells (90-92%). After swim-ups the proportion of sperm with midpiece + tail defect was increased (Fig. 48) compared to it in the thawed semen, and was 2-3-fold higher than after Percolls (Table 13, Fig. 47). The reason is not clear however it is possible that the negative effect of centrifugation after swim up could be one cause. This finding may support the theory of Shamsuddin et al. (1993) that swim up causes the spermatozoa to be more sensitive to centrifugation and as it is seen in my Experiment 2, susceptible spermatozoa can response with curving and bending of their tail for the stress effects of centrifugation.

P-CON and P-PX resulted in the most morphologically normal, intact sperm according to high proportion of normal cells, the most intact sperm and the best recovery rate within treatments. Incubation with PX is beneficial if Percoll® separation is delayed, but there is need to clarify its effect on acrosome exocytosis and the influence of absence of acrosome for further development of equine embryos produced by ICSI.

6.4 Experiment 4. Viability, acrosome integrity and morphology evaluation of sperm samples from subfertile stallions

Breeding stallions are selected primarily based on their pedigree, athletic performance, or other phenotypic characteristics. Fertility or fertility potential are usually secondary considerations, and in most cases assessment is limited to selecting out stallions that clearly do not possess the characteristics necessary for reasonable fertility (Colenbrander et al. 2003). Infertility or subfertility is the most common reproductive complaint of horse owners. Diagnosis of the reason of infertility causes a confusing problem for veterinarians. Owners may not keep accurate records, the stallion may have changed owners, the reproductive history of the mares bred may be unknown and overall reproductive management may be poor (Murchie 2005). The challenge is to determine which abnormalities are constitutive and reflect an individual stallion's intrinsic genetic ability, and which changes are extrinsic due to a disturbance (nutritional, hormonal, infectious, toxic, degenerative, neoplastic and idiopathic) in spermatogenesis. This is a complicated process because intrinsic and extrinsic factors concurrently influence spermatogenesis (Card 2005).

Semen analysis provides important data from which fertilizing ability of the stallion can be inferred for. However conventional laboratory techniques evaluate only one or two attributes of the viability or functionality of spermatozoa. In many cases a specified parameter (eg. a special morphologic defect) correlates to reduced fertility of the sperm however it doesn't mean that the examination of only this or another attribution provides obvious prognosis for fertility ability of the semen sample. One reason for that to establish carefully planned in vivo study and involve adequate number of mares (at least 100 mares/treatment) to the experiment is very expensive in horses. Another cause that fertilization is a very complex procedure and overall fertility of the stallion is influenced by many factors of the sire and also of the dam (optimal time of insemination, reproductive status and condition of the mare). The aim of most current research is to identify a combination of tests that together analyse the most important sperm function parameters simultaneously since multi-parametric analysis allows more accurate prediction of fertility (Colenbrander et al. 2003, Rodriguez-Martinez 2006).

Most of the viability staining is being used for only evaluating membrane-integrity of the head. TB/Giemsa or CSB/Giemsa staining method is an ideal laboratory test that could evaluate several attributes of the spermatozoa using simple light microscopy. Semen specimens of fertile and subfertile stallions were collected during the years of 2003-2008 which overlap with the period of the improvement and modification of staining

procedure, when the CSB was still being tested. Therefore in *Stallion 4* and *Stallion 10* only smears stained by TB were available, In the other cases I analysed the samples dyed with CSB because CSB/Giemsa staining provided more clearly differentiation of intact and damaged midpiece and tail membrane. Sperm smears from Stallion "A", "B" and "C", were delivered to me after viability staining with TB and fixing by the veterinarian at the breeding station, the other subfertile semen samples were stained by myself on the site of semen collection or the chilled-transported semen in the lab. In these cases, CSB was used.

In the previous studies (Tartaglione and Ritta 2004, Domes 2003) in which the authors found correlation between fertility and the results of TB/Giemsa viability assesment in combination with other methods, they analysed only membrane-integrity and acosome status, but not morphology. In our study we used the staining method for morphologic analysis alone and I also developed an evaluation system in which viability and acrosome integrity examination is combined with morphology. The results have pointed out to the importance of defining of the ratio of membrane-intact and morphologically normal spermatozoa. Taking this into account in determining the sperm number in the insemination dose is recommended. The further distribution of the ejaculate and allocating the number of mares inseminated had been based on this way in the case of Stallion "G", hence it was possible to achieve good pregnancy outcome in the breeding season and 75% pregnancy rate at the end of the season. Management also plays a critical part in reproductive performance and good, intensive management can substantially improve the 'apparent' fertility of many poorly performing stallions, primarily by ensuring insemination closer to the time of ovulation (Colenbrander et al. 2003). It is also important to define the type of abnormalities of spermatozoa, because the decision of further sperm manipulation methods depends on these results. If the abnormality is compensable (eg. microcephal head defect, DMR, bent, coiled tail), sperm concentration is satisfactory and a 20-30% of normal, viable sperm is also present in the ejaculate, the increasing of insemination dose could be solution for the problem. However for example the presence of high proportion of CDs may be more confusing problem, because of active enzymes of CDs which may have negative effects on the processes after fertilization of oocyte (Kuster et al. 2004, Fischer et al. 2005). ROS are also increased in SP in the presence of increased proportion of sperm with CDs. In these cases (eg. in Stallion "J") sperm separation may help to isolate normal viable spermatozoa from the defected sperm and also from the affected seminal plasma. This portion after dilution with semen extender can be used more effectively for AI immediately after preparation or after cooled-transportation.

Smearing stallion semen samples on the slide increased the proportion of detached sperm heads when compared to wet-mounts (Brito et al. 2011). Studies in bulls also reported an increase in the proportion of detached sperm heads and broken midpieces when eosin/nigrosin-stained smears where compared to wetmount preparations (Sekoni et al. 1981, Brito et al. 2011). During our staining procedure gently preparation of smears using two slides paralelly attached to each other and pulling to make two smears ensures less damage of spermatozoa which is proved by low proportion of detached heads counted in the samples (e.g 1.0 ± 0.6% of this category in fertile stallion semen). A common concern with eosin/nigrosin is the hypotonicity of the stain and the possibility of introduction of artifactual tail defects, e.g., bending and coiling. The use of warm slides and stain combined with quick drying of the smear by blowing to minimize the time of contact of sperm with the hypotonic stain prevented any increase in bent and coiled sperm tails in the study of Brito et al. 2011. Using CSB/TB stains which are otherwise isotonic, the smears are air dried nearly vertically at room temperature which results in very fast drying therefore minimize artefact being formed. In the study of Brito et al. (2011) there were significant differences among clinicians for all sperm morphology classification categories. Sperm morphology evaluation is subjective and results are largely dependent on the proficiency and experience of the evaluator. For utilizing of Kovács-Foote staining method practice in classifying spermatozoa is also indispensable. However with training and consistent evaluation of semen samples the technicians can reliably perform both of the viability and morphology assessment.

Semen quality also depends on the age of the stallion what is important to consider in sperm evaluation. A typical ejaculate of a pubertal stallion has a low concentration, low motility, high percentage of germinal cells and other defects such as head, midpiece and proximal droplets. A pubertal horse should be classified as a questionable breeding prospect. Re-evaluation in 2–4 months is suggested. At re-evaluation, the expectations are the followings; increases in: total sperm numbers, sperm concentration, motility and percentage of morphologically normal sperm, with a decrease in germinal epithelial cells, proximal droplets and head/midpiece defects (Card 2005). Idiopathic testicular degeneration usually affects older stallions and produces detectable changes on testicular size and consistency (Turner 2002). In testicular degeneration the typical changes in semen include low spermatozoa concentration and a high percentage of morphological defects, especially high number of premature germ cells, head defects and midpiece defects (Brito 2007). Regardless of the stallion is young or old, subfertile ones may participate in breeding in those special cases if they have extraordinary genetic value, outstanding sports results, or in

a small population of rare, native breeds (eg. Gidrán, Hucul) for the purpose of gene conservation.

Management of subfertile stallions

According to a special survival mechanism, the testis has strong ability to completely recover spermatogenic capability some time after the insult if it was ceased (Johnson et al. 1997). Several times after stress factors (e.g. sport competitions) discarded, body condition of the stallion improved or background disease treated the problem could be solved. However reduced fertility in most of the cases could not be cured neither by conventional treatment, nor using GnRH or GH therapy. In these cases the only alternative is enhanced management and changes in management of the stallions and the mares: decrese in number of mares mated/inseminated, determination of the optimal time of natural service or AI, changes in semen handling. Fertility parameters and spermiogram of subfertile stallions shows seasonal fluctuations (Card 2005). In the beginning and end of the season per cycle pregnancy rate can be 37% and in the middle of the season it may decrease to 25%. Fewer mares inseminated/mated may improve the fertility rate in these cases (Juhász and Nagy 2003). A number of investigations have indicated that seminal plasma (SP) has a detrimental effect on storage of equine sperm as either cooled or cryopreserved semen (Jasko et al. 1991). The presence of some SP seems to be necessary for semen storage and fertility, but it is beneficial to remove most of the SP by centrifugation before storage, at least for those stallions whose ejaculates have poor tolerance to cooling and storage (Brinsko et al. 2000, Love et al. 2005). Factors such as composition of extender, dilution ratio, storage temperatures and times, and centrifugation regimens affect on sperm survival during storage. SP affects on sperm longevity, and particularly in stallions that produce semen with limited tolerance to storage, semen quality can be improved through modifications of semen handling procedures (Kareskoski and Katila 2008). Semen of a subfertile stallion may be centrifuged and diluted with an extender which is more suitable to the given sample. Higher sperm number in the insemination dose in the worst cases generally does not improve the pregnancy result although the morphologically normal, viable spermatozoa can compensate many of the abnormal spermatozoa if the morphologic sperm abnormality was compensable. Frequency of the inseminations can be increased (in every 24 hours instead of 48 hours interval with fresh or chilled-transported sperm, or inseminations either before and after the ovulation using frozen semen) and also management of the mares can be more accurate (determination the most optimal time of the insemination, using ovulation induction).

Biomimetic selection of the best quality spermatozoa for AI or for cryopreservation could improve pregnancy rates and may help to reverse the decline in fertility seen in several domestic species over the recent decades, for example in dairy cattle and horses (Morrell and Rodriguez-Martinez 2010). Recently a new method, single layer centrifugation (SLC) through a species-specific colloid (Androcoll) was developed which uses only one layer of colloid. Thus, time is saved during preparation and the method can be scaled-up successfully to allow large volumes of semen (e.g. the whole stallion ejaculate) to be centrifuged to produce sufficient numbers of spermatozoa for stallion AI doses. SLC consistently improves the quality of stallion sperm samples in terms of motility, membrane-integrity, morphology and chromatin integrity (Morrell et al. 2009c, Morrell and Rodriguez-Martinez 2010, Morrell et al. 2011). Recently we reported case studies in which SLC was used to select the best spermatozoa from 'problem' ejaculates for subsequent use in AI. Pregnancies were obtained after using SLC-selected spermatozoa from the five subfertile stallions for AI. The results suggest that SLC can be used for preparing doses from some 'problem' ejaculates for conventional AI resulting in successful pregnancies (Morrell et al. 2011). Using subfertile frozen semen, in vitro sperm separation methods (Percoll, swim up, single layer centrifugation, glass wool centrifugation) then deep intrauterine insemination or in vitro fertilization (ICSI) may be another chance to result in pregnancy. However the complexity of the problem was shown in a study in which the authors conducted a fertility trial with a subfertile stallion whose semen was subjected to density-gradient centrifugation in an effort to improve semen quality prior to insemination. They inferred that semen treatment for the subfertile stallion yielded a spermatozoal population with quality similar to, or exceeding (based on motility values), that of the fertile control stallion. Nonetheless, when fertile mares were inseminated hysteroscopically with 20 x 10^6 progressively motile spermatozoa, the resulting pregnancy rates were 15/20 (75%) for the fertile stallion, as compared to 7/20 (35%) for the subfertile stallion. This demonstrates that spermatozoal motility does not provide absolute discrimination power and emphasizing that spermatozoal attributes other than motility play critical roles in spermatozoal fertilizing ability (Varner 2008).

In the *Experiment 4* I investigated weather some alterations would be detected either in viability or morphology evaluation in the "subfertile' stallion samples related to their decreased fertilization potency. At subfertile stallions in every cases some alterations were detected and quality of the sperm in respect of membrane-integrity or morphology was lagged behind those in fertile stallions were observed. In several cases serious morphologic abnormalities or dramatic reduction in the proportion of intact, viable, morphological normal sperm with an increase rate of different

membrane-damaged sperm categories were found in the ejaculate. Different morphologic abnormalities of spermatozoa of subfertile stallions are shown in microscopic pictures in the Appendix. Every semen samples of subfertile stallions were unsatisfactory regarding to the strict guidelines of the Hungarian Standard for breeding stallion semen (7034/1999) which allows ≤ 30% sperm with any morphologic aberrations, if less than half of these abnormal cells have primary defect.

Earlier suggestion of Colenbrander et al. (2003) could be realised, namely that using this multi-parametric semen analysis method, subfertile and infertile stallions would be identified and reason for decreased pregnancy results may be revealed. For this purpose the evaluation method would be installed into the annual control examination of the stallions' semen. In the case of high genetic stallions remained in breeding, with thorough examination of the horse and his semen, and use of complex evaluation system, changes in semen quality can be monitored and the management would be adjusted to these alterations. Besides of standard parameters of routine semen evaluations (volume, sperm concentration, total sperm number, motility and progressive motility) the complex staining method after collection or thawing in the case of frozen semen, and together with further longevity tests (evaluation after 24 hours storage at 4°C of fresh semen, or 1 and 2 hours storage at 38°C of frozen/thawed samples) alone or with additional DNA integrity analysis and a sperm functional test (ZP binding or hyaluronic acid binding or progesterone-induced acrosome reactions etc.) would be able for the prognosis of fertility potencial of stallion semen. Selection of subfertile and infertile sires or prospective breeding stallions would facilitate the improvement of semen quality used in artificial insemination industry and thus better results would appear in the pregnancy and foaling rates.

Since the improved staining method by CSB was published it has been successfully applied also for evaluation of ram, bull, dog, elephant and white rhino spermatozoa. However the complexity of the technique allows to classify spermatozoa in more different numbers and systems of categories, I recommend the eight, viability – combined morphology categories (Experiment 2 and 4) to use for routine quality control and also for experimental cases. The aim of the future innovation is to develop a solution for sperm dilution in which the sample can be stored for some hours without quality alterations for the field cases when there is no possibility to make smear at the site of sperm collection (e.g. semen collection from wild animals). It would be a very useful innovation also to work out computer aided automatised technique for evaluation the stained smears.

7. NEW SCIENTIFIC RESULTS

1. I improved the Kovács-Foote staining to distinguish different cell types more accurately: Chicago sky blue (CSB) resulted in similar sperm head, but better tail live/dead differentiation compared to trypan blue (TB). After staining with 0.16% CSB and 4 minutes fixation, 2-4 hours Giemsa staining at 25-40°C is recommended for stallion semen. I validated the improved technique: CSB/Giemsa staining showed good repeatability and high agreement with the standard TB/Giemsa method.

2. I developed an evaluation system combining the viability and acrosome integrity examination with morphology analysis in order to define the proportion of intact sperm with no morphological abnormalities and those with the most common morphologic aberrations (proximal-, distal cytoplasmic droplets and midpiece or tail defects). Altogether with different membrane-damaged spermatozoa, cells were classified in eight categories. The new evaluation system was used for monitoring changes during cryopreservation process, and to define detectable anomalies as causes of subfertility of different stallions. In all studied subfertile stallions relationship was found between qualitative sperm parameters and the degree of reduced fertility. I have verified that high proportion of sperm with cytoplasmic droplets among intact spermatozoa has a negative effect on the fertility of equine semen. Using this multi-parametric semen analysis method, subfertile and infertile stallions can be identified and reason for decreased pregnancy results may be revealed.

3. During the cryopreservation procedure, the proportion of all membrane-intact cells and the ratio of intact, morphologically normal sperm was not changed after centrifugation but was decreased significantly after freezing/thawing. Damages and depletion of acrosome of viable cells were uncharacteristic after freezing/thawing since the rate of IHITDA was lower than 1%. I found individual susceptibility to centrifugation which caused similar morphologic alterations (bent, coiled sperm tail) as induced by cold-, warm- and hypoosmotic shocks.

4. Percoll method was successfully modified by reducing the volume of separating media (Mini-Percoll: 0.4 ml 90% and 0.5 ml 45% Percoll in a 1.5 ml microcentrifuge tube), the time of centrifugation and use higher g-force (600 x g for 5 min) to increase the yield of viable sperm separation for ICSI when low volume and few numbers of equine sperm are available. Mini-Percoll separation without incubation and additional chemical supplementation (P-CON) or after incubation with 3.5 mM pentoxifylline (P-PX) resulted in the most morphologically normal, intact sperm according to high proportion of normal cells, the most intact sperm and the best recovery rate compared to mini-percoll after incubation of spermatozoa with 1 mg/ml hyaluronic acid (P-HA) and all swim-up treatments. Twenty five to 35% of the sperm with intact midpiece and tail membrane, - which are considered motile -, have damaged head or acrosome membranes after separations. This could affect on the success of ICSI procedures, in which selection of sperm is based on motility. Rate of viable sperm with damaged acrosome (IHITDA) was the highest after P-PX separation. These spermatozoa are loosing or already have lost acrosomal material what can be advantage in ICSI fertilization of oocyte.

8. SUMMARY

There are many different laboratory methods for evaluation of spermatozoon and semen quality. However most of the tests provide information from only one or two attributes of spermatozoa. A simple trypan blue (TB) -neutral red-Giemsa staining procedure for simultaneous evaluation of acrosome, sperm head, and tail membrane integrity and morphology has been used to evaluate mammalian spermatozoa. Since first introduction of the technique some special characteristics and problems have arisen in evaluating stallion semen. The main problem was the differentiation of intact vs. damaged sperm tails primarily in frozen and thawed samples. After freezing and thawing, a high percentage of spermatozoa with an unstained head and stained tail were observed. These cells are considered immotile. Therefore, unambiguous differentiation of intact vs. damaged sperm tail membrane is very important for evaluating semen quality. The aim of the **Experiment 1** was to improve the method using another viability stain, Chicago sky blue 6B (CSB) which molecule is very similar to TB and optimizing each steps of the staining procedure to distinguish more accurately the different cell types. CSB/Giemsa staining showed good repeatability and agreement with TB/Giemsa measurements. For densitometry analysis, individual digital images were taken from smears stained by CSB/Giemsa and by TB/Giemsa. A red-green-blue (RGB) histogram for each area of spermatozoa was drawn. Differences of means of RGB values of live vs. dead tails and separate live vs. dead heads from each photo were used to compare the two staining procedures. CSB produced similar live/dead sperm head differentiation and better tail differentiation. We concluded that TB could be replaced by CSB resulting in more reliable evaluation. After staining with 0.16% CSB and 4 min fixation, 2 - 4 h Giemsa staining at 25-40° C is recommended for stallion semen.

In **Experiment 2 and 3** the improved and validated complex staining method was used to evaluate sperm quality during and after two prominent sperm manipulation procedures: cryopreservation and sperm separation. In **Experiment 4** the technique was applied to define detectable anomalies in semen samples from stallions with reduced fertility.

Viability evaluation in combination with morphological assessment was utilized in **Experiment 2** in order to define the proportion of intact sperm with no morphological abnormalities and those with the most common morphologic aberrations (with cytoplasmic droplets; with midpiece or tail defects) after the technological steps of cryopreservation. Neither of “Intact” and IHITIA cells proportion was changed after centrifugation but both were decreased significantly ($p<0.01$) in the frozen/thawed semen (78±9; 78±8; 38±11% and 58±16; 58±15; 26±9%, in fresh, centrifuged and frozen sperm, respectively). After freezing/thawing not only the proportion of DHDTDA sperm was higher but IHDT also increased considerably compared to fresh and centrifuged semen (19±7% vs. 4±3; 4±3; $p<0.01$). Damages and depletion of acrosome of viable cells were uncharacteristic after freezing/thawing since the

proportion of IHITDA was less than 1 %. We found individual susceptibility also to centrifugation. Centrifugation may cause similar morphologic alterations (bent-looped, coiled tail of sperm) as induced by cold-, warm and hypoosmotic shock and this occurred intensively in some stallions. Increasing IBT cells were observed after centrifugation related to 3 stallions (19±4%, $p<0,01$), which had fresh semen also containing high percentage of this cell type (14±5%). Proportion of IBT was also high in the frozen/thawed semen (13±5%) in these 3 stallions, besides that rate of IHITIA sperm decreased considerable from 44% to 23%. Elevation of this defect can be more important in the frozen semen, because it could show higher or equal proportion among the viable sperm than the cells with normal morphology. In the combined categories all the sperm with CD-s (IDCD) were slightly decreased during the process (15±9; 13±8; 12±8 %) and sperm with midpiece- and tail defect (IDBT) were mildly increased after centrifugation (10±7; 12±10; 12±10 %). Proportion of IDCDBT didn't change during the freezing procedure (25±15; 26±15; 24±15% fresh, centrifuged and frozen respectively). The relative ratios of IDBT, IDCD and IDCDBT during the process might be explained in some cases with the effect of centrifugation which results in curve of the midpiece containing CD which is very often entrapped in the bend. The ratio of intact, viable spermatozoa is the most important parameter of the quality of frozen semen. However, for the further development of cryopreservation technologies or determination of freezability of individual stallion and usability of frozen semen, it is also important to define accurately the localization of cell injury during the cryopreservation process for which each of the part of the sperm need to be assessed. The staining method is well-applicable for subdomain-specific examination of spermatozoa.

Standard sperm separation methods are not always effective with low numbers of total and viable sperm. In addition, stallion spermatozoa are very sensitive to protracted procedures. In **Experiment 3** we reduced the volume of separating media and the time of centrifugation to increase the yield of viable sperm for ICSI when few sperm are available. The purpose of this study was to compare the effectiveness of mini-Percoll (P) and swim-up (SU) method for low numbers of sperm treated or non-treated with hyaluronic acid (HA) or pentoxifylline (PX). Numerous studies have previously been carried out to compare swim-up and Percoll® separation of spermatozoa with very varied results. I found Percoll separation superior compared to swim-up. Percoll-control (P-CON) and P-PX were the most effective separation procedures when beginning with low numbers of sperm. Twenty five to 35% of the sperm with intact midpiece and tail membrane, which possibly are motile have damaged head or acrosome membranes after separations. This could affect the success and results of ICSI procedures, in which final selection of sperm is based on motility. The results point out a weakness of this method since there is a quite high proportion of the sperm having intact tail but damaged head and acrosome consequently these cells could be also functionally damaged. All Percoll® separations resulted in more „normal", and less sperm with droplets (proximal + distal droplets) and midp + tail defect compared to all swim-ups (91-92% vs.71-78%; 1% vs. 4-7%; 6-7% vs.16-19% respectively, $p<0.01$). HA increased the recovery rate during swim-up, but not viability and proportion of normal cells in any of the treatments. The reason for good sperm

concentration (recovery rate) but poor viability (survival rate) and morphology of the SU-HA selected sperm may be attributed to the detrimental effect of the final washing procedure after SU. P-CON and P-PX resulted in the most morphologically normal, intact sperm according to high proportion of normal cells, the most intact sperm and the best recovery rate within treatments. PX is beneficial if Percoll® separation is delayed, but there is a need to clarify its effect on acrosome exocytosis and the influence of absence of acrosome for further development of equine embryos produced by ICSI.

Unlike bulls, stallions have been not selected by the artificial insemination (AI) industry for many years and generations based on semen production, sperm quality and freezability. This explains that there is a wide variation in semen characteristics among individuals and in remarkable rate the semen quality is not sufficient. Semen analysis provides important data from which fertilizing ability of the stallion can be inferred for. In **Experiment 4** we used the staining method for morphologic analysis alone and also viability and acrosome integrity examination was combined with morphology. The examined stallions had been categorized as “fertile” or “subfertile” previously by the veterinarians of the Breeding Stations based on pregnancy results of mares inseminated with sperm of the given stallion during the breeding season. At subfertile stallions in every case some alterations were detected and quality of the sperm in respect of membrane-integrity or morphology was lagged behind those in fertile stallions were observed. In several cases serious morphologic abnormalities or dramatic reduction in the proportion of intact, viable, morphological normal sperm with an increase rate of different membrane-damaged sperm categories were found in the ejaculate. Every semen samples of subfertile stallions were unsatisfactory regarding to the strict guidelines of the Hungarian Standard for breeding stallion semen (7034/1999).

Our results have pointed out to the importance of defining of the ratio of membrane-intact and morphologically normal spermatozoa. Taking this into account in determining the sperm number in the insemination dose is recommended. It is also important to define the type of abnormalities of spermatozoa, because the decision of further sperm manipulation methods and usage depends on these results. Besides of standard parameters of routine sperm evaluations (volume, sperm concentration, total sperm number, motility and progressive motility) using the complex staining method for analysis of fresh ejaculate and of 24-hours-chilled-stored semen (longevity test), subfertile and infertile stallions would be identified and reason for decreased pregnancy results may be revealed. For this intention the method would be installed into the annual control examination of the stallions’ semen.

9. ÖSSZEFOGLALÁS

A spermiumok értékelésére számos különböző laboratóriumi módszer ismeretes, mindemellett a tesztek többsége a spermiumok csak egy-egy tulajdonságát vizsgálja. Kovács és Foote (1992) tripánkék-neutrálvörös-Giemsa festést írt le az ondósejtek feji és farki részének élő/elhalt- és akroszóma állapotának kimutatására emlős spermiumokon, amely a morfológiai értékelést is biztosítja. A technika első bemutatása óta néhány speciális jellemző és probléma merült fel a ménspermiumok vizsgálatánál. A tripánkék (TB) - Giemsa festéssel – különösen a mélyhűtött mintákban – gondot jelentett az élő és elhalt farkak megkülönböztetése. Mélyhűtés/felolvasztás után az elhalt farkú, de ép fej-membránú ondósejtek aránya nagymértékben megnő. Ezek a sejtek bizonyára mozgásképtelenek, ezért az ép/sérült farkak elkülönítése nagyon fontos az ondóminőség értékelésénél. Az **első kísérlet** célja a komplex festési technika javítása és továbbfejlesztése volt, egyrészt egy másik élő/elhalt festék, a Chicago sky blue 6B (CSB) alkalmazásával, amelynek molekulaszerkezete hasonló a tripánkékhez; másrészt a festés egyes lépéseinek optimális beállításaival a különböző spermium kategóriák hatékonyabb és egyértelműbb elkülönítésének megoldása elsősorban mén spermiumok vizsgálata esetén. A CSB/Giemsa fetés jó ismételhetőséget és módszer-egyetértést mutatott a standard TB/Giemsa mérésekkel. A CSB festék hasonló spermium fej- és tökéletesebb ondósejt farok élő/elhalt differenciálást eredményezett a tripánkékhez képest. A szubjektív vizsgálat megállapítása denzitometriás analízissel megerősítést nyert. A TB festék biztonsággal felváltható a CSB vitális festékkel ménsperma esetén, a farok-membrán épségének pontosabb és könnyebb meghatározását biztosítva. Ménsperma esetén a 0,16%-os CSB-vel történő vitális festés utáni 4 perces fixálás és 25-40°C-on, 2-4 órás Giemsa festés ajánlott.

A **második vizsgálatban** az ép membránú sejteket morfológialag is értékelve kombinált kategóriákba soroltam az ondósejteket és nyomon követtem a sperma-minőség változását a mélyhűtési folyamat egyes lépései utáni analízissel. Az élő, ép akroszómájú sejtek aránya a centrifugálás során nem változott (78±9 vs. 78±8%), a felolvasztott spermában viszont szignifikánsan csökkent (38±11%). Ezen belül a normál morfológiájú sejtek aránya (IHITIA) ugyanazt a tendenciát mutatta (58±16; 58±15; 26±9%, $p<0,01$). Az IHDT sejtek aránya a feldolgozás során csak a fagyasztás/felolvasztás után növekedett (4±3; 4±3; 19±7%, $p<0,01$). Az élő sejtek akroszómájának sérülése, illetve leválása nem volt jellemző a fagyasztás után sem, az IHITDA sejttípus kevesebb, mint 1%-os arányban volt jelen. Vizsgálatainkban azt tapasztaltuk, hogy centrifugálás is okozhat a hidegsokkhoz és hipoozmotikus sokkhatáshoz hasonló morfológiai elváltozást és ez a hatás egyes méneknél fokozottan jelentkezett. Az esetek jellemzően 3 ménhez voltak köthetőek, amelyeknél már a friss spermában is magas arányban volt ez a sejttípus: 14±5%. A 3 mén centrifugált spermájában 19±4%-ra emelkedett ($p<0,01$), ami jellemzően a fagyasztás után is relatíve nagy arányt képviselt (13±5%), amellett, hogy a normál morfológiájú élő sejtek aránya

nagymértékben csökkent (44 %-ról 23%-ra). Ez jelentős aránybeli változást jelent és hatással lehet a mélyhűtött sperma a termékenyítő képességére.
Az összes plazmacseppes+farok-rendellenességet mutató sejtek aránya nem változott a feldolgozás során (25±15; 26±15; 24±15%). Az összes farok-rendellenességgel rendelkező sejtek aránya enyhén emelkedett (10±7; 12±10; 12±10), az összes plazmacseppel rendelkező sejtek aránya pedig enyhe csökkenést mutatott a feldolgozás során (15±9; 13±8; 12±8). Ez több esetben azzal magyarázható, hogy a plazmacseppes-középrészen a centrifugálás után visszahajlás alakul ki, a plazmacsepp pedig megreked a hajtűkanyarban.

A mélyhűtött sperma legfontosabb minőségi paramétere az élő ép ondósjtek aránya. Mindamellett a mélyhűtési technológiák további fejlesztése céljából, a mének egyedi sperma-mélyhűthetőségének feltérképezése és a fagyasztott sperma felhasználhatóságának szempontjából is fontos a mélyhűtési folyamat során a sejtkárosodás helyének pontos behatárolása, amihez a spermiumok egyes részeinek elkülönített értékelése szükséges. Az alkalmazott festési módszer jól használható az ondósejtek subdomain-specifikus vizsgálatára.

Alacsony teljes, vagy élő sejtszám esetén a standard spermium szeparációs módszerek nem mindig hatékonyak. A ménspermiumok ezen túl igen érzékenyek az elhúzódó eljárásokra. A **3. kísérletben** a Percoll szeparációs eljárást sikeresen módosítottam a médium térfogatának csökkentésével (Mini-Percoll), a centrifugálás időtartamának rövidítésével és magasabb g-érték használatával, hogy növeljem az életképes spermiumok kinyerésének hatékonyságát ICSI-re, kis térfogatú, alacsony sejtkoncentrációjú ménsperma elérhetősége esetén A kísérlet célja a mini-Percoll (P) és swim-up (SU) módszer összehasonlítása volt alacsony spermium-számú minták esetén, stimuláló vegyületekkel történő inkubáció nélkül (P-CON, P-NT és SU-NT), pentoxifillinel (PX), illetve hialuronsavval (HA) történő kezelést alkalmazva. Percoll-control (P-CON) és P-PX eredményezte a legtöbb morfológiailag normál, intakt spermiumot és a legjobb sejtkinyerési arányt. Spermium szeparálás után magas arányban fordult elő sérült fejű, ép farki részű spermium (DHIT). A szeparált ép farok membránú spermiumok (amelyek mozgásra képesek) 25-35%-ának sérült volt a feji vagy akroszóma membránja. Ez befolyásolhatja az ICSI eljárás sikerét, amelynek során a beinjektálásra kerülő spermium kiválasztása a mozgási képessége6n alapul a gyakorlatban. Az ép, de akroszóma-sérült sejtek (IHITDA) aránya P-PX kezelés után volt a legmagasabb. Ezek a spermiumok kiürülőben lévő, vagy már kiürült akroszómát tartalmaznak, ami előnyös is lehet az ICSI általi petesejt megtermékenyítés során. Az összes Percoll-szeparálásos kezelés több normál morfológiájú spermiumot és kevesebb plazmacseppet-, vagy közép+farokrész rendellenességet tartalmazó ondósejtet eredményezett, mint a swim up szeparálások (91-92% vs.71-78%; 1% vs.4-7%; 6-7% vs.16-19% sorrendben, $p<0.01$). A hialuronsav kezelés növelte a kinyerési arányt a swim up szeparálás során, viszont az élő és morfológiailag normális sejtek arányát egyik kezelési csoportnál sem. Ennek egyik oka a SU kezelés utáni centrifugálás károsító hatása lehet.

Méneknél, a bikákkal ellentétben nem történt spermatermelésre, laboratóriumi minőségre, mélyhűthetőségre és fertilitásra generációk óta folytatott szelekció. Ezzel magyarázható, hogy az egyes lovak spermájának e jellemzői igen változatosak, magas arányban nem megfelelőek. A **negyedik vizsgálatba** vont „fertilis" és „szubfertilis" méneket az általuk termékenyített és sikeresen vemhesült vagy üresen maradt kancák aránya alapján ítélték jó termékenyítő képességűnek, illetve csökkent fertilitásúnak a mesterséges termékenyítő állomást vezető állatorvosok. Célom az volt, hogy bemutassam az egyes mének spermájának minőségét külön morfológia és norfológiával kombinált membránintegritás alapján és összehasonlítsam vizsgálataim alá vont fertilis mének friss és hűtve-tárolt spermájának ezen paramétereivel. A szubfertilis méneknél a komplex festési módszerrel minden esetben kimutatható volt, hogy a sperma minősége membránintegritás szempontjából vagy az ondósejtek morfológiája szempontjából elmarad a fertilis mének eredményeitől. Sok esetben igen komoly morfológiai defektusokat mutattak, és/vagy az élő, ép membránú és normál morfológiájú spermiumok arányának drámai csökkenését tapasztaltam, más membrán-sérült sejtkategóriák emelkedése mellett. A szubfertilis mének eredményei minden esetben elmaradtak a magyar szabvány (7034/1999) ménspermára vonatkozó előírásától is.

Eredményeink rámutattak az ép membránú normális morfológiájú ondósejtek azonosításának fontosságára. Ajánlatos ezek arányának figyelembe vétele a termékenyítő adagok sejtszámának megállapításánál. Fontos a spermium rendellenességek típusainak meghatározása, mert ezeken alapulhat a további ondómanipulációs módszerek alkalmazása. A rutin spermaértékelés standard paraméterei (térfogat, sűrűség, összes sejtszám, motilitás és progresszív motilitás) mellett a komplex festési módszer további adalékkal szolgál a friss ejakulátum és a 24-órás hűtve tárolt sperma (eltartási próba) vizsgálatával. A szubfertilis és terméketlen mének felismerésével megelőzhetők a csökkent fertilitási eredmények. A módszert hasznos lenne bevezetni a mének spermájának évenkénti kontroll vizsgálatánál.

10. REFERENCES

1. Aalseth EA, Saacke RG. 1986. Vital staining and acrosomal evaluation of bovine sperm. Gamete Res. 15: 73-81.
2. Aitken RJ, Clarkson JS. 1988. Significance of reactive oxygen species and antioxidants in defining of the efficiency of sperm preparation technique. J. Androl. 9: 367–376.
3. Aitken RJ, Clarkson JS, Fishel S. 1999. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol. Reprod. 40: 183–197.
4. Aitken RJ, Fisher H. 1994. Reactive oxygen species generation and human spermatozoa. The balance of benefit and risk. Bioessays 16: 259-267.
5. Álamo D, Batista, M, Gonzalez F, Rodrıguez N, Cruz G, Cabrera F, Gracia A. 2005. Cryopreservation of semen in the dog: use of ultra-freezers of −152 °C as a viable alternative to liquid nitrogen. Theriogenology 63: 72–82.
6. Allen WR. 2005. The development and application of the modern reproductive technologies to horse breeding. Reprod. Dom. Anim. 40: 310-329.
7. Althouse GC. 1998. Cytoplasmic droplets on boar sperm cells. Swine Health Prod. 6: 128.
8. Alvarenga MA, Papa FO, Landim-Alvarenga FC, Medeiros AS. 2005. Amides as cryoprotectants for freezing stallion semen: a review. Anim. Reprod. Sci. 89: 105-113.
9. Alvarez JG, Lasso JL, Blasco L, Nunez RC, Heyner S, Caballero P, Storey BT. 1993. Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile life. Hum. Reprod. 8: 1087–1092.
10. Amann RP. 1993. Physiology and endocrinology. In: McKinnon AO, Voss J, editors. Equine reproduction. Philadelphia: Lea and Febiger; p. 658–673.
11. Amann RP. 2005. Weaknesses in reports of “fertility” for horses and other species. Theriogenology 63: 698-715,
12. Amann RP, Graham JK. 1993. Spermatozoal Function. In: Equine Reproduction, McKinnon, AO, Voss JL. (Eds.), Lea and Febiger, London, UK, 1993, pp: 715-745.
13. Amann RP, Hammerstedt RH. 1993. In vitro evaluation of sperm quality: an opinion. J. Androl. 14: 397–406.
14. Amann RP, Seidel GE, Mortimer RG. 2000. Fertilizing potential in vitro of semen from young beef bulls containing a high or low percentage of sperm with a proximal droplet. Theriogenology 54: 1499–1515.
15. Andersen Berg K, Filseth O, Engeland E. 1996. A sperm midpiece defect in a Hereford bull with variable semen quality and freezability. Acta Vet. Scand. 37: 367–373.
16. Anzar M, He L, Buhr MM, Kroetsch G, Pauls KP. 2002. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol Reprod. 66: 354–360.
17. Arai KY, Tanaka Y, Taniyama H, Tsunoda N, Nambo Y, Nagamine N, Watanabe G, Taya K. 2006. Expression of inhibins, activins, insulin-like growth factor-I and steroidogenic enzymes in the equine placenta. Domest. Anim. Endocrinol. 31: 19–34.
18. Arruda RP, Ball BA, Gravance CG, Garcia AR, Liu IKM. 2002. Effects of extenders and cryoprotectans on stallion sperm head morphometry. Theriogenology 58: 253-256.
19. Assumpcao MEOA, Buratini J Jr, Haipeck K, Lima AS, Mello MRB, Oliveira LJ, Oliveira VP, Tavares LMT, Visintin JA. 2000. In vitro capacitation of bovine sperm by heparin and calcium ionophore evaluated with different staining methods. Theriogenology 53: 485.
20. Aurich JE, Schonherr U, Hoppe H, Aurich C. 1997. Effects of antioxidants on motility and membrane integrity of chilled-stored stallion semen. Theriogenology 48: 185–92.

21. Avery B, Greve T. 1995. Impact of Percoll on bovine spermatozoa used for in vitro insemination. Theriogenology 44: 871-8.
22. Bailey JL, Bilodeau JF, Cormier N. 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl. 21: 1–7.
23. Ball BA. 2005 Infertility in the stallion. Proceedings of the 11th Italian Association of Equine Veterinarians Congress (SIVE), Pisa, Italy.
24. Ball BA. 2008. Diagnostic methods for evaluation of stallion subfertility: a review. J. Equine Vet. Sci. 28: 650-665.
25. Ball BA. 2009. Role of oxidative stress in normal and abnormal function of equine spermatozoa. Rev. Bras. Reprod. Anim. Supl. 6: 20-24 (www.cbra.org.br)
26. Ball BA, Gravance CG, Medina V, Baumber J, Liu IKM. 2000. Catalase activity in equine semen. Am. J. Vet. Res. 61: 1026–1030.
27. Ball BA, Little TV, Weber JA, Woods GL. 1989. Survival of day-4 embryos from young normal mares and aged subfertile mares after transfer to normal recipient mares. J. Reprod. Fertil. 85: 187–194.
28. Ball, BA, Medina V, Gravance CG, Baumber J. 2001. Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5 degrees C. Theriogenology 56: 577-89.
29. Ball BA, Mohammed HO. 1995. Morphometry of stallion spermatozoa by computer-assisted image analysis. Theriogenology 44: 367-377.
30. Ball BA, Vo A. 2001. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. J. Androl. 22: 1061-1069.
31. Barbacini S, Marchi V, Zavaglia G. 1999. Equine frozen semen: results obtained in Italy during the 1994–1997 period. Equine Vet. Educ. 11: 109–112.
32. Barker CAV. Gandier JCC. 1957. Pregnancy in the mare resulted from frozen epididymal spermatozoa. Can. J. Comp. Med. Vet. Sci. 21: 47–50.
33. Barrier-Battut I, Dacheux JL, Gatti JL, Rouviere P, Stanciu C, Dacheux F, Vidament M. 2005. Seminal plasma proteins and semen characteristics in relation with fertility in the stallion. Anim. Reprod. Sci. 89: 255–258.
34. Barroso G, Taylor S, Morshedi M, Manzur F, Gavino F, Oehninger S. 2006. Mitochondrial membrane potential integrity and plasma membrane translocation of phosphatidylserine as early apoptotic markers: a comparison of two different sperm subpopulations. Fertil. Steril. 85: 149–154.
35. Barth AD. 1994. Bull Breeding Soundness Evaluation. Textbook. Printed by The Western Canadian Association of Bovine Prectitioners, Saskatoon.
36. Barth AD, Oko RJ. 1989. Abnormal morphology of bovine spermatozoa. Iowa State University Press, Ames, Iowa.
37. Bataille B, Magistrini M, Palmer E. 1990. Analyse objective de la mobilite du sperme congele-decongele d'etalon. Essai decorrelation avec la fertilite. (Objective determination of sperm motility in frozen-thawed stallion semen. Correlation with fertility). Anim. Breed. Abstr. 96-106.
38. Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MC. 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. J. Androl. 21: 895–902.
39. Baumber J, Ball BA, Linfor JJ. 2005. Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants. Am. J. Vet. Res. 66: 772-779.

40. Baumber J, Ball BA, Linfor JJ, Meyers SA. 2003a. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J. Androl. 24: 621–628.
41. Baumber J, Sabeur K, Vo A, Ball BA. 2003b. Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. Theriogenology 60: 1239-1247.
42. Bedford SJ, Kurokawa M, Hinrichs K, Fissore RA. 2004. Patterns of intracellular calcium oscillations in horse oocytes fertilized by intracytoplasmic sperm injection: possible explanations for the low success of this assisted reproduction technique in the horse. Biol. Reprod. 70: 936–944.
43. Behr B, Hildebrandt TB, Kovács A, Rath D, Blottner S, Goeritz F, Reid CE, Hermes R. 2007. A simple staining method to assess sperm viability and complete morphology under field conditions in rhinoceros and elephant. Annual Conference of Physiology and Pathology of Reproduction and Mutual Conference on Veterinary and Human Reproductive Medicine 2007. Reprod. Dom. Anim. 42 (Suppl. 1): p.3.
44. Behr B, Rath D, Hildebrandt TB, Goeritz F, Blottner S, Portas TJ, Bryant BR, Sieg B, Knieriem A, De Graaf SP, Maxwell WMC, Hermes R. 2009a. Index of sperm sex sortability in elephant and rhinoceros. Reprod. Dom. Anim. 44: 273-277.
45. Behr B, Rath D, Mueller P, Hildebrandt TB, Goeritz F, Braun BC, Leahy T, De Graaf SP, Maxwell WMC, Hermes R. 2009b. Feasibility of sperm sex-sorting in rhinoceros species. Theriogenology 72: 353-264.
46. Bielanski W, Kaczmarski F. 1979. Morphology of spermatozoa in semen from stallions of normal fertility. J. Reprod. Fert. Suppl: 39-45.
47. Birkhead TR, Immler S. 2006. Making sperm: quality control and sperm competition. 10th International Symposium on Spermatology, Madrid, 17-22 Sept. 2006, Abstract in the proc.: p. 33.
48. Blanc G, Magistrini M, Palmer E. 1991. Use of Concanavalin A for coating the membranes of stallion spermatozoa. J. Reprod. Fert . Suppl. 44: 191-198.
49. Blanchard TL, Johnson L, Roser JF. 2000. Increased germ cell loss rates and poor semen quality in stallions with idiopathic testicular degeneration. J. Equine Vet. Sci. 20: 263–265.
50. Blanchard TL, Varner DD. 1993. Testicular degeneration. In: McKinnon AO, Voss JL eds. Equine Reproduction. Philadelphia: Lea & Febiger, 855-860.
51. Bland JM, Altman DG. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet i 8476: 307–310.
52. Blom E. 1950. Interpretation of spermatic cytology in bulls. Fertil. Steril. 1: 223–238.
53. Blom E. 1959. A rare sperm abnormality: corkscrew-sperm associated with sterility in bulls. Nature 183: 1280–1281.
54. Blom E. 1973. Ultrastucture of some characteristic sperm defects and a proposal for a new classification of the bull spermiogram. (in Danish) Nord. Vet. Med. 25: 383-391.
55. Blom E. 1977. Sperm morphology with reference to bull infertility. In: First All India Symposium Animal Reproduction. p. 61–81.
56. Blom E, Birch-Andersen A. 1962. Ultrastructure of the sterilizing knobbed sperm defect in the bull. Nature 194: 989–990.
57. Blottner S, Warnke C, Tuchscherer A, Heinen V, Torner H. 2001. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. Anim. Reprod. Sci. 65: 75-88.
58. Bölcsházy K, Mészáros I. Állatorvosi Szülészet 2, Mezőgazdasági Kiadó, Budapest. 1962. 194-199.
59. Bosard T, Love C, Brinsko S, Blanchard T, Thompson J, Varner D. 2005. Evaluation and diagnosis of acrosome function/dysfunction in the stallion. Anim. Reprod. Sci. 89: 215-

217.
60. Bowen JM. 1969. Artificial insemination in the horse. Equine Vet. J. 1: 98–110.
61. Boyle MS. 1999. Assessing the potential fertility of frozen stallion semen. In: Allen WR and Wade JF (eds.), Havemeyer Foundation Monograph Series No. 1. R & W Publications Ltd, Newmarket, p. 13–16.
62. Brandon CI, Heusner GL, Caudle AB, Fayrer-Hosken RA. 1999. Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility. Theriogenology 52: 863–873.
63. Brinsko SP, Crockett EC, Squires EL. 2000. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoa motility after cooling and storage. Theriogenology 54: 129–136.
64. Brinsko SP, Love CC, Bauer JE, Macpherson ML, Varner DD. 2005a. Cholesterol to phospholipid ratio in sperm of stallions with unexplained subfertility. Anim. Reprod. Sci. 89: 217-219.
65. Brinsko SP, Varner DD, Love CC, Blanchard TL, Day BC, Wilson ME. 2005b. Effect of feeding a DHA-enriched nutriceutical on the quality of fresh, cooled and frozen stallion semen. Theriogenology 63: 1519-1527.
66. British Standards Institution (1975) Precision of test methods. 1. Guide for the determination and reproducibility for a standard test method (BS 597, Part 1). London, BSI.
67. Brito LFC. 2007. Evaluation of stallion sperm morphology. Clin. Tech. Equine. Pract. 6: 249-264.
68. Brito LF, Greene LM, Kelleman A, Knobbe M, Turner R. 2011. Effect of method and clinician on stallion sperm morphology evaluation. Theriogenology 76: 745-750.
69. Brito LF, Kelleman A, Greene LM, Raz T, Barth AD. 2010. Semen characteristics in a sub-fertile Arabian stallion with idiopathic teratospermia. Reprod. Dom. Anim. 45: 354-358.
70. Brück I, Anderson GA, Hyland JH. 1993: Reproductive performance of thoroughbred mares on six commercial stud farms. Aust. Vet. J. 70: 299–303.
71. Brum AM, Sabeur K, Ball BA. 2008. Apoptotic-like changes in equine spermatozoa separated by density-gradient centrifugation or after cryopreservation. Theriogenology 69: 1041-1055.
72. Busch W., Holzmann A. (Eds.) 2001. Veterinary Medicine Andrology, Physiology, Pathology of Reproduction in Male Animals. Schattauer, Stuttgart.
73. Caiza de la Cueva FIC, Pujol MR, Rigau T, Bonet S, Miro J, Briz M, Rodriguez-Gill JE. 1997. Resistance to osmotic stress of horse spermatozoa: the role of ionic pumps and their relationship to cryopreservation success. Theriogenology 48: 947–968.
74. Calogero AE, Fishel S, Hall J, Ferrara E, Vicari E, Green S, Hunter A, Burrello N, Thornton S, D'Agata R. 1998. Correlation between intracellular cAMP content, kinematic parameters and hyperactivation of human spermatozoa after incubation with pentoxifylline. Hum. Reprod. 13: 911-915.
75. Calvete JJ, Nessau S, Mann K, Sanz L, Sieme H, Klug E, Töpfer-Petersen E. 1994. Isolation and biochemical characterization of stallion seminal-plasma proteins. Reprod. Dom. Anim. 29: 411-426.
76. Campbell RC, Hancock JL, Shaw IG. 1960. Cytological characteristics and fertilizing capacity of bull spermatozoa. J. Agric. Sci. 55: 91-99.
77. Card C. 2005 Cellular associations and the differential spermiogram: Making sense of stallion spermatozoal morphology. Theriogenology 64: 558-567.
78. Card C. 2010. Reproductive problems in the stallion. Proceedings of the 16th Annual Meeting of the Italian Association of Equine Veterinarians, Carrara, Italy January 29-31

2010. pp. 20-29. Publication is available in http://www.ivis.org
79. Carvajal G, Cuello C, Ruiz M, Vázquez JM, Martínez EA, Roca J. 2004. Effects of centrifugation before freezing on boar sperm cryosurvival. J. Androl. 25: 389-96.
80. Casey PJ, Gravance CG, Davis RO, Chabot DD, Liu IK. 1997. Morphometric differences in sperm head dimensions of fertile and subfertile stallions. Theriogenology 47: 575-582.
81. Casey PJ, Hillman RB, Robertson KR, Yudin AI, Liu IK, Drobnis EZ. 1993. Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. J. Androl. 14: 289-297.
82. Celeghini EC, de Arruda RP, de Andrade AF, Nascimento J, Raphael CF. 2007. Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. Reprod. Dom. Anim. 42: 479-488.
83. Celeghini ECC, Arruda RP, Andrade AFC, Raphael CF, Nascimento J. 2004. Simultaneous evaluation of the plasmatic, acrosomal, and mitochondrial membranes in equine spermatozoa. Proceedings of the 15th Internacional Congress of Animal Reproduction; 2004; p. 511. Porto Seguro, BA-Brazil.
84. Centola GM, Herko R, Andolina E, Weisensel S. 1998. Comparison of sperm separation methods: effect on recovery, motility, motion parameters, and hyperactivation. Fertil. Steril. 70: 1173–1175.
85. Chan SY, Chan YM, Tucker MJ. 1991. Comparison of characteristics of human spermatozoa selected by the multiple tube swim-up and simple discontinous Percoll® gradient centrifugation. Andrologia 23: 213–218.
86. Chen MJ, Bongso A. 1999. Comparative evaluation of two density gradient preparations for sperm separation for medically assisted conception. Hum. Reprod. 4: 759-764.
87. Cheng FP, Fazeli A, Voorhout WF, Marks A, Bevers MM, Colenbrander B. 1996. Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. J. Androl. 17: 674-682.
88. Cheng TC, Huang CC, Huang LS, Chen CI, Lee MS, Liu JY. 2004. Evaluation of mouse blastocyst implantation rate by morphology grading. Chin. J. Physiol. 47: 43-47.
89. Chenoweth PJ. 2005. Genetic sperm defects. Theriogenology 64:457-468,
90. Chenoweth PJ, Pascoe RR, Mc Dougall HL, Mc Cosker PJ. 1970. An abnormality of the spermatozoa of a stallion (Equus caballus). Brit. Vet. J. 126: 476–488.
91. Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT. 2006. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. J. Androl. 27: 53–59.
92. Choi YH, Love CC, Love LB, Varner DD, Brinsko S, Hinrichs K. 2002. Developmental competence in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen-thawed spermatozoa. Reproduction 123: 455-465.
93. Choi YH, Love CC, Varner DD, Hinrichs K. 2006. Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. Theriogenology 65: 808-819.
94. Christensen P, Parlevliet JM, van Buiten A, Hyttel P, Colenbrander B. 1995. Ultrastructure of fresh and frozen-thawed stallion spermatozoa. Biol. Reprod. Mono. 1: 769-777.
95. Clément F, Ladonnet Y, Magistrini M. 2001. Sperm morphology and fertility. Anim. Reprod. Sci. 68: 362–363.
96. Colenbrander B, Fazeli AR, van Buiten A, Parlevliet J, Gadella BM. 1992. Assessment of sperm cell membrane integrity in the horse. Acta. Vet. Scand. Suppl 88: 49-58.
97. Colenbrander B, Feitsma H, Grooten HJ. 1993. Optimizing semen production for artificial insemination in swine. J. Reprod. Fertil. Suppl. 48: 207–215.

98. Colenbrander B, Gadella, BM, Stout TAE. 2003. The predictive value of semen analysis in the evaluation of stallion fertility. Reprod. Dom. Anim. 38: 305–311.
99. Costa MZ, Oliveira LZ, Resende MV, Lucio AC, Perini AP, Miguel MCV, Lima VFMH. 2010. Induction of the acrosome reaction test to in vitro estimate embryo production in Nelore cattle. Arq. Bras. Med. Vet. Zootec. 62: 771-777.
100. Cross NL, Mahasreshti P. 1997. Prostasome fraction of human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist progesterone. Arch. Androl. 39: 39-44.
101. Cross NL, Meizel S. 1989. Methods for evaluating the acrosomal status of mammalian sperm. Biol Reprod 41: 635-641.
102. De Leeuw FE, Chen HC, Colenbrander B, Verkleij AJ. 1990. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. Cryobiology 27: 171–183.
103. Dell'Aqua Jr. JA, Papa FO, Alvarenga MA, Zahn FS. 2001. Effect of centrifugation and packing system on sperm parameters of equine frozen semen. Anim. Reprod. Sci. 68: 324-325.
104. De Vries PJ. 1993. Diseases of the testes, penis, and related structures. In: McKinnon AO, Voss JL eds. Equine Reproduction. Philadelphia: Lea & Febiger, 1993; 878-884.
105. Devireddy RV, Swandlund DJ, Olin T, Vincente W, Troedsson MHT, Bischof JC, Roberts, KP. 2002. Cryopreservation of equine sperm: optimal cooling rates in the presence and absence of cryoprotective agents determined using differential scanning calorimetry. Biol. Reprod. 66: 222-231.
106. Didion BA, Dobrinsky JR, Giles JR, Graves CN. 1989. Staining procedure to detect viability and true acrosome reaction in spermatozoa of various species. Gamete Res. 22: 51-57.
107. Dinger JE, Noiles EE, Hoagland TA. 1986. Effect of controlled exercise on semen characteristics in two-year-old stallions. Theriogenology 25: 525–535.
108. Dobrinski I, Thomas PGA, Ball BA. 1995. Cryopreservation reduces the ability of equine sperm to attach to oviductal epithelial cells and zona pellucida in vitro. J. Androl. 16: 536–542.
109. Domes U. 2003. Untersuchungen über die Bestimmung der Spermaqualität - insbesondere Motilität und Membranintegrität – und Zusammenhänge zur Fertilität von Besamungshengsten. Inaugural Dissertation zur Erlangung der tiermedizinischen Doktorwürde der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München.
110. Domes U, Stolla R. 2001. Staining of stallion spermatozoa with damaged tail membrane after freezing/thawing. Anim. Reprod. Sci. 68: 329-330.
111. Dott HM. 1975. Morphology of stallion spermatozoa. J. Reprod. Fertil. Suppl. 23: 41–46.
112. Dott HM, Dingle JT. 1968. Distribution of lysosomal enzymes in the spermatozoa and cytoplasmic droplets of bull and ram. Exp. Cell Res. 52: 523–540.
113. Dowsett KF, Knott LM. 1996. The influence of age and breed on stallion semen. Theriogenology 46: 397-412.
114. Dowsett KF, Osborne HG, Pattie WA. 1984. Morphological characteristics of stallion spermatozoa. Theriogenology 22: 463-472.
115. Dowsett KF, Pattie WA. 1982. Characteristics and fertility of stallion semen. J. Reprod. Fert. 32: 1–8.
116. Dudenhausen E, Talbot P. 1982. Detection and kinetics of the normal acrosome reaction of mouse sperm. Gamete Res. 6: 257-265.
117. Ecot P, Vidament M, de Mornac A, Perigault K, Cle´ment F, Palmer E. 2000. Freezing of stallion semen: interactions among cooling treatments, semen extenders and stallions. J

Reprod Fertil Suppl. 56: 141–150.
118. Egbase PE, al-Sharhan M, Ing R, Grudzinskas JG. 1997. Pregnancy rates after intracytoplasmic sperm injection in relation to sperm recovery techniques. J. Assist. Reprod. Genet. 14: 317-320.
119. Einarsson S, Dalin AM, Lundeheim N. 2009. Sperm production and sperm morphology of Swedish Warmblood stallions. Reprod. Domest. Anim. 44: 33-36.
120. Eliasson R, Treichl L. 1971. Supravital staining of human permatozoa. Fertil. Steril. 22: 134-137.
121. Englert Y, Lesage B, Van Vooren JP, Liesnard C, Place I, Vannin AS, Emiliani S, Delbaere A. 2004. Medically assisted reproduction in the presence of chronic viral disease. Hum. Reprod. Update 10: 149–162.
122. Englert Y, Van Den Bergh M, Rodesch C, Bertrand E, Biramane J, Legreve A. 1992. Comparative auto-controlled study between swim-up and Percoll® preparation of fresh semen samples for in vitro fertilization. Hum. Reprod. 7: 399–402.
123. Ericsson SA, Garner DL, Thomas CA, Dowling TW, Marshall CE. 1993. Interrelationships among fluorometric analysis of spermatozoal function, classical semen quality parameters and the fertility of frozen-thawed bovine spermatozoa. Theriogenology 39: 1009-1024.
124. Esteso MC, Soler AJ, Fernández-Santos MR, Quintero-Moreno AA, Garde JJ. 2006. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red deer (Cervus elaphus hispanicus) epididymal sperm samples. J. Androl. 27: 662-670.
125. Esteves SC, Spaine DM, Cedenho AP. 2007. Effects of pentoxifylline treatment before freezing on motility, viability and acrosome status of poor quality human spermatozoa cryopreserved by the liquid nitrogen vapor method. Braz. J. Med. Biol. Res. 40: 985-992.
126. Evenson DP, Darzynkiewicz Z, Melamed MR. 1980. Relation of mammalian sperm chromatin heterogenecity to fertility. Science 210:1131–1133.
127. Evenson DP, Darzynkiewicz Z, Melamed MR. 1982. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J. Histochem. Cytochem. 30: 279-280.
128. Farlin ME, Jasko DJ, Graham JK, Squires EL. 1992. Assessment of Pisum sativum agglutinin in identifying acrosomal damage in stallion spermatozoa. Mol. Reprod. Dev. 32: 23-27.
129. Fazeli AR, Steenweg W, Bevers MM, van den Broek J, Bracher V, Parlevliet J, Colenbrander B, 1995. Relation between stallion sperm binding to homologous hemizonae and fertility. Theriogenology 44: 751–760.
130. Fisch JD, Behr B, Conti M. 1998. Enhancement of motility and acrosome reaction in human spermatozoa: differential activation by type-specific phosphodiesterase inhibitors. Hum. Reprod. 13: 1248-1254.
131. Fischer KA, Van Leyen K, Lovercamp KW, Manandhar G, Sutovsky M, Feng D, Safranski T, Sutovsky P. 2005. 15-Lipoxygenase is a component of the mammalian sperm cytoplasmic droplet. Reproduction 130: 213-222.
132. Foote RH. 2002. The history of artificial insemination: Selected notes and notables. J Anim Sci. 80: 1-10.
133. Gabriel LK, Vawda AI. 1993. Preparation of human sperm for assisted conception: a comparative study. Arch. Androl. 30(1): 1-6.
134. Gadea J, Garcia-Vazquez F, Matas C, Gardon JC, Canovas S, Gumbao D. 2005. Cooling and freezing of boar spermatozoa: supplementation of the freezing media with reduced glutathione preserves sperm function. J. Androl. 26: 396-404.
135. Gadella BM, Miller NGA, Colenbrander B, van Golde LMG, Harrison RAP. 1999.

Flow cytometric detection of transbilayer movement of fluorescent phospholipid analogues across the boar sperm plasma membrane: elimination of labelling artefacts. Mol. Reprod. Dev. 53: 108–125.
136. Gadella BM, Rathi R, Brouwers JFHM, Stout TAE, Colenbrander B. 2001. Capacitation and the acrosome reaction in equine sperm. Anim. Reprod. Sci. 68: 249–265.
137. García-Roselló E, Matás C, Cánovas S, Moreira PN, Gadea J, Coy P. 2006. Influence of sperm pretreatment on the efficiency of intracytoplasmic sperm injection in pigs. J Androl. 27: 268-75.
138. Garner DL, Johnson LA, Yue ST, Roth BL, Haugland RP. 1994. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. J Androl. 15: 620-629.
139. Garner DL, Pinkel D, Johnson LA, Pace MM. 1986. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. Biol. Reprod. 34: 127- 138.
140. Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE. 1997. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol Reprod. 57: 1401-1406.
141. Garside DA, Charlton A, Heath KJ. 1996. Establishing the timing of implantation in the Harlan Porcellus Dutch and New Zealand white rabbit. Reg. Tox. Pharm. 23: 69–73.
142. Geraghty RJ, Morrell JM, Spencer L, Holmes PV. 2004. Removal of equine arteritis virus from stallion semen. Reprod. Fertil. Dev. 16. Abst. 195.
143. Gerlach T, Aurich JE. 2000. Regulation of seasonal reproductive activity in the stallion, ram and hamster. Anim. Reprod. Sci. 58: 197–213.
144. Gianaroli L, Magli MC, Ferraretti AP, Crippa A, Lappi M, Capitani S, Baccetti B. 2010. Birefringence characteristics in sperm heads allow for the selection of reacted spermatozoa for intracytoplasmic sperm injection. Fertil. Steril. 93: 807-813.
145. Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. 1996. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. J. Androl. 17: 276–287.
146. Gómez MC, Catt JW, Gillan L, Evans G, Maxwell WM. 1997. Effect of culture, incubation and acrosome reaction of fresh and frozen-thawed ram spermatozoa for in vitro fertilization and intracytoplasmic sperm injection. Reprod. Fertil. Dev. 9: 665-673.
147. Goolsby HA, Elanton JR, Prien SD. 2004. Preliminary comparisons of a unique freezing technology to traditional cryopreservation methodology of equine spermatozoa. J. Equine Vet. Sci. 24: 314–318.
148. Goto K, Kinoshita A, Takuma Y, Ogawa K. 1990. Fertilization of bovine oocytes by the injection of immobilised, killed spermatozoa. Vet. Rec. 127: 517 -520.
149. Gradil CM, Ball BA. 2000. The use of pentoxifyllin to impove motility of cryopreserved equine spermatozoa. Therigenology. 54: 1041-1047.
150. Graham JK, Kunze E, Hammerstedt RH. 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biol Reprod. 43: 55-64.
151. Gravance CG, Champion Z, Liu IK, Casey PJ. 1997. Sperm head morphometry analysis of ejaculate and dismount stallion semen samples. Anim. Reprod. Sci. 47: 149-155.
152. Gravance CG, Garner DL, Baumber J, Ball BA. 2000. Assessment of equine sperm mitochondrial function using JC-1. Theriogenology. 53:1691-703.
153. Gravance CG, Liu IK, Davis RO, Hughes JP, Casey PJ. 1996. Quantification of normal head morphometry of stallion spermatozoa. J. Reprod. Fertil. 108:41-46.
154. Grippo AA, Way AL, Killian GJ. 1995. Effect of bovine ampullary and isthmic

oviductal fluid on motility, acrosome reaction and fertility of bull spermatozoa. J. Reprod. Fertil. 105: 57–64.
155. Gualtieri R, Talevi R. 2000. In vitro-cultured Bovine oviductal cells bind acrosome-intact sperm and retain this ability upon sperm release. Biol. Reprod. 62: 1754–1762.
156. Hallap T, Nagy Sz, Jaakma U, Johannisson A, Rodriguez-Martinez H. 2005. Mitochondrial activity of frozen-thawed spermatozoa assessed by MitoTracker Deep Red 633. Theriogenology 63: 2311-2322.
157. Hallap T, Nagy Sz, Jaakma U, Johannisson A, Rodriguez-Martinez H. 2006. Usefulness of a triple fluorochrome combination Merocyanine 540/Yo-Pro 1/Hoechst 33342 in assessing membrane stability of viable frozen-thawed spermatozoa from Estonian Holstein AI bulls. Theriogenology 65: 1122-1136.
158. Hammerstedt RH, Graham JK. 1992. Cryopreservation of poultry sperm: the enigma of glycerol. Cryobiology 29: 26–38.
159. Hammerstedt RH, Graham JK, Nolan JP. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. J. Androl. 11: 73-88.
160. Hancock JL. 1957. The morphology of boar spermatozoa. J. R. Microsc. Soc. 76: 84-97.
161. Haraszti J, Zöldág L. 1993. A háziállatok szülészete és szaporodásbiológiája. Mezőgazdasági Könyvkiadó, Budapest.
162. Harrison RAP, Vickers SE. 1990. Use of fluorescent probes to assess membrane intergrity in mammalian spermatozoa. J. Reprod. Fertil. 88: 343-352.
163. Heape W. 1897. The artificial insemination of mammals and subsequent possible fertilization or impregnation of their ova. Proc. R. Soc. Lond. B. 61: 52–63.
164. Heitland AV, Jasko DJ, Squires EL, Graham JK, Pickett BW, Hamilton C. 1996. Factors affecting motion characteristics of frozen-thawed stallion spermatozoa. Equine Vet. J. 28: 47–53.
165. Held JP, Prater P, Stettler M. 1991. Spermatozoal head defect as a cause of infertility in a stallion. J. Am. Vet. Med. Assoc. 199: 1760-1761.
166. Hellander JC, Samper JC, Crabo BG. 1991. Fertility of a stallion with low sperm motility and a high incidence of an unusual tail defect. Vet. Rec. 128: 449–451.
167. Heninger NL, Staub C, Blanchard TL, Johnson L, Varner DD, Forrest DW. 2004. Germ cell apoptosis in the testes of normal stallions. Theriogenology 62: 283–97.
168. Henkel RR, Schill WB. 2003. Sperm preparation for ART. Reprod. Biol. Endocrinol. 1:108.
169. Hermes R, Behr B, Hildebrandt TB, Blottner S, Sieg B, Frenzel A, Knieriem A, Saragusty J, Rath D. 2009. Sperm sex-sorting in the Asian elephant (Elephas maximus). Anim. Reprod. Sci. 112: 390-396.
170. Hermo L, Dworkin J, Oko R. 1988. Role of epithelial clear cells of the rat epididymis in the disposal of the contents of cytoplasmic droplets detached from spermatozoa. Am. J. Anat. 183: 107–124.
171. Hershko A, Ciechanover A. The ubiquitin system. Ann. Rev. Biochem. 1998. 67: 425–479.
172. Hess MF, Roser JF. 2001. The effects of age, season and fertility status on plasma and intratesticular insulin-like growth factor I. concentration in stallions. Theriogenology 56: 723–733.
173. Hidalgo M, Rodríguez I, Dorado J, Soler C. 2008. Morphometric classification of Spanish thoroughbred stallion sperm heads. Anim. Reprod .Sci. 103: 374-378.
174. Hinrichs K. 2010. In vitro production of equine embryos: state of the art. Reprod. Domest. Anim. 45. Suppl 2: 3-8.
175. Hinting A, Lunardhi H. 2001. Better sperm selection for intracytoplasmic sperm injection with the side migration technique. Andrologia 33: 343-346.

176. Hoogewijs M, Rijsselaere T, De Vliegher S, Vanhaesebrouck E, De Schauwer C, Govaere J, Thys M, Hoflack G, Van Soom A, de Kruif A. 2010. Influence of different centrifugation protocols on equine semen preservation. Theriogenology 74: 118-126.
177. Horváth A, Kútvölgyi G, Molnár M, Pribenszky Cs, Harnos A, Szenci O. 2007. A magas hidrosztatikai nyomás alkalmazása a ménondó mélyfagyasztási protokolljában. 14. Szaporodásbiológiai Találkozó. 2007. október 5. Keszthely, Hungary, oral presentation in Hungarian
178. Householder DD, Pickett BW, Voss JL, Olar TT. 1981. Effect of extender, number of spermatozoa and hCG on equine fertility. Equine Vet. Sci. 1: 9-13.
179. Hurtgen JP, Johnson LA. 1982. Fertility of stallions with abnormalities of the sperm acrosome. J. Reprod. Fertil. Suppl. 32: 15–20.
180. Huszár G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E, Vigue L. 2003. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. Fertil Steril. 79 (suppl 3): 1616–1624.
181. Huszár G, Ozkavukcu S, Jakab A, Celik-Ozenci C, Sati GL, Cayli S. 2006. Hyaluronic acid binding ability of human sperm reflects cellular maturity and fertilizing potential: selection of sperm for intracytoplasmic sperm injection. Curr. Opin. Obstet. Gynecol. 18: 260-267.
182. Huszár G, Vigue L. 1994. Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. J. Androl. 15: 71-77.
183. Hyne RV, Stojanoff A, Clarke GN, Lopata A, Johnston WIH. 1986. Pregnancy from in vitro fertilization of human eggs after separation of motile spermatozoa by density gradient centrifugation. Fertil. Steril. 45: 93-96.
184. Ivanov II. 1922. On the use of artificial insemination for zootechnical purposes in Russia. J. Agric. Sci. 12: 244–256.
185. Jakab A, Sakkas D, Delpiano E, Cayli S, Kovanci E, Ward D, Revelli A, Huszar G. 2005. Intracytoplasmic sperm injection: a novel selection method for sperm with normal frequency of chromosomal aneuploidies. Fertil. Steril. 84: 1665-1673.
186. Janett F, Aebi L, Burger D, Imboden I, Hässig M, Kindahl H, Thun R. 2005. Influence of vedaprofen (Quadrisol) on quality and freezability of stallion semen. Theriogenology. 64: 1867-1877.
187. Janett F, Burkhardt C, Burger D, Imboden I, Hässig M, Thun R. 2006. Influence of repeated treadmill exercise on quality and freezability of stallion semen. Theriogenology 65: 1737-1749.
188. Janett F, Thun R, Niederer K, Burger D, Hässig M. 2003. Seasonal changes in semen quality and freezability in the Warmblood stallion. Theriogenology 60: 453-461.
189. Janett F, Thun R, Ryhiner A, Burger D, Hässig M, Hertzberg H. 2001. Influence of Eqvalan® (ivermectin) on quality and freezability of stallion semen.Theriogenology 55: 785-792.
190. Jansen T, Forster P, Levine MA, Oelke H, Hurles M, Renfrew C, Weber J, Olek K. 2002. Mitochondrial DNA and the origins of the domestic horse. Proc. Natl. Acad. Sci. USA. 99:10905-10910.
191. Jasko DJ. 1992. Evaluation of stallion semen. Vet. Clin. North. Am. Equine Pract. 8: 129-148.
192. Jasko DJ, Lein DH, Foote RH. 1990. Determination of the relationship between sperm morphologic classifications and fertility in stallions: 66 cases (1987–1988). J. Am. Vet. Med. Assoc. 197: 389–394.
193. Jasko DJ, Little TV, Smith K, Lein DH, Foote RH. 1988. Objective analysis of stallion sperm motility. Theriogenology 30: 1159-1167.

194. Jasko DJ, Moran DM, Farlin ME, Squires EL. 1991. Effect of seminal plasma dilution or removal on spermatozoa motion characteristics of cooled semen. Theriogenology 35: 1059–1067.
195. Jasko, DJ, Moran, DM, Farlin ME, Squires EL, Amann RP, Pickett BW. 1992. Pregnancy rates utilizing fresh, cooled and frozen-thawed stallion semen. In: Proceedings of the 38th Annual Convention of American Association on Equine Practice. Orlando, Florida, pp. 649–660.
196. Jayaprakash D, Kumar KS, Shivaji S, Seshagiri PB. 1997. Pentoxifylline induces hyperactivation and acrosome reaction in spermatozoa of golden hamsters: changes in motility kinematics. Hum. Reprod. 12: 2192-2199.
197. Jeyendran RS, van der Ven HH, Pérez-Peláez M, Crabo BG, Zaneveld LJD. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil. 70: 219-228.
198. Jeyendran RS, van der Ven HH, Zaneveld LJD. 1992. The hypoosmotic swelling test: an update. Arch Androl 29, 105–116.
199. Johnson D.E, Confino E, Jeyendran RS. 1996. Glass wool column filtration versus mini-Percoll gradient for processing poor quality semen samples. Fertil. Steril. 66(3): 459-462.
200. Johnson L, Blanchard TL, Varner DD, Scrutchfield WL. 1997. Factors affecting spermatogenesis in the stallion. Theriogenology 48: 1199-1216.
201. Juhász J, Nagy P, Kulcsár M, Huszenicza Gy. 2000. Methods for semen and endocrinological evaluation of the stallion: A Review, Acta Vet. Brno 69: 247-259.
202. Juhász J, Nagy P. 2003. A mének nemi működésének jellemzői. Mesterséges termékenyítés és sperma mélyhűtés. A Magyar Állatorvosi Kamara Lógyógyász Specialistája-Szakvizsga előkészítő képzésre készült jegyzet, Budapest, 2003.
203. Kankofer M, Kolm G, Aurich J, Aurich C. 2005. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5 degrees C. Theriogenology 63 :1354-1365.
204. Kaplan M, Russell LD, Peterson RN, Martan J. 1984. Boar sperm cytoplasmic droplets: their ultrastructure, their numbers in the epididymis and at ejaculation and their removal during isolation of sperm plasma membranes. Tissue Cell 16: 455–468.
205. Kareskoski AM, del Alamo MM, Güvenc K, Reilas T, Calvete JJ, Rodriguez-Martinez H, Andersson M, Katila T. 2011. Protein Composition of Seminal Plasma in Fractionated Stallion Ejaculates. Reprod. Dom. Anim. 46: 79-84.
206. Kareskoski M, Katila T. 2008. Components of stallion seminal plasma and the effects of seminal plasma on sperm longevity. Anim. Reprod. Sci. 107: 249-256.
207. Kasai T, Hoshi K, Yanagimachi R. 1999. Effect of sperm immobilisation and demembranation on the oocyte activation rate in the mouse. Zygote 7: 187-193.
208. Katayama M, Koshida M, Miyake M. 2002. Fate of the acrosome in ooplasm in pigs after IVF and ICSI. Hum. Reprod. 17: 2657 -2664.
209. Katayama M, Sutovsky P, Yang BS, Cantley T, Rieke A, Farwell R, Oko R, Day BN. 2005. Increased disruption of sperm plasma membrane at sperm immobilization promotes dissociation of perinuclear theca from sperm chromatin after intracytoplasmic sperm injection in pigs. Reproduction 130: 907–916.
210. Katila, T. 2001a. In vitro evaluation of frozen-thawed stallion semen: a review. Acta. Vet. Scand. 42: 199-217.
211. Katila T. 2001b. Sperm-uterine interactions: a review. Anim. Reprod. Sci. 68: 267-272.
212. Katila T, Koskinen E, Andersson M. 2000a. Evaluation of frozen-thawed semen. A Dorothy Russell Havemeyer Foundation Workshop, Advanced Current Topics in Stallion Veterinary Practice 54-56.

213. Katila T, Kuisma P, Andersson M. 2000b. Evaluation of frozen stallion semen. Proceeding of the First Meeting of the European Equine Gamete Group (EEGG) (Edited by: Allen WR, Wade JF). Havemeyer Foundation Monograph Series 1: 19-21.
214. Katkov II, Mazur P. 1998. Influence of centrifugation regimes on motility, yield and cell associations of mouse spermatozoa. J. Androl. 19: 232–241.
215. Kavak A, Johannisson A, Lundeheim N, Rodriguez-Martinez H, Aidnik M, Einarsson S. 2003a. Evaluation of cryopreserved stallion semen from Tori and Estonian breeds using CASA and flow cytometry. Anim. Reprod. Sci. 76: 205-216.
216. Kavak A, Lundeheim N, Aidnik M, Einarsson S. 2003b. Testicular measurements and daily sperm output of Tori and Estonian breed stallions. Reprod. Dom. Anim. 38: 167–169.
217. Kavak A, Lundeheim N, Aidnik M, Einarsson S. 2004. Sperm morphology in Estonian and Tori Breed stallions. Acta Vet. Scand. 45: 11-18.
218. Kawarsky SJ, Stubbings RB, Basrur PK, King WA. 1995. Pyriform bovine spermatozoa and fertilization potential. Can. Vet. J. 36: 434-436.
219. Kenney RM, Bergman RV, Cooper WL, Morse GW. 1975. Minimal contamination techniques for breeding mares: techniques and preiminary findings. In:Proc.21st Annu. Conv. Am. Assoc. Equine Pract. p327.
220. Kenney RM, Evenson DP, Garcia MC, Love CC. 1995. Relationship between sperm chromatin structure, motility, and morphology of ejaculated sperm and seasonal pregnancy rate. Biol. Reprod. Monogr. Ser. 1. Equine Reprod. 4: 647–653.
221. Kenney RM, Hurtgen J, Pierson R, Withersponn D, Simons J. 1983. Manual for clinical fertility evaluation of the stallion. J. Soc. Theriogenology 9: 3-100.
222. Kenney RM, Hurtgen JP, Pierson RA, Witherspoon D, Simons J. 1990. Society for Theriogenology manual for clinical fertility evaluation of the stallion. Hastings, Nebraska: Society for Theriogenology.
223. Kenney RM, Kent MG, Garcia MC, Hurtgen JP. 1991. The use of DNA index and karyotype analyses as adjuncts to the estimation of fertility in stallions. J. Reprod. Fertil. Suppl. 44: 69–75.
224. Keyser-Tracqui C, Blandin-Frappin P, Francfort HP, Ricaut FX, Lepetz S, Crubézy E, Samashev Z, Ludes B. 2005. Mitochondrial DNA analysis of horses recovered from a frozen tomb (Berel site, Kazakhstan, 3rd Century BC). Anim Genet. 36: 203-209.
225. Kirk ES, Squires EL, Graham JK. 2005. Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. Theriogenology 64:1422-1439.
226. Koehler JK. 1985. Mammalian sperm membranes: Their mosaic form and differential sensitivities. In: Deep freezing of boar semen. Ed: Johnson LA, Larsson K. Uppsala, Sweden, 37-60.
227. Kovacic B, Vlaisavljevic V, Reljic M. 2006. Clinical use of pentoxifylline for activation of immotile testicular sperm before ICSI in patients with azoospermia. J. Androl. 27: 45-52.
228. Kovács A, Foote RH. 1992. Viability and acrosome staining of bull, boar and rabbit spermatozoa. Biotech. Histochem. 67: 119-124.
229. Kovács A, Foote RH, Nagy Sz, Boersma A, Leidl W, Stolla R, Domes U. 2000. Live/dead and acrosome staining of stallion spermatozoa. 14th International Congress on Animal Reproduction, Stockholm. 2-6 July 2000. p. 82.
230. Kovács A., Nagy Sz., Kútvölgyi G., Behr B., Hermes R. 2007a. Különböző vad- és állatkerti emlősök ondósejtjeinek diagnosztikai festése (Diagnostic staining of spermatozoa from different wild and exotic mammals); Diagnostics in wild animal medicine – Annual Conference of Zoo Veterinarians, March 9-11, 2007, Budapest. Abstract published in the proceedings: p.77-79.

231. Kovács A, Tumennasan KH, Demberel SH, Nagy Sz, Kútvölgyi G, Oláh J, Jávor A. 2007b. Argáli spermiumok mélyhűtése /Előzetes közlemény/ Magyar Állatorvosok Lapja (Hungarian Veterinary Journal): 306-309.
232. Koyago M, Nakada K, Tsunoda N, Moriyoshi M, Sawamukai Y. 2008. Spermatozoa morphology during the breeding season in Thoroughbred stallions in Japan J. Vet. Med. Sci. 70: 1121-1124.
233. Kuisma P, Andersson M, Koskinen E, Katila T. 2006. Fertility of frozen-thawed stallion semen cannot be predicted by the currently used laboratory methods. Acta. Vet. Scand. 48:14.
234. Kuster CE, Hess RA, Althouse GC. 2004. Immunofluorescence reveals ubiquitination of retained distal cytoplasmic droplets on ejaculated porcine spermatozoa. J. Androl. 25: 340–347.
235. Kusunoki H, Yasui T, Kato S, Kanda S. 1984. Identification of acrosome-reacted goat spermatozoa by a simplified triple-stain technique. Jap. J. Zootech. Sci. 55: 832-837.
236. Kútvölgyi G, Horváth A, Oláh I, Pálinkás P, Merész L, Molnár M, Pribenszky Cs, Szenci O. 2008. High hydrostatic pressure treatment of fresh bull semen increases the proportion of cells surviving the freezing-thawing procedure in a Hungarian Holstein-Friesian bull population. Presented at the 25th Congress of the World Association for Buiatrics, July 6 - 11, 2008, Budapest, Hungary.
237. Lachaud C, Tesarik J, Cañadas ML, Mendoza C. 2004. Apoptosis and necrosis in human ejaculated spermatozoa. Human Reproduction 19: 607-610.
238. Landim-Alvarenga FC, Fernandes CB, Devito LG, Derussi AAP, Blanco IDP, Alvarenga MA. 2008. New assisted reproductive technologies applied to the horse industry: successes and limitations. Anim. Reprod. 5: 67-82.
239. Lange J, Matheja S, Klug E, Aurich C, Aurich JE. 1997. Influence of training and competition on the endocrine regulation of testicular function and on semen parameters in stallions. Reprod. Dom. Anim. 32: 297–302.
240. Lee HL, Kim SH, Ji DB, Kim YJ. 2009. Comparative study of Sephadex, glass wool and Percoll separation techniques on sperm quality and IVF results for cryopreserved bovine semen. J. Vet. Sci. 10: 249-255.
241. Leeuwenhoek A. 1678. De natis e` semine genitali animalculis. R. Soc. (Lond.) Philos. Trans. 12: 1040–1043.
242. Lefebvre R, Chenoweth PJ, Drost M, LeClear CT, MacCubbin M, Dutton JT. 1995. Characterization of the oviductal sperm reservoir in cattle. Biol. Reprod. 53: 1066–1074.
243. Lefebvre R, Samper JC. 1993. Interaction between stallion spermatozoa and oviductal epithelial cells in vitro. Equine Vet. J. 15: 39–41.
244. Leibo SP. 2006. Cryobiology of spermatozoa: principles, species differences and individual variations. 10th International Symposium on Spermatology, Madrid, 17-22 September 2006, Abstract in the proceedings: p.48.
245. Lewis-Jones I, Aziz N, Seshadri S, Douglas A, Howard P. 2003. Sperm chromosomal abnormalities are linked to sperm morphologic deformities. Fertil Steril. 79: 212-215.
246. Lillie RD. 1977. H. J. Conn's Biological Stains, 9th ed., Williams & Wilkins Co., Baltimore. pp. 158-163.
247. Linfor J, Meyers SA. 2002. Assessing DNA integrity of cooled and frozen-thawed equine sperm using single cell gel electrophoresis. J. Androl. 23: 107-113.
248. Loomis PR. 2001. The equine frozen semen industry. Anim. Reprod. Sci. 68: 191-200.
249. Loomis PR, Amann RP, Squires EL, Pickett BW. 1984. Fertility of unfrozen and frozen stallion spermatozoa extended in EDTA-lactose-egg yolk and packaged in straws. J. Anim.Sci. 56: 687–693.
250. Loomis PR, Graham JK. 2008. Commercial semen freezing: individual male variation

in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim. Reprod. Sci. 105: 119-128.
251. López-Fernández C, Crespo F, Arroyo F, Fernández JL, Arana P, Johnston SD, Gosálvez J. 2007. Dynamics of sperm DNA fragmentation in domestic animals II. The stallion. Theriogenology 68: 1240-1250.
252. Love CC, Blanchard JA, Brinsko SP, Rigby SL, Blanchard TL, Lowry VK, Varner DD. 2003. Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. Theriogenology 60: 1127–1138.
253. Love CC, Brinsko SP, Rigby SL, Thompson JA, Blanchard TL, Varner DD, 2005. Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. Theriogenology 63: 1584–1591.
254. Love CC, Kenney RM. 1994. Relationship of sperm chromatin to fertility in the stallion. In: Proceedings of the 40th Annual Convention AAEP, pp. 21–22.
255. Love CC, Kenney RM. 1998. The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. Theriogenology 50: 955–972.
256. Love CC, Varner DD, Thompson JA. 2000. Intra and inter-stallion variation in sperm morphology and their relationship with fertility. J. Reprod. Fertil. Suppl. 56: 93-100.
257. Lundkvist O, Ljungkvist I. 1977. Morphology of the rat endometrial stroma at the appearance of the pontamine blue reaction during implantation after an experimental delay. Cell Tissue Res. 184: 453-466.
258. Macías García B, Morrell JM, Ortega-Ferrusola C, González-Fernández L, Tapia JA, Rodriguez-Martínez H, Peña FJ. 2009. Centrifugation on a single layer of colloid selects improved quality spermatozoa from frozen-thawed stallion semen. Anim. Reprod. Sci. 114: 193-202.
259. Macpherson ML, Simmen RC, Simmen FA, Hernandez J, Sheerin BR, Varner DD, Loomis P, Cadario ME, Miller CD, Brinsko SP, Rigby S, Blanchard TL. 2002. Insulin-like growth factor-I and insulin-like growth factor binding protein-2 and -5 in equine seminal plasma: association with sperm characteristics and fertility. Biol. Reprod. 67: 648–654.
260. Magistrini M, Guitton E, Levern Y, Nicolle JC, Vidament M, Kerboeuf D, Palmer E. 1997. New staining methods for sperm evaluation estimated by microscopy and flow cytometry. Theriogenology 48: 1229- 1235.
261. Mahadevan M, Baker G. 1984. Assessment and preparation of semen for in vitro fertilization. In Clinical In Vitro Fertilization. Edited by Wood C, Trounson A. Springer-Verlag, Berlin; 1984: 83-97.
262. Malcuit C, Maserati M, Takahashi Y, Page R, Fissore RA. 2006. Intracytoplasmic sperm injection in the bovine induces abnormal [Ca2+]i responses and oocyte activation. Reprod. Fertil. Dev. 18: 39–51.
263. Mari G, Iacono E, Rizzato G, Merlo B, Belluzzi S. 2005. Effects of hyaluronic acid supplementation on motility of stallion spermatozoa after cryopreservation. Anim. Reprod. Sci. 89: 284-285.
264. Martin G, Sabido O, Durand P, Levy R. 2004. Cryopreservation induces an apoptosis like mechanism in bull sperm. Biol. Reprod. 71: 28–37.
265. Martin JC, Klug E, Gunzel AR. 1979. Centrifugation of stallion semen and its storage in large volume straws. J. Reprod. Fertil. 27 (Suppl.): 47–51.
266. Matsukawa K, Akagi S, Adachi N, Sato F, Hasegawa T, Takahashi S. 2007. In vitro development of equine oocytes from preserved ovaries after intracytoplasmic sperm injection. J. Reprod. Dev. 53: 877-885
267. Matsukawa K, Takahashi S. and Hanada A. 2002. Assay for penetrability of frozen-thawed stallion spermatozoa into zona-free hamster oocytes. J. Reprod. Dev. 48: 539-544.

268. Mazur P, Leibo SP, Chu EH. 1972. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. Exp. Cell Res. 71: 345–355.
269. McCue PM, Moore AI, Bruemmer JE. 2003. Refreezing Stallion Spermatozoa for Assisted Reproduction (personal communication, a manuscript, not published).
270. McKinnon AO, Voss JL eds. Equine Reproduction. Philadelphia: Lea & Febiger, 1993.
271. McNutt T, Killian G. 1991. Influence of bovine follicular and oviduct fluids on sperm capacitation in vitro. J. Androl. 12: 244–252.
272. Medeiros ASL. 2003. Cryopreservation of stallion sperm utilizing different amides. Master Thesis. University of Sao Paulo State-UNESP, Botucatu, 123 pp.
273. Medeiros ASL, Gomes GM, Carmo MT, Papa FO, Alvarenga MA. 2002. Cryopreservation of stallion sperm using different amides. Theriogenology 58: 273–276.
274. Mehmood A, Anwar M, Naqvi SM. 2009. Motility, acrosome integrity, membrane integrity and oocyte cleavage rate of sperm separated by swim-up or Percoll gradient method from frozen-thawed buffalo semen. Anim. Reprod. Sci. 111: 141-148.
275. Mendes JOB, Burns PD, De La Torre-Sanchez JF, Seidel GE. 2003. Effect of heparin on clevage rates and embryo production with four bovine sperm preparation protocols. Theriogenology 60: 331–340.
276. Menkveld R, Swanson RJ, Kotze TJ, Kruger TF. 1990. Comparison of a discontinuous Percoll gradient method versus a swim-up method: effects on sperm morphology and other semen parameters. Andrologia 22: 152–158.
277. Menzies PI. 1999. Reproductive health management programs. In: Youngquist, RS (ed.), Current Therapy in Large Animal Theriogenology. W.B. Saunders Co., Philadelphia, pp. 643–649.
278. Merkies K, Chenier T, Plante C, Buhr MM. 2000. Assessment of stallion spermatozoa viability by flow cytometry and light microscope analysis. Theriogenology 54: 1215–1224.
279. Mészáros I. 1951. A mesterséges termékenyítés eredményeinek javítása a lótenyésztésben. Magyar Állatorvosok lapja 6: 16-18.
280. Mészáros I, Pásztor L, Rozenberg I. 1951. A Kuhaylan Zaid arab törzs szaporaságára vonatkozó vizsgálatok. Magyar Állatorvosok Lapja 6: 375-380.
281. Metcalf ES. 1995. Maximizing reproductive efficiency in private practice: the management of mares and the use of cryopreserved semen. Proc. Ann. Conf. Soc. Theriogenol., San Antonio, Texas, pp.155–159.
282. Meyers SA, Overstreet JW, Liu IK, Drobnis EZ. 1995. Capacitation in vitro of stallion spermatozoa: comparison of progesterone-induced acrosome reactions in fertile and subfertile males. J. Androl. 16: 47-54.
283. Minelli A, Moroni M, Martinez E, Mezzasoma I, Ronquist G. 1998. Occurrence of prostasome-like membrane vesicles in equine seminal plasma, J. Reprod. Fertil. 114: 237–243.
284. Moffet PD, Bruemmer JE, Card C, Squires EL. 2003. Comparison of dimethyl formamide and glycerol for cryopreservation of equine spermatozoa. Proceedings of Annual Conference of Society for Theriogenology, p. 42.
285. Molnár A, Sarlós P, Fáncsi G, Rátky J, Nagy Sz, Kovács A. 2001. A sperm tail defect associated with infertility in a goat - a case report. Acta Vet. Hung. 49: 341-348.
286. Moore AI, Squires EL, Graham JK. 2005a. Effect of seminal plasma on the cryopreservation of equine spermatozoa. Theriogenology 63: 2372–2381.
287. Moore AI, Squires EL, Graham JK. 2005b. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology 51(3): 241-249.
288. Morales P, Vantman D, Barros C, Vigil P. 1991. Human spermatozoa selected by Percoll gradient or swim-up are equally capable of binding to the human zona pellucida

and undergoing the acrosome reaction. Hum. Reprod. 6: 401–404.
289. Moran DM, Jasko DJ, Squires EL and Amann RP. 1992. Determination of temperature and cooling rate induced cold shock in stallion spermatozoa. Theriogenology 38: 999–1012.
290. Morozumi K, Shikano T, Miyazaki S and Yanagimachi R. 2006. Simultaneous removal of sperm plasma membrane and acrosome before intracytoplasmic sperm injection improves oocyte activation/embryonic development. Proc. Natl. Acad. Sci. U S A. 103: 17661–17666.
291. Morozumi K, Yanagimachi R. 2005. Incorporation of the acrosome into the oocyte during intracytoplasmic sperm injection could be potentially hazardous to embryo development. Proc. Natl. Acad. Sci. U S A. 102: 14209–14214.
292. Morrell JM. 2006. Update on semen technologies for animal breeding. Reprod. Dom. Anim. 41: 63-67.
293. Morrell JM, Dalin AM, Rodriguez-Martinez H. 2009a. Comparison of density gradient and single layer centrifugation of stallion spermatozoa: yield, motility and survival. Equine. Vet. J. 41:53-58.
294. Morrell JM, Johannisson A, Dalin AM, Rodriguez-Martinez H 2009b. Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. Reprod. Dom. Anim. 44: 512-517.
295. Morrell JM, Johannisson A, Dalin AM, Rodriguez-Martinez H. 2009c. Single-layer centrifugation with Androcoll-E can be scaled up to allow large volumes of stallion ejaculate to be processed easily. Theriogenology. 72: 879-84.
296. Morrell JM, Saravia F, van Wienen M, Wallgren M, Rodriguez-Martinez H. 2009d Selection of boar spermatozoa using centrifugation on a glycidoxypropyltrimethoxysilane-coated silica colloid. J. Reprod. Dev. 55: 547-552.
297. Morrell JM, Mari G, Kútvölgyi G, Meurling S, Mislei B, Iacono E, Rodriguez-Martinez H. 2011. Pregnancies following artificial insemination with spermatozoa from problem stallion ejaculates processed by Single Layer Centrifugation with Androcoll-E. Reprod. Dom. Anim. 46: 642-645.
298. Morrell JM, Rodriguez-Martinez H. 2010. Practical applications of sperm selection techniques as a tool for improving reproductive efficiency. Vet. Med. Int. Aug 4; 2011. pii: 894767.
299. Morris GJ, Faszer K, Green JE, Draper D, Grout BW, Fonseca F. 2007. Rapidly cooled horse spermatozoa: loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. Theriogenology 68: 804-812.
300. Morris ID, Ilott S, Dixon L, Brison DR. 2002. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Hum. Reprod. 17: 990-998.
301. Morris LHA, Allen WR. 2002. Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket. Equine Vet. J. 34: 51-60.
302. Morris LHA, Tiplady C, Allen WR. 2003. Pregnancy rates in mares after a single fixed time hysteroscopic insemination of low numbers of frozen-thawed spermatozoa onto the uterotubal junction. Equine Vet. J. 25: 197–201.
303. Morte MI, Rodrigues AM, Soares D, Rodrigues AS, Gamboa S, Ramalho-Santos J. 2008. The quantification of lipid and protein oxidation in stallion spermatozoa and seminal plasma: seasonal distinctions and correlations with DNA strand breaks, classical seminal parameters and stallion fertility. Anim. Reprod. Sci. 106: 36-47.
304. Murchie T. 2005. Stallion infertility. Proceeding of the NAVC North American Veterinary Conference Jan. 8-12, , Orlando, Florida. pp. 267-269. Publication is available in http://www.ivis.org

305. Nagy Sz, Házas G, Bali Papp Á, Iváncsics J, Szász F, Szász F Jr, Kovács A, Foote RH. 1999. Evaluation of sperm tail membrane integrity by light microscopy. Theriogenology 52: 1153-1159.
306. Nagy Sz, Jansen J, Topper EK 2003a. Validation of a light microscopic analysis of bull sperm quality by flow cytometry. Reprod. Dom. Anim. 38. p334. Abstract P3.
307. Nagy Sz, Jansen J, Topper EK, Gadella BM. 2003b. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in the presence of egg-yolk particles. Biol. Reprod. 68: 1828–1835.
308. Nagy Sz, Kovács A, Zubor T, Zomborszky Z, Tóth J, Horn P. 2001. Evaluation of frozen/thawed deer spermatozoa: short communication. Acta Vet. Hung. 49: 223-227.
309. Nagy Sz, Qi X, Han J, Kovács A. 2000. Light microscopic investigations on frozen-thawed yak semen-a pilot study. Third Int. Congr. Yak (ICY), Lhasa, P.R. China. Proc.: 356-358.
310. Nagy Sz. 2007. Bikaspermiumok vizsgálata doménspecifikus fluoreszcens jelöléssel. Magyar Állatorvosok Lapja (Hungarian Veterinary Journal). 129: 464-467.
311. Nagy Sz. 2002. Emlős-spermiumok membránintegritas-vizsgálatai (Irodalmi áttekintés). Állattenyésztés és Takarmányozás (Hungarian Journal of Animal Production) 51: 607-616.
312. Nakai M, Ito J, Sato K, Noguchi J, Kaneko H, Kashiwazaki N, Kikuchi K. 2011a. Pre-treatment of sperm reduces success of ICSI in the pig. Reproduction 142: 285-293.
313. Nakai M, Kashiwazaki N, Ito J, Maedomari N, Ozawa M, Shino M, Noguchi J, Kaneko H, Kikuchi K. 2011b. Factors affecting fertilization and embryonic development during intracytoplasmic sperm injection in pigs. J. Reprod.Dev. 57: 183-187.
314. Nakai M., Kashiwaszaski N., Takizawa A., Hayashi Y, Nakatsukasa E, Fuchimoto D., Noguchi J., Kaneko H, Shino M., Kikuchi K. 2003. Viable piglets generated from porcine oocytes matured in vitro and fertilized by Intracytoplasmic Sperm Head Injection. Biol. Reprod. 68: 1003-1008. Erratum in: Biol Reprod. 2003. 68:1941.
315. Neild DM, Brouwers JFHM, Colenbrander B, Aguero, A, Gadella BM. Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. 2005. Mol. Reprod. Dev. 72: 230-238.
316. Neild DM, Chaves MG, Flores M, Miragaya MH, Gonzalez E, Aquero A. 2000. The HOS test and its relationship to fertility in the stallion. Andrologia 32: 351-355.
317. Neild DM, Chaves MG, Flores MC, Mora NG, Beconi MT, Agüero A. 1999. Hypoosmotic test in equine spermatozoa. Theriogenology 51: 721–727.
318. Neild DM, Gadella BM, Chaves MG, Miragaya MH, Colenbrander B, Aguero A. 2003. Membrane changes during different stages of a freeze-thaw protocol for equine semen cryopreservation. Theriogenology 59: 1693-1705.
319. Ng SC, Bongso TA, Sathananthan H, Tok VCN, Ratnam SS. 1990. Microcentrifugation of human spermatozoa: its effect on fertilization of hamster oocytes after micro-insemination spermatozoal transfer. Hum. Reprod. 5: 209–211.
320. Nie GJ, Johnson KE, Wenzel JG. 2003. Pregnancy outcome in mares following insemination deep in the uterine horn with low numbers of sperm selected by glass wool/Sephadex filtration, Percoll separation or absolute number. Anim. Reprod. Sci. 79: 103-109.
321. Nie GJ, Wenzel JGW, Johnson KE. 2002. Comparison of pregnancy outcome in mares among methods used to evaluate and select spermatozoa for insemination. Anim. Reprod. Sci. 69: 211–222.
322. Nishikawa Y. 1962. Fifty years of artificial insemination of farm animals in Japan. English Bull. 2. Kyoto University, Japan.

323. Ord T, Patrizio P, Marello E, Balmaceda J, Asch RH 1990. Mini-Percoll: A new method of semen preparation for IVF in severe male factor infertility. Hum. Reprod. 5: 987-989.
324. Organization for Hungarian Standards. Hungarian Standard for breeding stallion semen (MSZ 7034/1999).
325. Ortega-Ferrusola C, García BM, Gallardo-Bolaños JM, González-Fernández L, Rodríguez-Martinez H, Tapia JA, Peña FJ. 2009. Apoptotic markers can be used to forecast the freezeability of stallion spermatozoa. Anim. Reprod. Sci. 114: 393-403.
326. Ortega-Ferrusola C, Sotillo-Galán Y, Varela-Fernández E, Gallardo-Bolaños JM, Muriel A, González-Fernández L, Tapia JA, Peña FJ. 2008. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. J. Androl. 29: 213-221.
327. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. 2007. Mitochondria, oxidative stress and cell death. Apoptosis 12: 913–922.
328. Ottier DA, Curtis EF. 2005. The effect of sodium hyaluronate on sperm function of cryopreserved equine spermatozoa. Anim. Reprod. Sci. 89: 239-242.
329. Outram AK, Stear NA, Bendrey R, Olsen S, Kasparov A, Zaibert V, Thorpe N, Evershed RP. 2009. The earliest horse harnessing and milking. Science 323: 1332-1335.
330. Palmer E, Magistrini M. 1992. Automated analysis of stallion semen post-thaw motility. Acta Vet . Scand. Suppl. 88: 137-152.
331. Parent S, Lefièvre L, Brindle Y, Sullivan R. 1999. Bull subfertility is associated with low levels of a sperm membrane antigen, Mol. Reprod. Dev. 52: 57–65.
332. Parks JE, Graham JK. 1992. Effects of cryopreservation on sperm membranes. Theriogenology 38: 209–222.
333. Parks JE, Lynch DV. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion and rooster sperm membranes. Cryobiology 29: 255–266.
334. Parlevliet JM, Colenbrander B. 1999. Prediction of first season stallion fertility of 3-year-old Dutch Warmbloods with prebreeding assessment of percentage of morphologically normal live sperm. Equine Vet. J. 31: 248-251.
335. Parlevliet JM, Kemp B, Colenbrander B. 1994. Reproductive characteristics and semen quality in maiden Dutch warmblood stallions. J. Reprod. Fert. 101: 183–187.
336. Parrish JJ, Krogenaes A, Susko-Parrish JL. 1995. Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. Theriogenology 44: 859-869.
337. Pécsi T. Házi emlősállatok mesterséges termékenyítése. Mezőgazdasági Kiadó, Budapest, 2007.
338. Peña AI, Barrio M, Becerra JJ, Quintela LA, Herradón PG. 2006. Infertility in a dog due to proximal cytoplasmic droplets in the ejaculate. 10th International Symposium on Spermatology, Madrid, 17-22 September 2006, abstract book: P2-40, P.93.
339. Peña AI., Johannisson, A., Linde-Forsberg, C. 1999. Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. Theriogenology 52: 965–980.
340. Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. 2003. Assessment of fresh and frozen-thawed boar semen using an annexin-V assay: a new method of evaluating sperm membrane integrity. Theriogenology 60: 677–689.
341. Peña FJ, Johannisson A, Wallgren M, Rodriguez-Martinez H. 2004. Effect of hyaluronan supplementation on boar sperm motility and membrane lipid architecture status after cryopreservation. Theriogenology 61: 63-70.
342. Pesch S, Bergmann M. 2006. Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. Micron 37: 597-612.
343. Pesch, S., Bergmann, M., Bostedt, H. 2006a. Determination of some enzymes and macro- and microelements in stallion seminal plasma and their correlations to semen

quality. Theriogenology 66: 307–313.
344. Pesch S, Bostedt H, Failing K. 2006b. Advanced fertility diagnosis in stallion semen using transmission electron microscopy. Anim. Reprod. Sci. 91: 285-298.
345. Petrie A, Watson P. 1999. Statistics for Veterinary and Animal Science. Blackwell Science Ltd., London. p243.
346. Petrunkina AM, Gehlhaar R, Drommer W, Waberski D, Topfer-Peterson E. 2001. Selective sperm binding to pig oviductal epithelium in vitro. Reproduction 121: 889–896.
347. Pharmacia Biotech 1996: Important notice: Percoll® NOT to be used in Assisted Reproduction Technologies in Humans. Pharmacia Biotech. Inc. December 12, 1996.
348. Pickett BW. Factors affecting sperm production and output. In: Mckinnon AO, Voss JL. Equine Reproduction. Lea and Febiger, Philadelphia, 1993a. 689-704.
349. Pickett BW. Reproductive evaluation of the stallion. In: McKinnon AO, Voss JL (eds), Equine Reproduction. Philadelphia, London: Lea and Febiger, 1993b. 755-768.
350. Pickett BW, Amann RP, 1993. Cryopreservation of semen. In: McKinnon AO, Voss JL (eds), Equine Reproduction. Lea & Febiger, Philadelphia, PA, pp. 769–789.
351. Pickett BW, Voss IL, Squires EL, Vanderwall DK, McCue PM, Bruemmer JE. 2000. Collection, Preparation and Insemination of Stallion Semen. Bulletin No. 10. Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO, pp. 1–15.
352. Polge C, Rowson LEA. 1952. Fertilizing capacity of bull spermatozoa after freezing at -79ºC. Nature 169: 626-627.
353. Pommer AC, Rutlant J, Meyers SA. 2002. The role of osmotic resistance on equine spermatozoal function. Theriogenology 58: 1373–1384.
354. Presicce GA, Révay T, Nagy Sz, Dinnyés A, Kovács A. 2003. Complex staining of water buffalo (Bubalus bubalis) spermatozoa. Bubalus bubalis II/2003: 55-60.
355. Pribenszky Cs, Molnár M, Horváth A, Kútvölgyi G, Harnos A, Szenci O, Dengg J, Lederer J. 2007. Improved post-thaw motility, viability and fertility achieved by hydrostatic pressure treated bull semen. 33rd Annual Conference of the IETS, January 7-9, 2007, Kyoto, Japan, Abstract published in the Reproduction, Fertility and Development. 2007. 19: 181 – 182.
356. Rathi R, Colenbrander B, Bevers MM, Gadella BM. 2001. Evaluation of in vitro capacitation of stallion spermatozoa. Biol. Reprod. 65: 462–470.
357. Rathi R, Nielen M, Cheng FP, van Buiten A, Colenbrander B. 2000. Exposure of progesterone receptors on the plasma membranes of stallion spermatozoa as a parameter for prediction of fertility. J. Reprod. Fertil. Suppl. 56: 87-91.
358. Révay T, Kopp C, Flyckt A, Taponen J, Ijäs R, Nagy S, Kovács A, Rens W, Rath D, Hidas A, Taylor JF, Andersson M. 2010. Diploid spermatozoa caused by failure of the second meiotic division in a bull. Theriogenology 73: 421-428.
359. Révay T, Nagy S, Kopp C, Flyckt A, Rens W, Rath D, Hidas A, Kovács A, Johannisson A, Rodriguez-Martinez H, Andersson M. 2009. Macrocephaly in bull spermatozoa is associated with nuclear vacuoles, diploidy and alteration of chromatin condensation. Cytogenet. Genome Res. 126: 202-209.
360. Révay T, Nagy S, Kovács A, Edvi ME, Hidas A, Rens W, Gustavsson I. 2004. Head area measurements of dead, live, X- and Y-bearing bovine spermatozoa. Reprod Fertil Dev. 16(7): 681-687.
361. Ricker JV, Linfor JJ, Delfino WJ, Kysar P, Scholtz EL, Tablin F, Crowe JH, Ball BA, Meyers SA. 2006. Equine sperm membrane phase behavior: the effects of lipid-based cryoprotectants. Biol Reprod. 74: 359-365.
362. Roasa LM, Choi YH, Love CC, Romo S, Varner DD, Hinrichs K. 2007. Ejaculate and type of freezing extender affect rates of fertilization of horse oocytes in vitro.

Theriogenology 68: 560-566.
363. Rodríguez-Martínez H. 2003. Laboratory Semen Assessment and Prediction of Fertility: still Utopia? Reprod. Dom. Anim. 38: 312–318.
364. Rodríguez-Martinez H. 2006. Can we increase the estimative value of semen assessment? Reprod. Dom. Anim. 41(suppl 2): 2-10.
365. Rodriguez-Martinez H, Larsson B, Pertoft H. 1997. Evaluation of sperm damage and techniques for sperm clean-up. Reprod. Fertil. Dev. 9: 297–308.
366. Roldan ER. 2006. Better intracytoplasmic sperm injection without sperm membranes and acrosome. Proc. Natl. Acad. Sci. USA. 103: 17585-17586.
367. Roser JF. 1995. Idiopathic subfertility/infertility in stallions: new endorcrine methods of diagnosis. Proc. Soc. Therio. 136–146.
368. Roser JF. 2001. Endocrine diagnostics for stallion infertility. In: Ball BA, ed. Recent Advances in Equine Reproduction. International Veterinary Information Service, (www.ivis.org), Ithaca, New York, USA.
369. Roser JF. 2007. Endocrine diagnostics and therapeutics for the stallion with declining fertility. In: Samper JC, Pycock JF, McKinnon AO. (Eds.). Current Therapy in Equine Reproduction. Saunders Elsevier, St. Louis, pp. 244–251.
370. Roser JF. 2008. Regulation of testicular function in the stallion: An intricate network of endocrine, paracrine and autocrine systems. Anim. Reprod. Sci. 107: 179-196.
371. Rousset H, Chanteloube PH, Magistrini M, Palmer EJ. 1987. Assessment of fertility and semen evaluations of stallions. J. Reprod. Fertil. Suppl. 35: 25–31.
372. Saacke RG, Dalton JC, Nadir S, Nebel RL, Bame JH. 2000. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim. Reprod. Sci. 2: 663–667.
373. Saacke RG, DeJarnette JM, Bame JH. 1998. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated super and single-ovulating cattle? Theriogenology 50:117-128,
374. Samardzija M, Karadjole M, Matkovic M, Cergolj M, Getz I, Dobranic T, Tomaskovic A, Petric J, Surina J, Grizelj J, Karadjole T. 2006. A comparison of BoviPure and Percoll on bull sperm separation protocols for IVF. Anim. Reprod. Sci. 91: 237-247.
375. Samper JC. 1992. Evaluation of cryopreserved semen: An alternative assay. Acta Vet. Scand. 88: 59-65.
376. Samper JC. 2001. Management and fertility of mares bred with frozen semen. Anim. Reprod. Sci. 58: 219–228.
377. Samper JC, Hellander JC, Crabo BG. 1991. Relationship between the fertility of fresh and frozen stallion semen and semen quality. J. Reprod. Fert. Suppl. 44: 107-114.
378. Saragusty J, Gacitua H, Pettit MT, Arav A. 2007. Directional freezing of equine semen in large volumes. Reprod. Dom. Anim 42: 610-615.
379. Saragusty J, Hildebrandt TB, Behr B, Knieriem A, Kruse J, Hermes R. 2009. Successful cryopreservation of Asian elephant (Elephas maximus) spermatozoa. Anim. Reprod. Sci. 115: 255-266.
380. Sarhaddi F, Iváncsics J, Gergátz E. 1995. Effect of handling on viability and acrosome status of ram spermatozoa. Acta Agron. Ovárien. 37: 193-198.
381. Sarlós P, Molnár A, Kókai M, Gábor G, Rátky J. 2002. Comparative evaluation of the effect of antioxidants in the conservation of ram semen. Acta Vet. Hung. 50: 235-45.
382. Schembri MA, Major DA, Suttie JJ, Maxwell WM, Evans G. 2002. Capacitation-like changes in equine spermatozoa throughout the cryopreservation process. Reprod. Fertil. Dev. 14: 225-33.
383. Scott JW. 1973. Lipid metabolism of spermatozoa. J. Reprod. Fertil. 18(Suppl): 65–76.
384. Scott MA, Liu IKM, Overstreet JW. 1995. Sperm transport to the oviducts:

Abnormalities and their clinical implications. Proc. Am. Assoc. Eq. Pract. 41: 1-2.
385. Seita Y, Ito J, Kashiwazaki N. 2009. Removal of acrosomal membrane from sperm head improves development of rat zygotes derived from intracytoplasmic sperm injection. J. Reprod. Dev. 55: 475-479.
386. Sekoni VO, Gustafsson BK, Mather EC. 1981. Influence of wet fixation, staining techniques, and storage time on bull sperm morphology. Nord. Vet. Med. 33: 161– 166.
387. Shamsuddin M, Rodriguez-Martinez H. 1994. A simple, non-traumatic swim up method for the selection of spermatozoa for in vitro fertilization in the bovine. Anim. Reprod. Sci. 36: 61-75.
388. Shamsuddin M, Rodriguez-Martinez H, Larsson B. 1993. Fertilizing capacity of bovine spermatozoa selected after swim-up in hyaluronic acid-containing medium. Reprod. Fertil. Dev. 5: 307-315
389. Shekarriz M, De Wire DM, Thomas AJ Jr, Agarwal A. 1995. A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. Eur. Urol. 28:31–35.
390. Sieme H, Harrison RA, Petrunkina AM. 2008. Cryobiological determinants of frozen semen quality, with special reference to stallion. Anim. Reprod. Sci. 107: 276-292.
391. Sieme H, Martinsson G, Rauterberg H, Walter K, Aurich C, Petzoldt R, Klug E. 2003. Application of techniques for sperm selection in fresh and frozen-thawed stallion semen. Reprod. Dom. Anim. 38: 134-140.
392. Slotte H, Akerlof E, Pousette A. 1993. Separation of human spermatozoa with hyaluronic acid induces, and Percoll inhibits the acrosome reaction. Int. J. Androl. 16: 349-354.
393. Smith AU, Polge C. 1950. Survival of spermatozoa at low temperatures. Nature 166: 668-669.
394. Smith S, Hosid S, Scott L. 1995. Use of postseparation sperm parameters to determine the method of choice for sperm preparation for assisted reproductive technology. Fertil. Steril. 63: 591-597.
395. Somfai T, Bodó Sz, Nagy Sz, Gócza E, Iváncsics J, Kovács A. 2002a. Simultaneous evaluation of viability and acrosome integrity of mouse spermatozoa using light microscopy. Biotech. & Histochem. 77: 117-120.
396. Somfai T, Bodó Sz, Nagy Sz, Papp AB, Iváncsics J, Baranyai B, Gócza E, Kovács A. 2002b. Effect of swim-up and Percoll treatment on viability and acrosome integrity of frozen–thawed bull sperm. Reprod. Dom. Anim. 37: 285–290.
397. Spallanzani L. Opuscoli di fiscal e vegetabile. Opuscolo II. Observazioni e esperienze intorno ai vermicelli spermatici dell' homo e degli animali. (Observations upon the spermatozoa of humans and animals). Modena, 1776. Cited by Watson PF, 1979.
398. Spallanzani L. 1784. Dissertations relative to the natural history of animals and vegetables. Trans. by T. Beddoes in Dissertations Relative to the Natural History of Animals and Vegetables. Vol. 2:195–199. J. Murray, London.
399. Squires EL. 2005. Integration of future biotechnologies into the equine industry. Anim. Reprod. Sci. 89: 187–198.
400. Squires EL, Keith SL, Graham JK. 2004. Evaluation of alternative cryoprotectants for preserving stallion spermatozoa. Theriogenology 62: 1056–1065.
401. Squires EL, Wilson JM, Kato H, Blaszczyk A. 1996. A pregnancy after intracytolasmic sperm injection into equine oocytes matured in vitro. Theriogenology 45: 306.
402. Stanic P, Sonicki Z, Suchanek E. 2002. Effect of pentoxifyllin on motility and membrane integrity of cryopreserved human spermatozoa. Int. J. Androl. 25: 286-190.
403. Steiner J. 2002. Breeding Soundness Examination of the Stallion. http://www.breeding-stallions.com

404. Stewart BL, Roser JF. 1998. Effects of age, season, and fertility status on plasma and intratesticular immunoreactive (IR) inhibin concentrations in stallions. Domest. Anim. Endocrinol. 15: 129–139.
405. Stewart DL. 1951. Storage of bull spematozoa at low ternperatures. Vet. Rec. 63:65-66.
406. Stich K, Brinsko S, Thompson J, Love C, Miller C, Blanchard T, Varner D. 2002. Stabilization of extragonadal sperm reserves in stallions. Application for determination of daily sperm output. Theriogenology 58: 397–400.
407. Stokes JE, Carnevale EM, Squires EL, Seidel GE Jr. 2004. Preparation of bovine and equine sperm by Percoll® or swim up for injection into bovine oocytes. Proc. 15th Int. Congress Animal Reproduction, Brazil, Abstracts Vol. 2, p. 471.
408. Sullivan JJ, Turner PC, Self LC, Gutteridge HB, Bartlett DE. 1975. Survey of reproductive efficiency in the quarterhorse and thoroughbred. J. Reprod. Fertil. Suppl. 23: 315–318.
409. Sullivan R, Saez F, Girouard J, Frenette G. 2005. Role of exosomes in sperm maturation during the transit along the male reproductive tract. Blood Cells, Molecules, and Diseases 35: 1–10.
410. Sutovsky P, Terada Y, Schatten G. 2001. Ubiquitin-based sperm assay for the diagnosis of male factor infertility. Hum. Reprod. 16: 250–258.
411. Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodriguez-Martinez H. 2002. Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma. Int. J. Androl. 25: 84–93.
412. Swanson EW, Boyd LJ. 1962. Factors affecting coiled-tail spermatozoa in the bull. Am. J. Vet. Res. 23: 300-309.
413. Sybracia M, Grasso J, Sayme N, Stronk J, Huszar G. 1997. Hyaluronic acid substantially increases the retention of motility in cryopreserved/thawed human spermatozoa. Hum. Reprod. 12: 1949-1954.
414. Talbot P, Chacon RS. 1981. A triple-stain technique for evaluating normal acrosome reaction of human sperm. J. Exp. Zool. 215: 201- 208.
415. Talevi R, Gualtieri R. 2004. In vivo versus in vitro fertilization. Eur. J. Obstet. Gynecol. Reprod. Biol. 115. Suppl 1: 568-71.
416. Tarlatzis BC, Kolibianakis EM, Bontis J, Tousiou M, Lagos S, Mantalenakis S. 1995. Effect of pentoxifylline on human sperm motility and fertilizing capacity. Arch. Androl. 34: 33-42.
417. Tartaglione CM, Ritta MN. 2004. Prognostic value of spermatological parameters as predictors of in vitro fertility of frozen-thawed bull semen. Theriogenology 62: 1245-1252.
418. Tea NT, Jondet M, Scholler R. 1984. A migration-gravity sedimentation method for collecting motile spermatozoa from human semen. In Vitro Fertilization, Embryo Transfer and Early Pregnancy. Edited by Harrison RF, Bonnar J, Thompson W. MTP Press ltd., Lancaster; pp. 117-120.
419. Terriou P, Hans E, Giorgetti C, Spach JL, Salzmann J, Urrutia V, Roulier R. 2000. Pentoxifyllin initiates motility in spontaneously immotile epididymal and testicular spermatozoa and allows normal fertilization, pregnancy, and birth after intracytoplasmic Sperm Injection. J. Assist. Reprod. Gen. 17: 194-199.
420. Tesarik J, Mendoza C, Carreras A 1992. Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reaction in human sperm. Fertil Steril. 58: 1185-1189
421. Tesarik J, Mendoza C. 1999. In vitro fertilization by intracytoplasmic sperm injection. Bioassays 21: 791-801
422. Thomas AD, Meyers SA, Ball BA. 2006. Capacitation-like changes in equine

spermatozoa following cryopreservation. Theriogenology 65: 1531-1550.
423. Thomas PG, Ball BA, Miller PG, Brinsko SP, Southwood L. 1994. A subpopulation of morphologically normal, motile spermatozoa attach to equine oviductal epithelial cell monolayers. Biol. Reprod. 51: 303-309
424. Thomas PGA, Ball BA, Brinsko SP. 1995. Changes associated with induced capacitation influence the interaction between equine spermatozoa and oviduct epithelial cells monolayers. Biol. Reprod. Monogr. 1: 697–705.
425. Thundathil J, Meyer R, Palasz AT, Barth AD, Mapletoft RJ. 2000. Effect of the knobbed acrosome defect in bovine sperm on IVF and embryo production. Theriogenology 54: 921–934.
426. Thundathil J, Palasz AT, Barth AD, Mapletoft RJ. 2001. The use of in vitro fertilization techniques to investigate the fertilizing ability of bovine sperm with proximal cytoplasmic droplets. Anim. Reprod. Sci. 65: 181–192.
427. Thundathil J, Palasz AT, Barth AD, Mapletoft RJ. 2002. Plasma membrane and acrosomal integrity in bovine spermatozoa with the knobbed acrosome defect. Theriogenology 58: 87–102.
428. Thundathil J, Palasz AT, Mapletoft RJ. 1999. An investigation of the fertilizing characteristics of pyriform-shaped bovine spermatozoa. Anim. Reprod. Sci. 57: 35-50.
429. Thys M, Vandaele L, Morrell JM, Mestach J, Van Soom A, Hoogewijs M, Rodriguez-Martinez H. 2009. In vitro fertilizing capacity of frozen-thawed bull spermatozoa selected by single-layer (glycidoxypropyltrimethoxysilane) silane-coated silica colloidal centrifugation. Reprod. Dom. Anim. 44: 390-394.
430. Tian JH, Wu ZH, Liu L, Cai Y, Zeng SM, Zhu SE, Liu GS, Li Y, Wu CX. 2006. Effects of oocyte activation and sperm preparation on the development of porcine embryos derived from in vitro-matured oocytes and intracytoplasmic sperm injection. Theriogenology 66: 439–448.
431. Töpfer-Petersen E, Petrunkina AM, Waberski D, Ekhlasi-Hundrieser M, Günzel-Apel AR. 2006. Sperm quality and fertility in domestic animals. 10th International Symposium on Spermatology, Madrid, 17-22 September 2006, Abstract in the proceedings: p. 16.
432. Troedsson MH, Liu IK, Crabo BG. 1998. Sperm transport and survival in the mare: a review. Theriogenology 50: 807-818.
433. Turner RM. 2002. Testicular degeneration in stallions. In: Proceedings Society for Theriogenology Conference, Society for Theriogenology, Colorado Springs, 9 pp.
434. Turner RM, McDonnell SM. 2007. Mounting expectations for Thoroughbred stallions. J. Am. Vet. Med. Assoc. 230: 1458-1460,
435. van Buiten A, Remmen JLAM, Colenbrander B. 1998. Fertility of Shetland pony stallions used in different breeding systems: a retrospective study. Vet. Quart. 20: 100–103.
436. van Buiten A, van den Broek J, Schukken YH, Colenbrander B. 1999. Validation of non-return rate as a parameter for stallion fertility. Livestock Prod. Sci. 60: 13–19.
437. van der Ven HH, Jeyendran RS, Al-Hasani S, Pérez-Peláez M, Diedrich K, Zaneveld LJD. 1986. Correlation between human sperm swelling in hypoosmotic medium (hypoosmotic swelling test) and in vitro fertilisation. J. Androl. 7: 190–196.
438. van der Ven HH, Jeyendran RS, Al-Hasani S, Tunnerhoff A, Hoebbel K, Diedrich K, Krebs D, Perez-Pelaez M. 1988. Glass wool column filtration of human semen: relation to swim-up procedure and outcome of IVF. Hum. Reprod. 3: 85-88.
439. van der Zwalmen P, Bertin-Segal G, Geerts L, Debauche C, Schoysman R. 1991. Sperm morphology and IVF pregnancy rate: comparison between Percoll® gradient centrifugation and swim-up procedures. Hum. Reprod. 6: 581-588.
440. Varner DD. 2008. Developments in stallion semen evaluation. Theriogenology 70: 448–

462.
441. Varner DD, Blanchard TL, Brinsko SP, Love CC, Taylor TS, Johnson L. 2000. Techniques for evaluating selected reproductive disorders of stallions. Anim. Reprod. Sci. 60-61: 493-509.
442. Varner DD, Bowen JA, Johnson L. 1993. Effect of heparin on capacitation/acrosome reaction of equine sperm. Arch. Andr. 31: 199-207.
443. Varner DD, Schumacher J, Blanchard TL, Johnson L. 1991. Reproductive Anatomy and Physiology. In: Pratt PW, (ed) Diseases and Management of Breeding Stallions. Goleta, CA: American Veterinary Publications, 1991: l-59.
444. Varner, DD, Ward CR, Storey BT, Kenney RM. 1987. Induction and characterization of acrosome reaction in equine spermatozoa. Am. J. Vet. Res. 48: 1383-1389.
445. Vartorella HA. 2003. Cryopreservation of equine and porcine spermatozoa with an unique freezing technology (UFT). A PhD dissertation. Texas Tech. Iniversity.
446. Veeramachaneni DN, Moeller CL, Sawyer HR. 2006. Sperm morphology in stallions: ultrastructure as a functional and diagnostic tool. Vet. Clin. North Am. Equine Pract. 22: 683-692.
447. Vidament M. 2005. French field results (1985–2005) on factors affecting fertility of frozen stallion semen. Anim. Reprod. Sci. 89: 115–136.
448. Vidament M, Ecot P, Noue P, Bourgeois C, Magistrini M, Palmer E. 2000. Centrifugation and addition of glycerol at 22° C instead of 4° C improve post-thaw motility and fertility of stallion spermatozoa. Theriogenology 54: 907-919.
449. Vidament M, Magistrini M, Palmer E, Clément F. 1999. Equine artificial insemination in French National Studs. 3rd Conference of the European Society for Domestic Animal Reproduction, Auger, France In: Reprod. Dom. Anim. Suppl. 6: 61-66.
450. Vidament M, Yvon JM, Couty I, Arnaud G, Nguekam-Feugang J, Noue P, Cottron S, Le Tellier A, Noel F, Palmer E, Magistrini M. 2001. Advances in cryopreservation of stallion semen in modified INRA82. Anim. Reprod. Sci. 68: 201-218.
451. Voss JL, Pickett BW, Squires EL. 1981. Stallion sperm morphology and motility and their relationships to fertility. J. Am. Vet. Med. Assoc. 178: 287-289.
452. Waberski D, Meding S, Dirkson G, Weitz KF, Leiding C, Hahn R. 1994. Fertility of long-term-stored boar semen: influence of extender (Androhep and Kiev), storage time, and plasma droplets in the semen. Anim. Reprod. Sci. 36: 145–151.
453. Watson PF. 1979. The preservation of semen in mammals. In: Finn CA, ed. Oxford Reviews of Reproductive Biology. Oxford, UK: Clarendon Press. 1: 283-350.
454. Watson PF. 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60-61: 481-492.
455. Weiss S, Janett F, Burger D, Hässig M, Thun R 2004. The influence of centrifugation on quality and freezability of stallion semen. Schweiz. Arch. Tierheilkd. 146:285-293.
456. Wilhelm KM, Graham JK, Squires EL. 1996. Comparison of the fertility of cryopreserved stallion spermatozoa with sperm motion analyses, flow cytometric evaluation, and zona-free hamster oocyte penetration. Theriogenology 46: 559-578.
457. Woods GL, Banker CB, Baldwin JL, Ball BA, Bilinski J, Cooper WL, Ley WB, Mank EC, Erb HN. 1987. Early pregnancy loss in brood mares J. Reprod. Fertil. Suppl. 35: 455-459.
458. World Health Organization laboratory manual for the examination and processing of human semen. WHO Press, 2010.
459. Yagci A, Murk W, Stronk J, Huszar G. 2010. Spermatozoa bound to solid state hyaluronic acid show chromatin structure with high DNA chain integrity: an acridine orange fluorescence study. J Androl. 31: 566-572.
460. Yanagimachi R. 1994. Mammalian fertilization. In: Knobil E, Neill JD. (Eds.), The

Physiology of Reproduction. Raven Press, New York, pp. 189–317.
461. Yogev L, Gamzu R, Botchan A, Hauser R, Paz G, Yavetz H. 2000. Zona pellucida binding improvement effect of different sperm preparation techniques is not related to changes in sperm motility characterizations. Fertil. Steril. 73: 1120-1125.
462. Zavos PM, Abou-Abdallah M, Aslanis P, Correa JR, Zarmakoupis-Zavos PN. 2000. Use of the multi-ZSC one-step standardized swim-up method: recovery of high-quality spermatozoa for intrauterine insemination or other forms of assisted reproductive technologies. Fertil. Steril. 74: 834-835.
463. Zhang JJ, Boyle, MS, Smith CA, Moore HDM. 1990. Acrosome reaction of stallion spermatozoa evaluated with monoclonal antibody and zona-free hamster eggs. Mol. Reprod. Dev. 27: 152–158.
464. Zimmerman ER, Robertson KR, Kim H, Drobnis EZ, Nakajima ST. 1994. Semen preparation with the Sperm Select System versus a washing technique. Fertil. Steril. 61: 269-275.
465. Zirkin BR, Awoniyi C, Griswold MD, Russell LD, Sharpe R. 1994. Is FSH required for adult spermatogenesis? J. Androl. 15: 273–276.
466. Zirkler H, Gerbes K, Klug E, Sieme H, 2005. Cryopreservation of stallion semen collected from good and poor freezers using a directional freezing device (Harmony CryoCare – Multi thermal Gradient 516). Anim. Reprod. Sci. 89: 291–294.

11. PUBLICATIONS IN THE FIELD OF THE THESIS

Scientific papers published in reviewed journals

1. **Kútvölgyi G.**, Nagy Sz., Czimber Gy., Balogh A., Stefler J., Kovács A. (2003) *Ménspermiumok élő/elhalt és akroszóma festése* (Viability and acrosome staining of stallion spermatozoa); Állatenyésztés és Takarmányozás (Hungarian Journal of Animal Production) 52. 2. 137-143. (in Hungarian, with English summary).

2. **Kútvölgyi G.**, Stefler J., Kovács A. (2006) *Viability and acrosome staining of stallion spermatozoa by Chicago sky blue and Giemsa*. Biotech. Histochem. Vol. 81. (4-6) p.109 – 117. Erratum in: Biotech. Histochem. 2007. 82: 45.

3. Morrell JM., Mari G., **Kútvölgyi G.**, Meurling S., Mislei B., Iacono E., Rodriguez-Martinez H. (2011). *Pregnancies following artificial insemination with spermatozoa from problem stallion ejaculates processed by Single Layer Centrifugation with Androcoll-E*; Reproduction in Domestic Animals. 46 (4): 642-645.

Poster and oral presentations in Conferences

1. **Kútvölgyi G.**, Balogh A., Nagy Sz., Czimber Gy., Stefler J., Kovács A. (2003) *Shorter (2 hours) live/dead and acrosome staining of stallion spermatozoa*; Reproduction in Domestic Animals 38: p340. Abstract P24. (ESDAR Congress, September 4-6, 2003; Dublin).

2. **Kútvölgyi G.**, Czimber Gy., Nagy Sz., Stefler J., Kovács A. (2004) *An unusual response of spermatozoa to centrifugation in case of an Arabian stallion*; 15th International Congress on Animal Reproduction (ICAR), 2004 August, Porto Seguro, Brazil, Abstracts. Vol 2. p.499.

3. **Kútvölgyi G.**, Suh T., Carnevale E., Seidel G. Jr. (2005) *Use of pentoxifylline and hyaluronic acid for stallion sperm separation*; The 31st Annual Conference of the International Embryo Transfer Society, Copenhagen, Denmark, 8-12 January 2005; Abstr. in: Reproduction, Fertility and Development. 17 (1,2) p.310.

4. **Kútvölgyi G.,** Suh T., Carnevale E., Seidel G. Jr. (2005) *Morphologic evaluation after using pentoxifylline and hyaluronic acid for stallion sperm separation*; The 9th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR), Murcia, Spain, 1-3 September, 2005; Abstract in: Reproduction in Domestic Animals. Vol. 40: p407. (Abstract P271).

5. **Kútvölgyi G.**, Reiczigel J., Stefler J., Kovács A. (2006) *Effect of Morinda citrifolia on the membrane integrity of stallion spermatozoa*; 10th International

Symposium on Spermatology Madrid, 17-22 September 2006, Abstract in the proceedings: P3-28, p.110.

6. **Kútvölgyi G.**, Czimber Gy., Nagy Sz., Jancsik V., Kovács A., Stefler J. (2006) *Mén ondósejtek károsodásainak elemzése a mélyhűtési folyamat során* (Analysis of the injuries of stallion spermatozoa during the whole freezing procedure); Állatbiotechnológiai kutatások Magyarországon konferencia (Animal-Biotechnology Research in Hungary, Hungarian Academy of Sciences Conference), September 29. 2006., Budapest, oral presentation, abstract in the proceedings: p.23 (in Hungarian).

7. Horváth A., **Kútvölgyi G.**, Molnár M., Pribenszky Cs., Harnos A., Szenci O. (2007) *A magas hidrosztatikai nyomás alkalmazása a ménondó mélyfagyasztási protokolljában*; 14. Szaporodásbiológiai Találkozó. 2007. október 5. Keszthely, Hungary. (Oral presentation by Horváth A. in Hungarian)

8. Mari G., Iacono E., **Kútvölgyi G.**, Mislei B., Rodriguez-Martinez H., Morrell JM. (2010) *Stallion spermatozoa prepared by single layer centrifugation with androcoll^{TM}-E are capable of fertilization in vivo*; (Poster presentation at ESDAR 2010, Eger, Hungary) Abstract in: Reproduction in Domestic Animals 45. supplement 3., p.97.

12. PUBLICATIONS OUT OF THE FIELD OF THE THESIS

Scientific papers published in reviewed journals

1. Kovács A., Tumennasan, Kh., Demberel, Sh., Nagy Sz., **Kútvölgyi G.**, Oláh J., Jávor A. (2007) *Argáli spermiumok mélyhűtése /Előzetes közlemény/* (*Cryopresevation of Argali spermatozoa* /Pilot study/); Magyar Állatorvosok Lapja (Hungarian Veterinary Journal) 2007 (5.) 306-309.

Poster and oral presentations in Conferences

1. Kovács A., Molnár A., Kukovics S., **Kútvölgyi G**. (2005) *Mouflon x British Milksheep Hybrids*; Poster No. 8. Workshop Materials. Hair Sheep Workshop. June 21-23, 2005. Virginia State University Petersburg, VA 23806.

2. Pribenszky Cs., Molnár M., Horváth A., **Kútvölgyi G.**, Harnos A., Szenci O., Dengg J., Lederer J. (2006) *Substantial increase of post-thaw survival of frozen bull semen by hydrostatic pressure assisted freezing*; 18th Annual Meeting of the AI-Vets, Boras, Sweden, October 11-14, 2006. Abstract published in the proceedings: Chapter 13.

3. Pribenszky Cs., Molnár M., Horváth A., **Kútvölgyi G.**, Harnos A., Szenci O., Dengg J., Lederer J. (2007) *Improved post-thaw motility, viability and fertility achieved by hydrostatic pressure treated bull semen*; 33rd Annual Conference of the International Embryo Transfer Society, January 7-9, 2007, Kyoto, Japan, Abstract published in the Reproduction, Fertility and Development. 2007. 19(1)181 – 182.

4. Kovács A., Nagy Sz., **Kútvölgyi G.**, Behr B., Hermes R. (2007) *Különböző vad- és állatkerti emlősök ondósejtjeinek diagnosztikai festése* (*Diagnostic staining of spermatozoa from different wild and exotic mammals)*; Diagnosztika a vadállatorvoslásban – Magyar Vad- és Állatkerti Állatorvosok Társaságának éves konferenciája(Diagnostics in wild animal medicine – Annual Conference of Zoo Veterinarians), March 9-11, 2007, Budapest. Abstract published in the proceedings: p.77-79.

5. Kovács A., Tumennasan, Kh., Demberel, Sh., Nagy Sz., **Kútvölgyi G.**, Oláh J., Jávor A.(2007) *Argáli spermiumok mélyhűtése* (*Cryopresevation of Argali spermatozoa)*; Diagnosztika a vadállatorvoslásban – Magyar Vad- és Állatkerti Állatorvosok Társaságának éves konferenciája (Diagnostics in wild animal medicine –Annual Conference of Zoo Veterinarians), March 9-11, 2007, Budapest. Abstract published in the proceedings: p.80-81.

6. **Kútvölgyi G.**, Molnár M., Horváth A., Horváth G. and Pribenszky Cs. (2008) *Effect of hydrostatic pressure pulse on the life-time of fresh extended boar semen at 15ºC storage and following insemination on pregnancy rate and litter characteristics*. Poster presentation at the 16th International Congress on Animal Reproduction (ICAR, Budapest, 2008), Abstract in: Reproduction in Domestic Animals. 43. Supplement 3, p119.

7. **Kútvölgyi G**, Horváth A, Oláh I, Pálinkás P, Merész L, Molnár M, Pribenszky Cs, Szenci O. (2008) *High hydrostatic pressure treatment of fresh bull semen increases the proportion of cells surviving the freezing-thawing procedure in a Hungarian Holstein-Friesian bull population*. Presented at the 25th Congress of the World Association for Buiatrics, July 6 - 11, 2008, Budapest, Hungary**.**

8. Pribenszky Cs., Molnár M., **Kútvölgyi G**., Harnos A., Horváth A. and Héjja I. (2008) *Sublethal stress treatment of fresh boar semen with high hydrostatic pressure, inserted into the routine insemination procedure improves average live litter size*; (Oral Presentation at ESDAR 2008 by Pribenszky Cs.) Abstract in: Reproduction in Domestic Animals 43. supplement 5.

9. Pribenszky Cs., Molnár M., **Kútvölgyi G.,** Harnos A., Horváth A. and Héjja, I. (2009) *Sublethal hydrostatic pressure treatment improves fresh and chilled boar semen quality in vitro and in vivo.* Annual Conference of the International Embryo Transfer Society, San Diego, California, USA, 3–7 January 2009, Abstract in: Reproduction, Fertility & Development 21. p.107.

13. CURRICULUM VITAE

Relevant education, Doctoral and Postgraduate Studies:
2007-2008: *Homeopathy in Veterinary Medicine*, IAVH certificate: *Nov 2008*
2003-2005: *Equine Specialist of the Hungarian Veterinary Chamber***,** HVC and WIFI certificate*: October 2005*
2002-2005: *PhD student*, PhD School of Animal Science, Kaposvár University
1991-1996: *Univ. of Veterinary Sci.*, Budapest, *veterinarian diploma***:** April 1997
1985-1989: *ELTE Radnóti Miklós Secondary School*, biology-chemistry faculty
Workplaces and employments:
May-Aug 2010, 2011, 2012: Stall Rishaug, Ler, Norway: *Stud veterinarian*
May-Aug 2008, 2009: Alebäck Stud Farm, Lidköping, Sweden: *Stud veterinarian*
2007-2008: Cryo-Innovation Ltd, Budapest: *researcher-veterinarian*
2000-: Private horse veterinary practice: *veterinarian*
1998-2000: Mőbinter Ltd., Simonpuszta: *veterinarian*
1998/99: Univ. Veterinary Sci., Bp: participating in the Hungarian and English *education* of obstetrics and reproductive biology (equine reproduction)
1998–1999: Experimental Institute of University of Veterinary Science, Budapest; Equine Artificial Insemination (AI) Station, Üllő: *Stud veterinarian*
1997–1998: *Postgradual Trainee Year*: Equine AI. Station, Mezőhegyes; University of Veterinary Science, Budapest
Relevant study trips abroad:
Dec-Jan 2009: *Alyose Horse Clinic, Sala, Sweden*: Alternative medicine studies
April-June 2004: *Colorado State University*, Department of Biomedical Sciences, Animal Reproduction and Biotechnology Laboratory, Fort Collins, Co. USA: Equine embryo and oocyte transfer studies, spermatology research
July 1999: *Hochmoor Horse Clinic*, Germany: clinical studies
March 1999: *I.N.R.A., Nouzilly, France*, Dept. of Mammalian Physiology and Reproduction: equine reproduction and embryo-transfer studies
Oct.- Nov 1996: *Haydon Veterinary Clinic*, Bridport, Dorset, UK: clinical studies
Other experiences, participation in home and foreign funded projects:
2006-2008. *Developing of an innovative method for stress-tolerance augmentation of gametes and embryos with hydrostatic pressure-impulse treatment.*
-Cryo-Innovation Ltd. (Irinyi János Program and Kozma László project, NKTH)
2001-2006. Participating in *parentage control* of Trotter and Thoroughbred foals in the inland breeding farms (National Institute for Agricultural Quality Control)
2001-2002. *Improving stallion semen freezing* (KF-29/4)
1998-1999. *Embryo-Transfer in the Hungarian Sport Horse breeding.* (Project funded by the Hungarian Ministry of Agriculture and Rural Development)
Memberships**:** Hungarian Veterinary Chamber (HVC)-Equine Specialist Section; HVC-Alternative Medicine Section; Hungarian Veterinary Clinical Society-Hungarian Equine Veterinary Association, Reviewer of Journal of Reproduction in Domestic Animals

14. KÖSZÖNETNYILVÁNÍTÁS

Mindenekelőtt hálás köszönetemet szeretném kifejezni Témavezetőimnek, Dr. Stefler Józsefnek és Dr. Kovács Andrásnak, megértő türelmükért és a disszertációm elkészítéséhez nyújtott fáradhatatlan segítségükért.

Szeretném megköszönni az Állattenyésztési és Takarmányozási Kutatóintézet vezetőinek Dr. Fésűs Lászlónak és Dr. Rátky Józsefnek, hogy lehetőséget nyújtottak a vizsgálatok elvégzéséhez és mindazon kollégának, különösen Dr. Nagy Szabolcsnak, Dr. Kovács Katalinnak és Dr. Egerszegi Istvánnak, akik technikai segítséget nyújtottak. Külön köszönet Dr. Nagy Szabolcsnak a szakmai támogatásért, értékes, építő kritikákért és a statisztikai elemzésben nyújtott segítségért.

Köszönöm állatorvos kollégáimnak: Dr. Czimber Gyulának (Mosonmagyaróvár), Dr. Balogh Attilának (Budapest), Dr. Horváth Daniellának (Bábolna), Dr. Fekete Zoltánnak (Kecskemét), Dr. Török Erzsébetnek (Rádiháza), Dr. Magosi Zoltánnak (Mezőhegyes) és Dr. Horváth Andrásnak (Üllő) a vizsgált ménekkel kapcsolatos információk biztosítását és a kísérletekben felhasznált ménsperma mintákat.

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Szeretném megköszönni Dr. Jane Morrell-nek az Uppsalai Agrártudományi Egyetemről, hogy svédországi klinikai munkám során is lehetőséget kaphattam egy hasznos spermatológiai kísérletben való részvételre és a szakmai támogatásáért.

Szeretném megköszönni családomnak, barátaimnak a támogatást, sok-sok segítséget, és biztatást.

És végül, de nem utolsósorban a munkahelyi vitára felkért opponenseimnek: Dr. Barna Juditnak és Dr. Zomborszky Zoltánnak hálás köszönetemet szeretném kifejezni áldozatos munkájukért, amelyet a disszertáció-tervezetem alapos végigolvasásával, bírálatával, építő, segítő és hasznos kritikai észrevételeikkel, végeztek.

15. APPENDIX

List of abbreviations

AI	artificial insemination
ALH	amplitude of lateral head displacement
ASMA	computer-assisted sperm head morphometry
BSE	breeding soundness examination
BSI	British Standards Institution
CASA	Computer Assisted Sperm Analysis
CD	cytoplasmic droplet
coiled	coiled tail defect
CSB	Chicago sky blue 6B
CTC	chlortetracycline
DBT	damaged sperm with bent, curved, broken midpiece or tail
DCD	damaged spermatozoa with cytoplasmic droplet
DD	distal cytoplasmic droplet
detached	detached head
DGC	discontinuous density gradient centrifugation
DHA	docosahexaenoic acid
DHDTDA	damaged head, tail, acrosome membrane
DHIT	damaged head, intact tail membrane
DMF	dimethyl-formamide
DMR	distal midpiece reflex
DNA	desoxyribonucleic acid
DSO	daily sperm output
EM	electron microscopy
EY-SM	egg-yolk-skim-milk
FITC-PNA	fluorescein isothiocyanate-labeled peanut agglutinin
FITC-PNA/PI	fluorescein isothiocyanate-conjugated peanut agglutinin/propidium iodide
FSH	follicle stimulating hormone
GH	growth hormone
GIFT	gamete intrafallopian transfer
GnRH	gonadotropin-releasing hormone
GPX	glutathione peroxidase
GW	glass wool filtration
HA	hyaluronic acid
HCDM	Hepes-buffered chemically defined handling medium
head	head abnormalities
HOST	hypo-osmotic swelling test
HSP	Horse Seminal Protein

IBT	intact head, tail and acrosome; bent, broken midpiece or tail
ICDBT	intact sperm with droplet + Intact sperm with bent tail
ICSI	intracytoplasmic sperm injection
IDBT	All sperm with bent, curved, broken midpiece or tail
IDCDBT	All spermatozoa with droplet or bent tail
IDCD	All (intact and damaged) spermatozoa with droplet
IDD	intact head, tail and acrosome; distal cytolasmic droplet
IGF	insulin-like growth factor
IHDT	intact head, intact acrosome, damaged tail
IHITDA	intact head, tail, damaged (loose-, lost-) acrosome
IHITIA	intact head, tail and acrosome; normal morphology
INH	inhibin
Intact	intact head, tail and acrosome membrane
IPD	intact head, tail and acrosome; proximal cytoplasmic droplet
IUI	intra-uterine insemination
IVF	in vitro fertilisation
LH	luteinizing hormone
LIN	Linearity /linearity of the curvilinear trajectory
LM	light microscope
LOX	Lipoxygenase
*L*p	water permeability or hydraulic conductivity
LPO	lipid peroxidation
LS means	Least squares means
MF	methyl-formamide
midp	midpiece defect
midp+tail	midpiece and tail defect
MIF	macrophage migration inhibitory factor
MOT	motility of the sperm
multiple	multiple forms (eg. double midpiece, head, tail)
NFDSM	non-fat dry skim milk
OEC	oviductal epithelial cells
PBS	phosphate-buffered saline
P-CON	Percoll-control
PD	proximal cytoplasmic droplet
PDE	phosphodiesterase
PDE	phosphodiesterase
PE-PNA	phycoerythrin-conjugated peanut agglutinin
PG	Percoll®-based density gradient
P-HA	Percoll- hyaluronic acid treatment
PI	propidium iodide
PLCζ	phospholipase Cζ
PM	progressive motility, progressive motile
PMS	progressive motile sperm

PNA	Peanut Agglutinin
P-NT	Percoll non-treated
P-PX	Percoll-pentoxifylline treatment
PR	pregnancy rate
PSA	Pisum Sativum Agglutinin
PUFA	polyunsaturated fatty acids
PVP	polyvinylpyrrolidone
PX	Pentoxifylline
R123	rhodamine-123
RAP	rapidity of the sperm
ROS	reactive oxygen species
SCD	chromatin dispersion test
SCD	sperm chromatin dispersion
SCSA	Sperm Chromatin Structure Assay
sDFI	DNA fragmentation index
SE	standard error of the mean
SEM	scanning electron microscopy
SLC	Single Layer Centrifugation
SOD	superoxide dismutase
SP	Seminal plasma
SU	swim-up
SU-HA	swim up- hyaluronic acid treatment
SU-NT	swim up- non-treated
SU-PX	swim up- pentoxifylline treatment
SUTI	Sperm Ubiquitin Tag Immunoassay
tail	tail abnormalities
TALP	Tyrode's albumin lactate pyruvate
TB	trypan blue
TEM	transmission electron microscopy
TM	total motility
TSN	total spermatozoa number
UFT	unique freezing technique
VCL	curvilinear velocity

Laboratory Stock solutions

STOCK	Formula/comp.	Mol. Weight	mM or % (w/v)	Quantity (g)	H2O ml (up to)	0.9% saline ml (up to)	Phenol red (g)	Filter (Y/N)	Storage time	Storage temp	How to store
ALA-GLU	Alanyl-Glutamine	217.2	100	0.434	20			Y		4 °C	50 ml tube
B	$NaHCO_3$	84.01	250	2.100	100		0.005	Y	1 month	4 °C	50 ml tube
BSA	Bov. Ser. Album.	~55.000	10%	2.000	20			Y	2 weeks	4 °C	50 ml tube
C	$CaCl_2 \times 2H_2O$	147	200	0.588	20			Y	3 months	4 °C	50 ml tube
CAFN	Caffeine	194.2	50	0.194	20			Y	1 month	4 °C	50 ml tube
CYSTMN	Cysteamine	113.6	10	0.011		10		N	3 months	-20 °C	.5 ml vial
CDM:	NaCl	58.44	710	4.149							
W-phosphate	KCl	74.55	60	0.447							
	KH_2PO_4	136.1	10	0.136							
	Na-Citrate.$2H_2O$	294.1	5	0.147							
	TOTAL:		785		100			Y	3 months	4 °C	50 ml tube
E(3)	Vitamin E	472.8	200	0.473	5			Y	1 month	4 °C	1 ml vial

EAA'S	BME AA sol 50X		73.56						1 year	4 °C	100 ml bottle
EDTA(4)	$C_{10}H_{16}N_2O_8$	292.2	1	0.029	100			Y	3 months	4 °C	50 ml tube
F	D-Fructose	180.2	200	0.721	20			Y	1 month	4 °C	50 ml tube
G	D-Glucose	180.2	200	0.721	20			Y	1 month	4 °C	50 ml tube
GLYCN	Glycine	75.07	490	0.736	20			Y	1 month	4 °C	50 ml tube
GLTMN	Glutamine	146.1	100	0.292	20			Y	3 weeks	4 °C	10 ml tube
H-1:1	HEPES free ac.	238.3	100	2.383							
	HEPES Na salt	260.3	100	2.603							
	TOTAL:		200		100		0.005	Y	1 month	4 °C	50 ml tube
HP	Heparin Na salt	5000	2	0.020		2		N	1 month	4 °C	5 ml tube
I(1)	Glacial Acetic Acid	60.05	166	0.100							
	Insulin	6000	1.66	0.100							
	TOTAL		167.66		10			Y	1 Year	-20 °C	.05 ml vial
MI	myo-Inositol	180.2	277	0.998	20			Y	1 month	4 °C	50 ml tube

L-D/L(2)	Na-Lactate (60% syrup)	112.1	1000	5.619 (ml)	40			Y	1 month	4 °C	50 ml tube
L-L	sodium L-lactate	112.1	1000	1.121	10			Y	1 month	4 °C	50 ml tube
MS	$MgSO_4$	120.4	50	0.120	20			Y	3 months	4 °C	50 ml tube
NaCl	NaCl	58.44	5000	5.844	20			Y	3 months	4 °C	50 ml tube
NEAA'S	MEM AA sol 100X		67.04						1 year	4 °C	100 ml bottle
P	Na-Pyruvate	110	50	0.110	20			Y	2 weeks	4 °C	50 ml tube
PH	Penicillamine	149.2	2.5	0.037							
	Hypotaurine	109.1	1	0.011							
	TOTAL:		3.5		100			Y	3 months	-20 °C	1 ml vial
T	Taurine	125.1	10	0.025	20			Y	3 months	-20 °C	1 ml vial
VITS	BME 100x vits		145.04								

H-CDM-1

CSU CHEMICALLY DEFINED MEDIUM

Stock component	25 ml	50 ml	75 ml	100 ml	mM Stock	% in final medium	Molarity (mM)	Dissociation factor	Osmolarity (mOsm)
H_2O	16.89	33.78	50.66	67.55	0	67.55%	0.00	1	0.00
CDM	2.50	5.00	7.50	10.00	785	10.00%	78.50	2	157.00
B	0.50	1.00	1.50	2.00	250	2.00%	5.00	2	10.00
C	0.25	0.50	0.75	1.00	200	1.00%	2.00	2	4.00
G	0.063	0.125	0.188	0.250	200	0.25%	0.50	1	0.50
Glycn	0.25	0.50	0.75	1.00	490	1.00%	4.90	1	4.90
ALA-GLU	0.25	0.50	0.75	1.00	100	1.00%	1.00	1	1.00
H (1:1)	2.50	5.00	7.50	10.00	200	10.00%	20.00	1.5	30.00
L-L	0.25	0.50	0.75	1.00	1000	1.00%	10.00	2	20.00
P	0.25	0.50	0.75	1.00	50	1.00%	0.50	2	1.00
MS	0.25	0.50	0.75	1.00	50	1.00%	0.50	2	1.00
NEAA'S	0.25	0.50	0.75	1.00	67.04	1.00%	0.67	1	0.67
NaCl 5M	0.11	0.23	0.34	0.45	5000	0.45%	22.50	2	45.00
BSA FAF	0.63	1.25	1.88	2.50	0.002	2.50%	0.00	1	0.00
Mix. adjust pH (7.3 - 7.4) and sterilize with a 0.22 µ membrane filter									
Finally add:									
Gentamycine 25 µg/ml	0.06	0.13	0.19	0.25	0	0.25%	0.00	1	0.00
TOTAL:	25.00	50.00	75.00	100.00		100.00%	146.07		275.07
Store at 4 °C for 3 weeks.									

Microscopic pictures

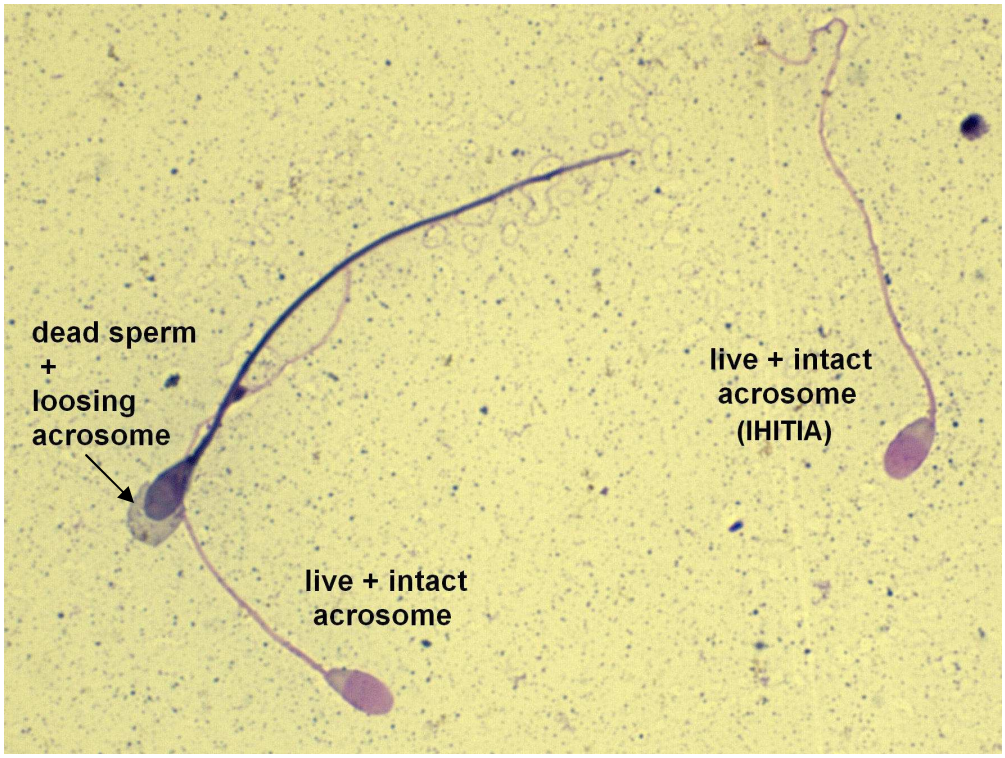

Fig. 1. Microscopic picture of different sperm types, CSB/Giemsa staining

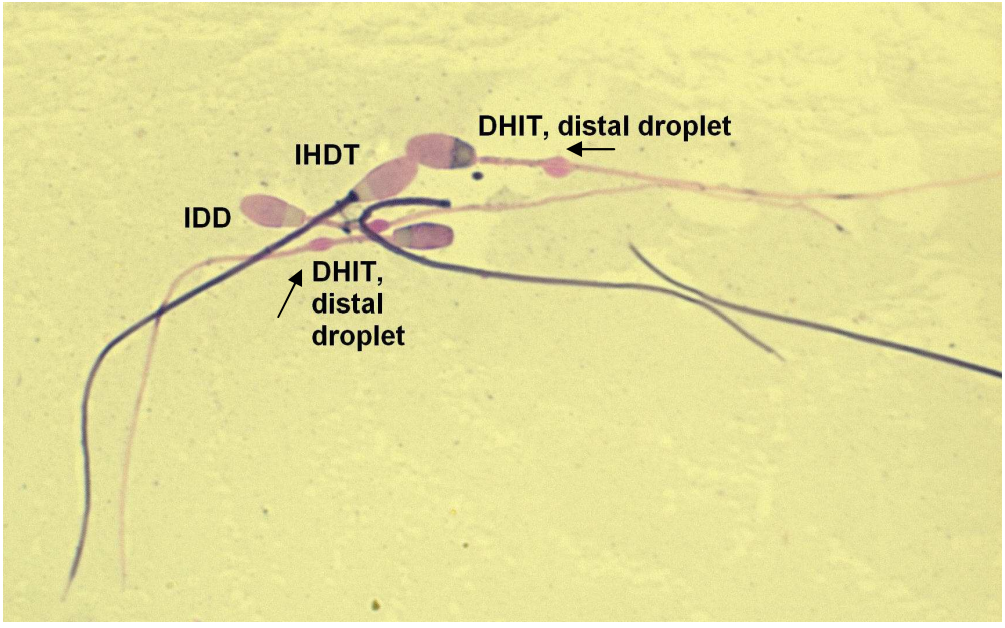

Fig. 2. Microscopic picture of different sperm types, CSB/Giemsa staining

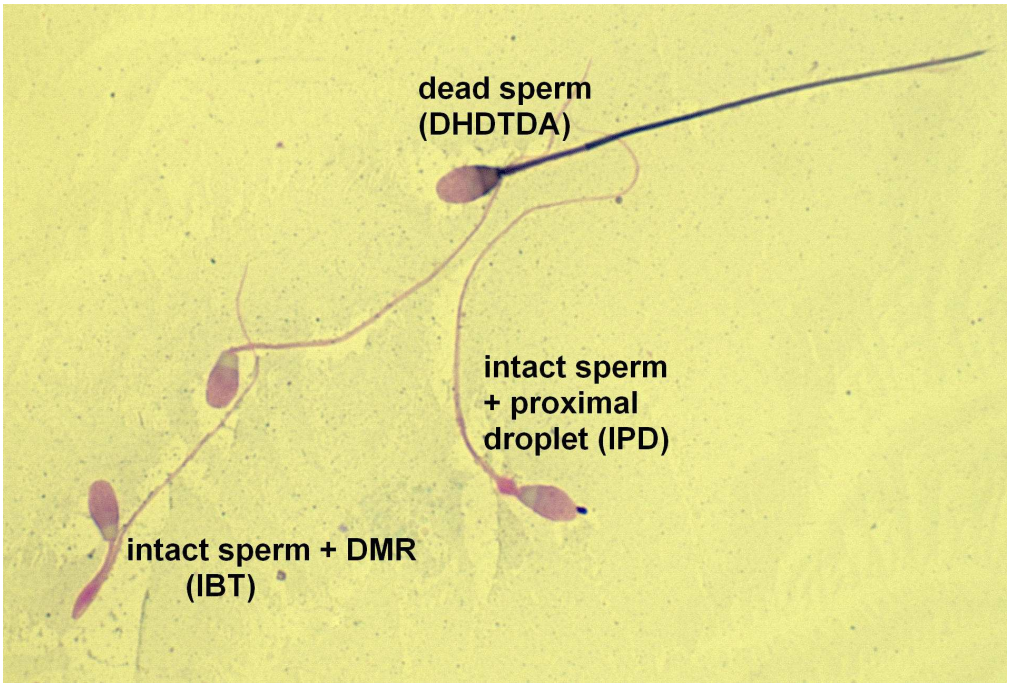

Fig. 3. Microscopic picture of different sperm types, CSB/Giemsa staining

Subfertile stallions

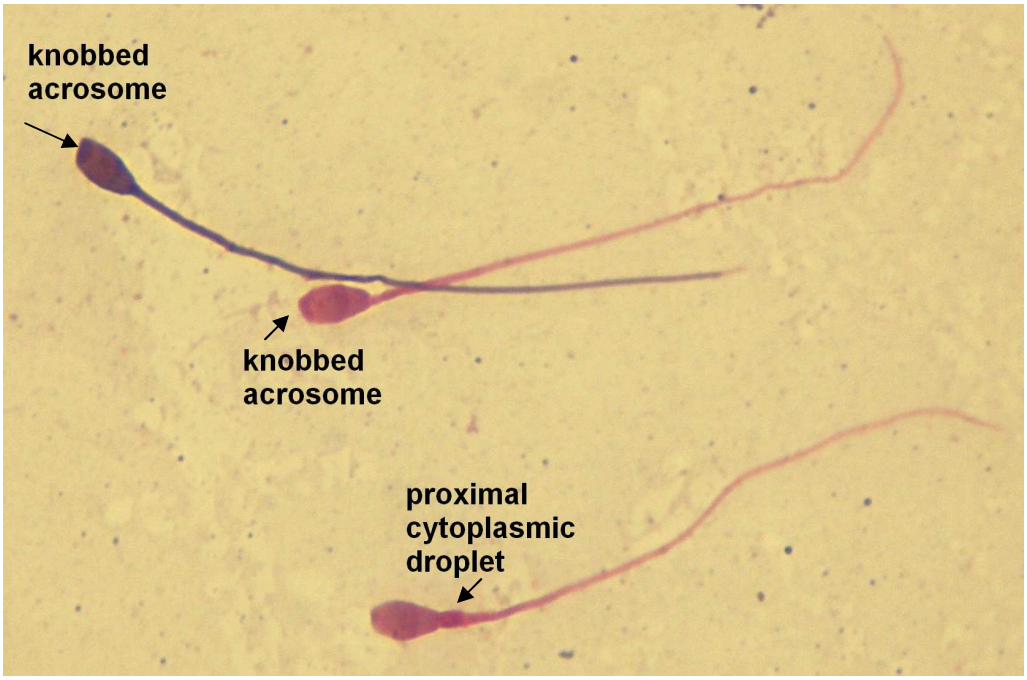

Fig. 4. Morphological defects of Stallion "A" spermatozoa, TB/Giemsa staining

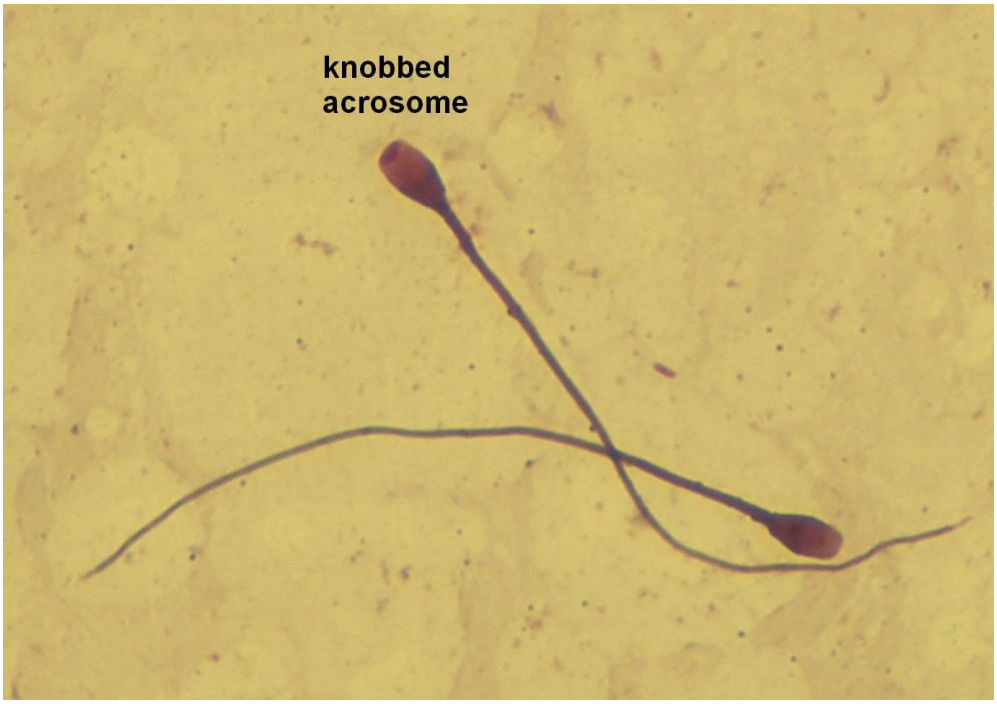

Fig. 5. Knobbed acrosome defect in Stallion "A" sperm, TB/Giemsa staining

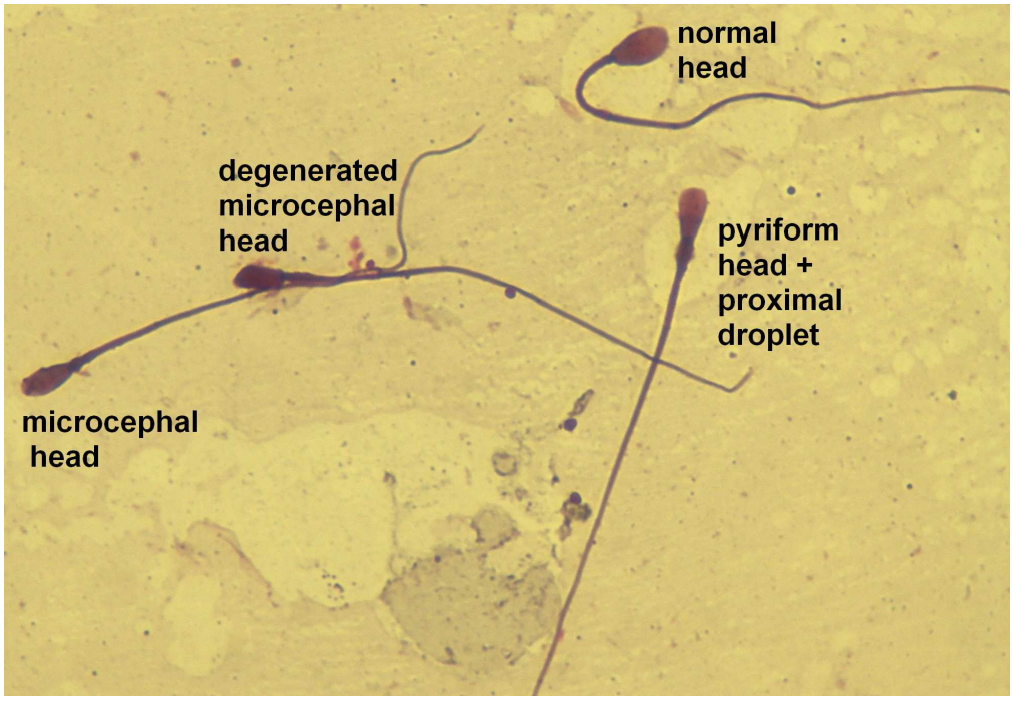

Fig. 6. Different head abnormalities of Stallion "A" spermatozoa, TB/Giemsa staining

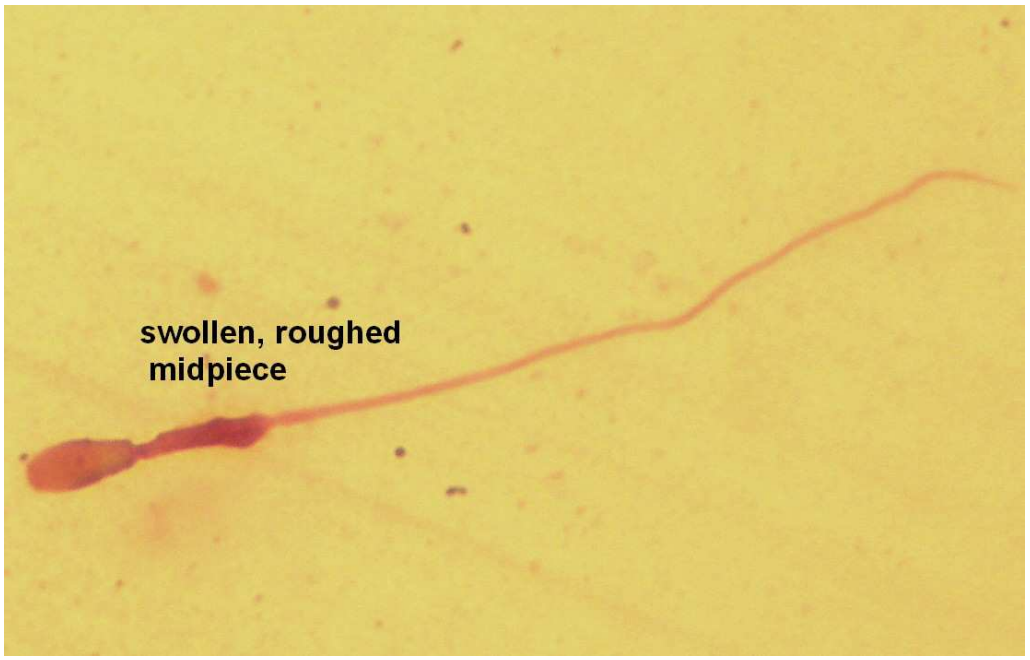

Fig. 7. Spermatozoon with midpiece defect in Stallion “C” semen, TB/Giemsa staining

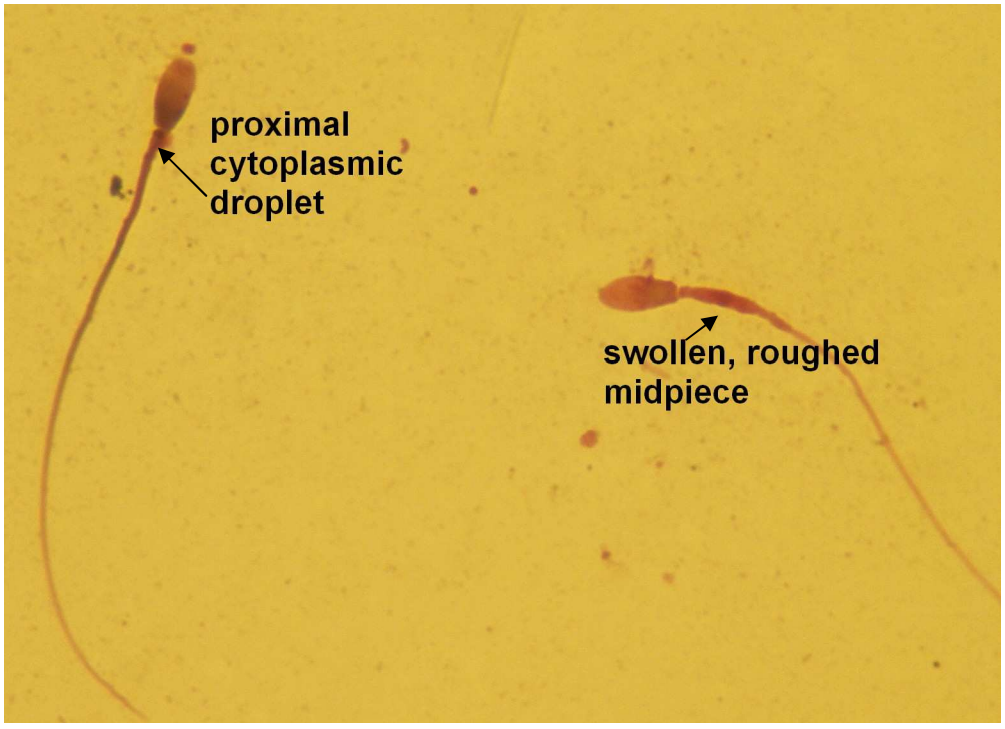

Fig. 8. Morphological defects of Stallion “C” spermatozoa, TB/Giemsa staining

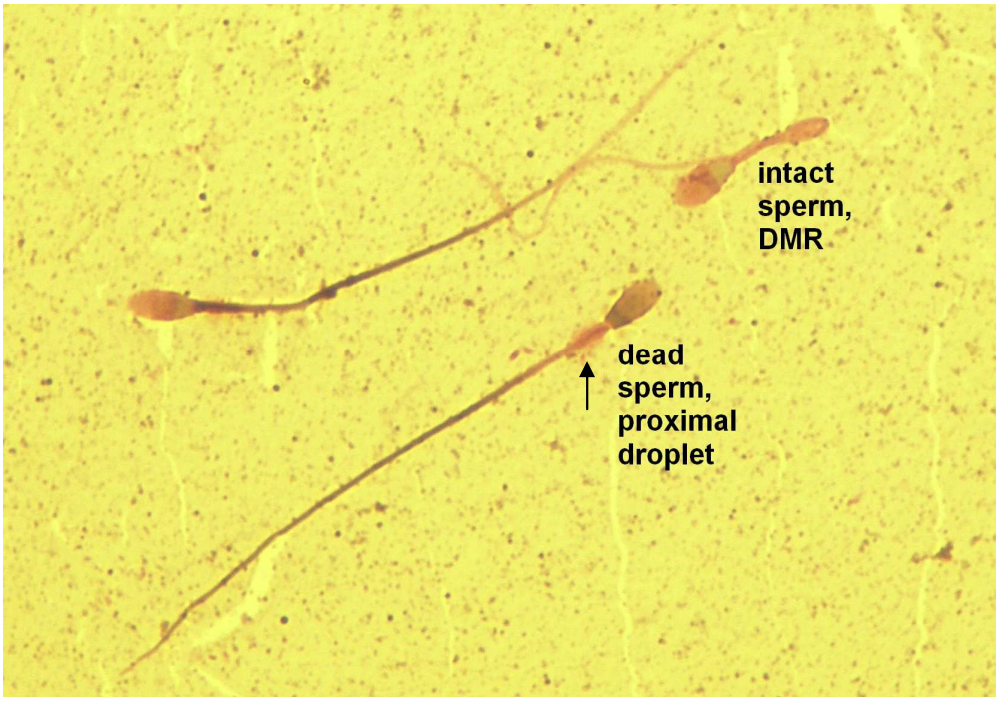

Fig. 9. Different morphological defects of Stallion "E" spermatozoa, CSB/Giemsa staining

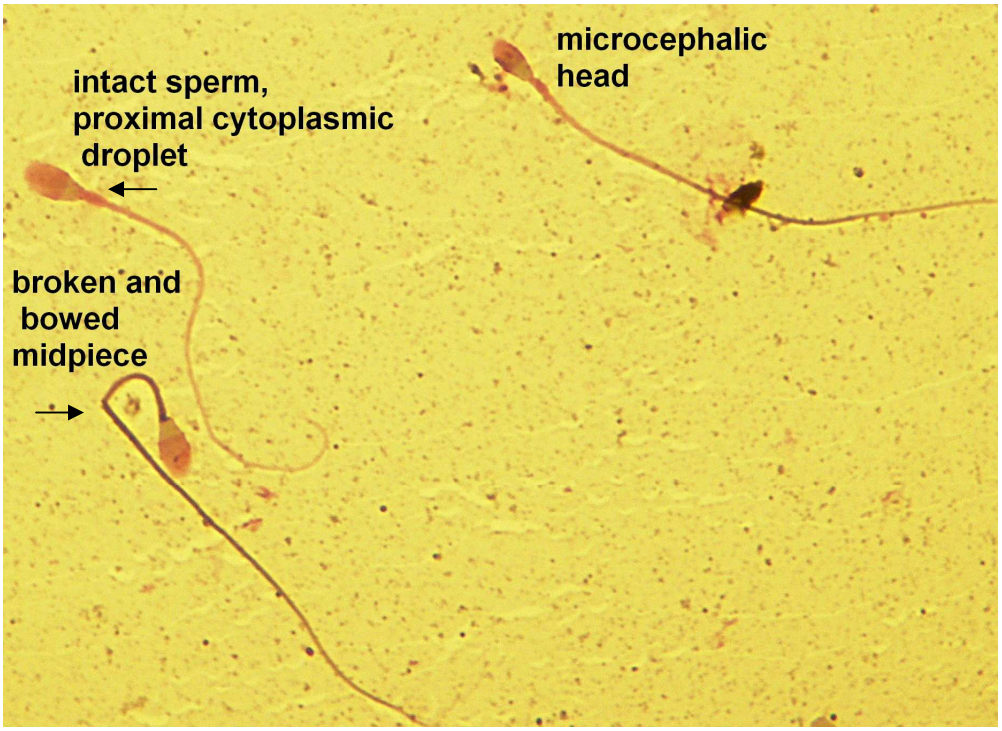

Fig. 10. Different morphological defects of Stallion "E" spermatozoa, CSB/Giemsa staining

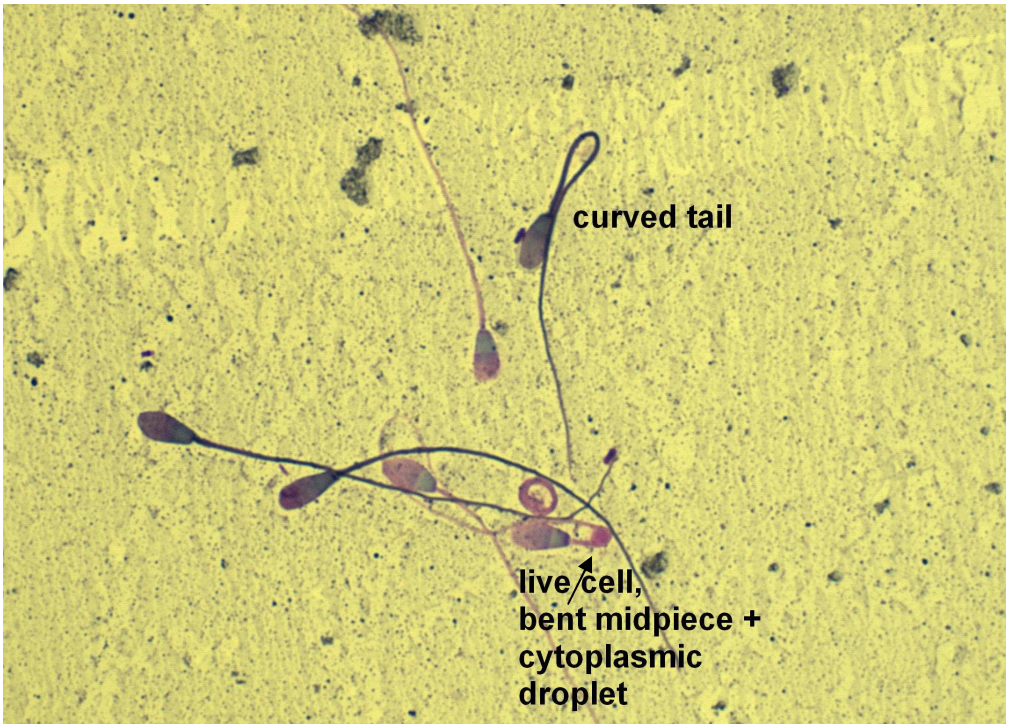

Fig. 11. Midpiece and tail defects in Stallion "I" sperm, CSB/Giemsa staining

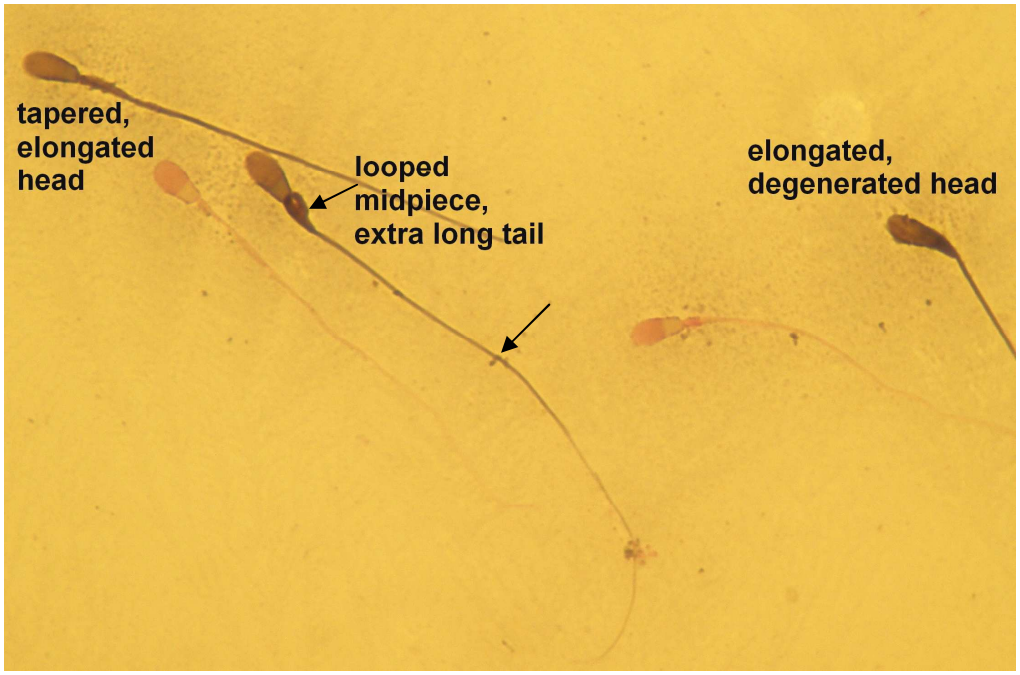

Fig. 12. Different morphological defects of Stallion "F" spermatozoa, CSB/Giemsa staining

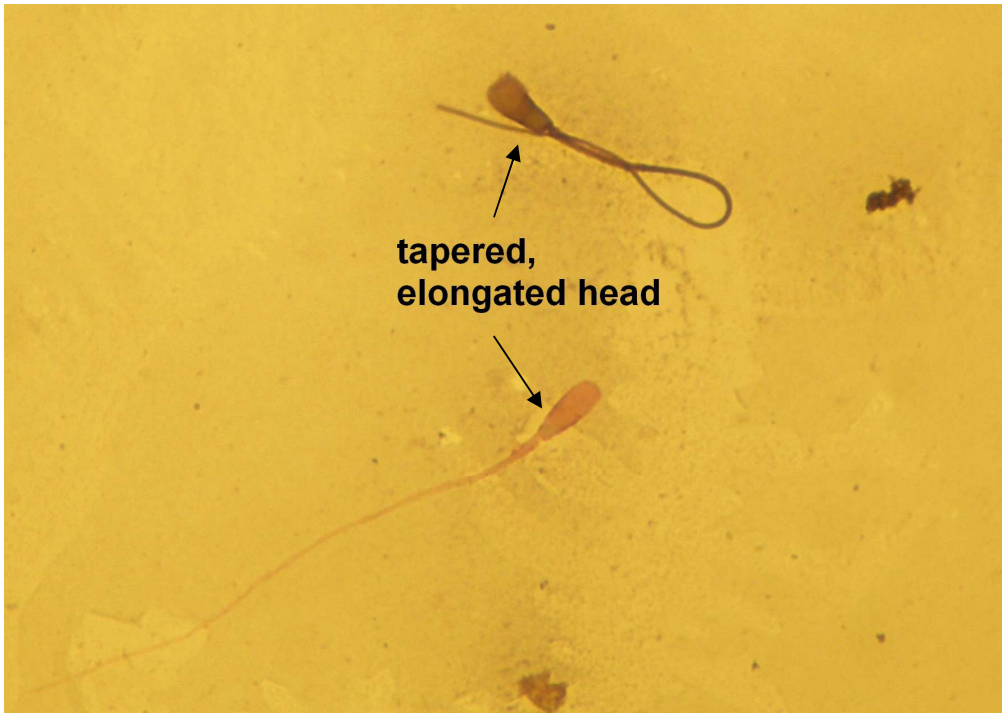

Fig. 13. Spermatozooa with head abnormalities in Stallion "F" semen, CSB/Giemsa staining

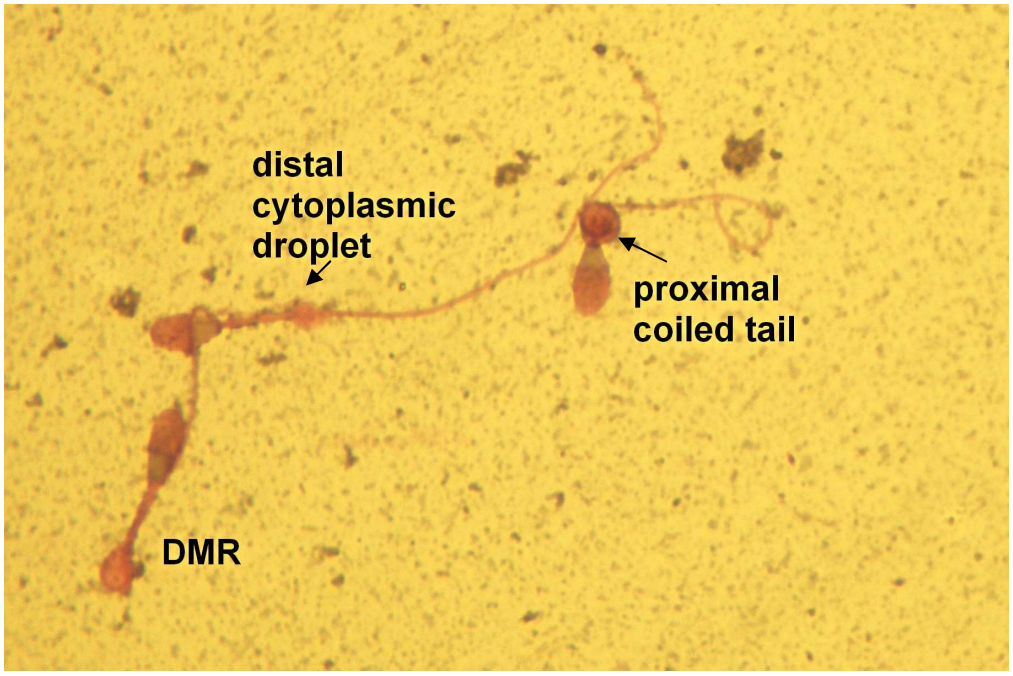

Fig. 14. Live spermatozoa with different morphological defects in Stallion "J" semen, CSB/Giemsa staining