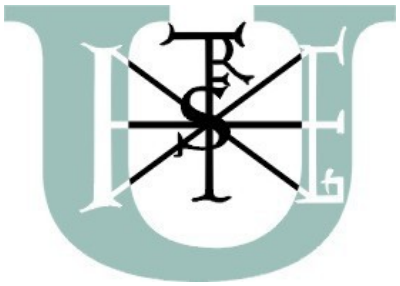

Szent István University

CRYOPRESERVATION OF POULTRY SPERMATOZOA AND GONADAL TISSUES FOR GENE CONSERVATION PURPOSES

Thesis of PhD dissertation

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1. ANTECEDENTS AND OBJECTIVES OF THE WORK

1.1. Introduction, importance of the subject

The preservation of biodiversity is of broad interest all over the world. Because of the persistent food shortage, it is becoming more difficult to preserve the genetic diversity of domestic animal species. Unfortunately the indigenous poultry breeds are pushed into the background. The genetic resources of indigenous breeds can serve as a basis of diversity in order to create breeds adaptable to the local environmental conditions.

The importance of gene preservation and biodiversity has resulted in the creation and improvement of gene and databanks, which have three different types. In the case of ***in situ*** gene banks (1) the populations are kept in their original habitat, but in the case of ***ex situ in vivo*** gene banks (2) the nucleus populations are kept and bred on artificial farms. Due to the vulnerability of the life population ***ex situ in vitro*** gene banks are also necessary, where the valuable genetic materials (spermatozoa and oocytes, embryos, embryonic cells, early gonadal tissues, DNA samples) are cryopreserved for long-term storage.

At present, semen cryopreservation is the most practical method of *in vitro* gene preservation in poultry species. In the case of semen cryopreservation, only the male genome is conserved as in birds the female is the heterogametic sex. However, by 6-8 generations backcrossing, almost 100% of the desired genotype can be retained. Certainly, elaborations of new alternative methods are needed to preserve the whole genome. The cryopreservation of oocytes could make it possible to retain the W chromosome, but in birds such techniques cannot be used due to the biophysical characteristics of the megalecithal egg. The use of embryonic cells (blastoderm cells, primordial germ cells) for the creation of chimeric birds can be a solution for conserving of the whole genetic material, but this method is not sufficiently effective and too expensive to be used for large programs of genetic conservation. An alternative approach to preserving the female genome can be the cryopreservation and transfer of early ovarian tissue to recipient animals. For the application of this method in gene preservation programs an effective, practical freezing and transplantation method is needed. These studies are in progress in several laboratories including our institute.

Since 2014, besides the *ex situ in vivo* gene bank an *in vitro* gene bank has also been under development in the Poultry Reproduction Laboratory of the Centre for Farm Animal Gene Conservation. In this *in vitro* gene bank the storage of cryopreserved semen samples of indigenous Hungarian poultry breeds is in progress at the moment.

1.2. Objectives

1. The evaluation of species-specific cryopreservation methods which are applicable in practice in a simple and environment-friendly manner with less investment and effective usage. The aim of my studies was to improve and compare the programmable freezing method and ultrafast techniques pellet method and freezing in nitrogen vapour of *domestic fowl, guinea fowl* and *domestic goose* spermatozoa, and to create the most practical method for these poultry species.

2. Another aim of my work was to cryopreserve *early gonadal tissues* of domestic fowl using the pellet method, freezing in nitrogen vapour and vitrification as ultrafast freezing techniques. The efficiency of these methods will be compared using *in vitro* methods such as histology and tissue culture.

2. MATERIALS AND METHODS

2.1. Cryopreservation of domestic fowl spermatozoa

In the experiment the efficiency of standard programmable freezing using glycerol and the pellet method was tested by *in vitro* and *in vivo* assessments.

Twenty one-year-old partridge-colour Hungarian chicken males were kept in individual cages. During a two-month period, semen was collected twice a week by dorso-abdominal massage as described by Burrows and Quinn. *In vitro* assessment of semen quality was done before freezing and after thawing (fresh and frozen/thawed semen). During sperm qualification the classical spermatological parameters (sperm volume, motility, concentration, morphological abnormalities, and live/dead cell ratio) were determined.

The efficiency of the freezing methods was tested *in vivo* by artificial insemination. Three groups of hens were created: the control group was inseminated with fresh, diluted semen; the 'freezing' groups were inseminated with frozen/thawed (programmable and pellet method) semen three times in the first week and subsequently twice a week for three weeks. Fertility was checked on the 7^{th} day of incubation by candling and the ratios of true fertility, early embryonic mortality (embryos dying in the oviduct), death during incubation and normal development were also determined.

2.2. Cryopreservation of guinea fowl spermatozoa

During the freezing experiment four protocols - slow and fast programmable method, freezing in nitrogen vapour, and pellet method - were evaluated in order to find the most effective freezing method. The efficiency of three cryoprotectants - 10% ethylene glycol (EG), 6% dimethylformamide (DMF), 6% dimethylacetamide (DMA) - was also examined.

Thirty one-year-old rural Hungarian guinea fowl males were kept in individual cages. Sperm collection and qualification were made as described for domestic fowl.

The two most effective freezing methods were tested by the use of artificial insemination. Ten females (controls) were inseminated with fresh, diluted semen and the remaining 20 birds with frozen/thawed semen processed by the slow (10% EG) and the pellet method (10 birds per protocol) twice a week for three weeks. The fertility and the embryonic status of eggs after candling were determined in a manner similar to that used in the case of domestic fowl.

2.3. Cryopreservation of domestic goose spermatozoa

During this study a programmable protocol and an own-developed method with freezing in nitrogen vapour were compared *in vitro*. Various non-permeable osmoprotectants (betaine, trehalose, sucrose) were also tested.

Thirty 2-year-old Landes ganders at the start of their spring production period were placed in individual cages. Sperm collection and qualification were done as described previously for domestic fowl. Two basic freezing methods - a programmable protocol and freezing in nitrogen vapour - and four various cryo- and osmoprotectants (DMF in two concentrations, betaine, trehalose-sucrose combination) were tested.

The most effective freezing methods were tested by the use of artificial insemination. Twenty females serving as controls were inseminated with fresh and diluted, while 20 females with frozen/thawed semen from the simple nitrogen vapour protocol. Artificial insemination was done twice a week for three weeks. Fertility and the embryonic status of eggs after candling were determined in a similar manner as described for domestic fowl.

2.4. Cryopreservation of early gonadal tissues

Three ultrafast freezing methods were tested in cryopreservation of early gonadal tissues. The efficiency of freezing in nitrogen vapour (using cryovials), by the pellet method and the vitrification technique (using acupuncture needles) was examined. The aim was to elaborate a freezing method that can be used for the transplantation of cryopreserved gonadal tissue and the integration of the female genome into the gene preservation programs.

Autosex Tetra SL day-old chickens were used as donors. The removed gonads were collected in a small Petri dish containing sterile DMEM (Dulbecco's Modified Eagle's Medium) solution at 0°C until freezing (10-40 minutes). The integrity of the frozen gonads was studied both histologically and by examination in tissue culture.

3. RESULTS

3.1. Cryopreservation of domestic fowl spermatozoa

No significant differences were detected between the two freezing methods in the parameters studied. The sperm quality after freezing/thawing showed that the slow, programmable and the pellet method resulted in 12.7% and 14.1% live sperm ratio as well as 8.1% and 10.6% live, intact sperm ratio, respectively. The survival rates of live, intact spermatozoa by the use of these methods were 9.3% and 12%, respectively.

Similarly no significant difference was found between the two freezing groups in fertilizing ability. By insemination with fresh, diluted semen 88.8% fertility was achieved, while the use of frozen/thawed semen by the slow, programmable and the pellet method resulted in 32.2%, and 44.2% fertility, respectively. Consistently with our earlier findings, a strong correlation was detected between the number of live, intact spermatozoa used for insemination and the ratio of embryos showing normal development. Insemination with fresh, diluted semen produced a 82.3% rate of normal embryos, while frozen/thawed semen by the pellet method resulted in a normal embryo rate of 22.6%, which was significantly ($p \leq 0.05$) higher than that obtained by the slow, programmable method (14.1%).

3.2. Cryopreservation of guinea fowl spermatozoa

The quality of spermatozoa after freezing and thawing shows that the worst live cell ratios were obtained in samples originating from nitrogen vapour (16.8% with EG and 14.8% with DMF) and the fast programmable protocol (24.5% with EG and 21.7% with DMF). The pellet method and the slow, programmable protocol with 10% EG resulted in the highest live sperm ratio (31.4% and 41%, respectively). Although the slow protocol resulted in the highest number of live cells, a significantly ($p \leq 0.05$) higher ratio of abnormal spermatozoa were detected among them as compared to the pellet method (23% vs. 10%, respectively). Thus, the highest live, intact cell ratio was found with the pellet method.

The study of sperm abnormalities shows that the cryoprotectant DMF produced a significantly ($p \leq 0.01$) higher rate of acrosome abnormality at all cooling rates. The pellet method produced a significantly ($p \leq 0.01$) the higher rate of head abnormalities and lower rate of tail abnormalities compared to the other protocols; however, the pellet method resulted in the lowest number of abnormalities among all protocols. The best survival rates of live, intact spermatozoa

were obtained by the pellet method (28.6%) and the slow programmable protocol with 10% EG (23.5%). The pellet method resulted in the highest survival rate ($p \leq 0.05$).

Spermatozoa cryopreserved by the two most successful methods and fresh, diluted semen were used for artificial insemination in three experimental groups. Up to the end of the 3-week insemination period, fertility rates obtained by the use of fresh samples (control), slow and pellet protocols were 91.7%, 29.1% and 63.6%, respectively. The rate of embryos that died in the oviduct was significantly ($p \leq 0.05$) higher following insemination with cryopreserved semen compared to the control group.

3.3. Cryopreservation of domestic goose spermatozoa

The quality of spermatozoa after freezing and thawing shows that the programmable methods resulted in 52.5-59%, while the nitrogen vapour methods produced a 48.3-54.9% live cell ratio. In the case of programmable freezing the 7% DMF protocol resulted in a significantly ($p \leq 0.01$) lower survival rate of live, intact spermatozoa than did protocols 2, 3 and 4 (42.6% 47.9% 48.5% and 50.3%, respectively). No significant differences in survival rates (43-46%) were found among the nitrogen vapour protocols. The osmoprotectants did not improve sperm survival in any of the freezing methods.

As no significant differences were found in survival rate between the programmable and the nitrogen vapour methods, the efficiency of the more practical nitrogen vapour protocol (9% DMF) was tested by artificial insemination. Although insemination with fresh, diluted semen resulted in a significantly ($p \leq 0.01$) higher fertility rate (81.8%), the frozen/thawed sperm also yielded high fertility (58.5%). The rate of embryos that died in the oviduct was significantly ($p \leq 0.05$) higher following insemination with cryopreserved semen (12.8%) than in control group (3.4%).

3.4. Cryopreservation of early gonadal tissues

A total of 104 ovaries and 175 testes were cryopreserved using the three different freezing methods. Histological sections of frozen gonads cryopreserved by different methods and fresh (control) gonads were compared. In the case of frozen testes the vitrification method better preserved the structure of the original testis than did the other freezing methods. By vitrification the ratio of tubules and interstitium in the testes was similar to the normal testis structure, while in the case of freezing in nitrogen vapour and by the pellet method this was not observed.

Histological examination of frozen ovaries cryopreserved by the three different freezing methods confirmed that the structures of the ovaries are well conserved even if the kidney tissue is already damaged.

During the creation of tissue cultures fibroblasts were grown from the frozen tissues originating from the three freezing methods of both types of gonads. This observation confirms that the gonads survived the freezing/thawing procedure.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Cryopreservation of spermatozoa

Studies conducted in the last 20-30 years provided evidence that species-specific freezing protocols are needed for the various avian species. At the moment only the cryopreservation of chicken semen can be found in the FAO guideline for creating gene banks. Therefore it is important to compare the efficiency of different freezing methods for other species as well.

According to our results on ***domestic fowl*** sperm freezing, there are no significant differences between the two different freezing methods either in the survival rate of live, intact spermatozoa or in their fertility. Since the efficiency of the simplified and fast pellet method was similar to that of the classical, slow programmable protocol, the application of the pellet method is recommended in the case of rare, endangered species which either produce a lower sperm volume and/or no programmable freezing machine is available for them.

Earlier trials on the cryopreservation of ***guinea fowl*** sperm were lacking. In the present comparative study the slow programmable and the pellet method produced the highest survival rate of live, normal spermatozoa. By their comparison a significantly higher survival rate was found with the pellet method which resulted in 63.6% fertility. According to the existing data of the special literature the above result was the best in the world, up to that time. This method can be used for sperm bank storage, especially for species that produce a low sperm volume.

Programmable freezing methods are used in the few laboratories working on ***gander*** sperm freezing. Frozen/thawed sperm originating from the most practical and economical nitrogen vapour method produced almost 60% fertility rate. The method is applicable not only for gene conservation purposes but also in goose breeding practice because its efficiency is nearly as good as that of insemination with fresh, diluted semen (70-80%). Using this method, cryopreservation can be performed also in goose breeding farms especially in the case of breeding stocks of higher level since it does not require expensive equipment.

During our freezing experiments in the last few years it was observed that the rate of the very early embryonic deaths (embryos dying in the oviduct) was significantly higher in the first week of insemination period in the case of frozen/thawed semen as compared to the fresh, diluted semen and to other early embryo mortalities. This phenomenon may be due to the low number of intact frozen/thawed spermatozoa which was not sufficient to fill the sperm storage tubules completely, and thus to support normal embryo development. For the above reason we recommend the use of more frequent inseminations with higher insemination doses in the first week of the insemination period.

4.2. Cryopreservation of early gonadal tissues

In the case of the female gamete, cryopreservation and transfer of frozen/thawed *ovarian tissues* to recipients is the only solution for conservation purposes. In certain cases, it might be necessary to replace cryopreservation of spermatozoa with *testicular tissue* freezing.

According to our studies, the freezing one-day-old *testes* by the vitrification method is suitable for preserving the genetic material of valuable males. The application of this method is recommended in the case of some genetically valuable males, where sperm collection and preservation cannot be accomplished. The normal structure of the frozen/thawed *ovaries* of day-old chickens was preserved in all cases when the above-mentioned three different freezing methods were used.

The intact character of frozen-thawed gonads was confirmed by tissue culture examinations, which verified that early gonads survived the cryopreservation procedure.

In the present studies on *in vitro* gene conservation, efficient alternatives of species-specific sperm freezing protocols were elaborated in three poultry species (domestic fowl, guinea fowl and domestic goose), In addition, through the adaptation and development of cryopreservation of early ovarian tissues of domestic fowl, the conservation of the avian female genome has become accessible for the future.

5. NEW SCIENTIFIC RESULTS

1. I proved that the pellet method is efficiently applicable in the case of partridge-colour Hungarian chicken, in the same way as the classical slow programmable method. The method can be applied in the case of rare, endangered species, which either produce a lower sperm volume and/or for which no programmable freezing machine is available.

2. I elaborated, for the first time a freezing protocol for guinea fowl semen which produced 63.6% fertility, which is conducive to the *in vitro* gene preservation of the species.

3. I verified that our own-developed nitrogen vapour method elaborated for gander sperm freezing resulted in nearly 60% fertility. The success of insemination with cryopreserved semen approached the efficiency of artificial insemination with fresh, diluted semen.

4. I determined that when using insemination with frozen-thawed semen the rate of early embryonic deaths (embryos dying in the oviduct) increases significantly compared to other embryo mortalities and to inseminations with fresh, diluted semen.

5. I verified that the normal structure of the frozen-thawed gonads can be preserved by cryopreservation in both sexes. This was confirmed by both histological and tissue culture examinations.

6. PUBLICATIONS RELATED TO THE DISSERTATION

Publications in peer-reviewed journals

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- **Váradi, É**., Végi, B., Liptói, K., Barna, J. (2013): Methods for cryopreservation of guinea fowl sperm. *Plos One, April 2013 Vol. 8 Issue 4 e62759.*
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- Barna, J., Patakiné Várkonyi, E., Liptói, K., Sztán, N., **Váradi, É.** (2013): Present situation in bio-banking management of indigenous poultry species in Hungary. *International Forum on Avian Germplasm 2013. Seoul, South Korea, October 25-28, 2013. pp.60-62.*
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