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VITRIFICATION OF FISH SPERM AND SPERMATOGONIA

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1. INTRODUCTION AND AIMS

1.1. Introduction

Nowadays the role of gene conservation is growing: many species are classified as endangered, critically endangered or extinct year by year. In parallel, genetic reserves of domesticated species are constantly narrowed as a result of inbreeding. Fish are the most varied and largest group of vertebrates with 32 000 discovered species. According to the information by FAO (Food and Agriculture Organization of the United Nations), more than a thousand species are threatened by overfishing. In order to preserve the diversity of alleles, it is recommended to maintain *ex situ in vitro* gene conservation in parallel to *in situ* conservation. The genetic information can be preserved long-term with cryopreservation of the cells.

Cryopreservation of fish gametes is an important research area in terms of both economic and gene preservation point of views. Cryopreservation of fish sperm facilitates the trade and transport of gametes. Cryopreservation procedures have a significant role during the creation of gene banks, with which endangered species can be saved from extinction, and also plays a key role in preserving transgenic lines. In the animal model zebrafish *(Danio rerio)*, sperm cryopreservation is also an important and developing area. The species used as laboratory animals, such as zebrafish and green swordtail *(Xiphophorus hellerii)* are characterized by small body size, and as a consequence of it, the production of sperm is relatively low.

Cryopreservation of a few microliter volume of liquid with conventional cryopreservation techniques is difficult to implement, vitrification can be a solution to this problem. For most fish species, the

commonly used sperm cryopreservation process is traditional (slow) freezing, altough many researchers have recently started investigating the topic of vitrification of fish sperm. Despite a number of studies conducted over the past three decades, cryopreservation of fish oocytes (eggs) and embryos is not feasible with slow freezing, nor with vitrification. The main reason for this is the relatively large size of egg and embryo, their high cooling sensitivity, and the large amount of yolk into which cryoprotectants are not able to penetrate. A number of research deals with the solution of this problem, including the better understanding and changing of the membrane channels and partially removing of the yolk. More and more researchers state that the cryopreservation of gonadal stem cells can be a substitute solution. Vitrification has been successfully applied in different fish species for cooling of gonadal stem cells. This process represent a breakthrough in genetic conservation of fish, since with cryopreservation of gonadal stem cells (spermatogonia, oogonia) genome of both sexes can be preserved. With the transplantation of primordial primordial stem cells isolated from vitrified zebrafish embryos into a sterile recipient yielded fertile zebrafish which contained the genetic stock of the donor vitrified embryos. Based on these it can be stated that for genetic preservation of fish species (eg. endangered species) gonadal stem cell freezing methods can be developed, thus it is possible to carry out *ex situ in vitro* gene preservation of the species without embryo or oocyte cryopreservation.

1.2. Aims

The aim of my research was to develop species-specific sperm vitrification protocols for the following fish species:

- Salmonids *(Salmonidae)*: grayling *(Thymallus thymallus)*, brown trout *(Salmo trutta m. fario)*, marble trout *(Salmo marmoratus),*
- Cyprinids *(Cyprinidae)*: common carp *(Cyprinus carpio)*, tench *(Tinca tinca),* zebrafish *(Danio rerio),*
- Percids *(Percidae)*: Eurasian perch *(Perca fluviatilis),*
- Eel *(Anguillidae)*: European eel *(Anguilla anguilla)*.

To evaluate the effectiveness of my experiments the following quantitative and qualitative parameters had been examined on vitrified-hawed sperm:

- progressive motility,
- viability (membrane integrity),
- morphometric parameters,
- fertilizing capacity.

In parallel with sperm vitrification studies, spermatogonia (testis tissue) vitrification protocols had been tested in zebrafish and carp. The effectiveness of these protocols was evaluated with tripan blue membrane integrity staining.

2. MATERIAL AND METHOD

2.1. Vitrification of fish sperm

Fish sperm was obtained by stripping. Fish were stripped in anesthesia, according to anesthetic methodology used in practice (eel: 60 mg/l benzocaine, perch and zebrafish: 150 mg L-1 MS-222, other species: 0.4 ml/l 2-phenoxyethanol). In case of carp, tench, perch and eel species sperm production was triggered by hormonal induction. Following stripping, fish were placed into anesthetic-free, oxygenated water for recovery. Sperm was kept on ice until analysis (up to 30 min). Progressive motility of fresh sperm samples was evaluated with computer assisted sperm analysis (CASA) following activation with species-specific media containint BSA (0.01 g/ml). The CASA system uses the Sperm Vision® software which recognizes the motile and static sperm cells. Progressive motility values were used to characterize sperm quality in all CASA measurements before and after vitrification. Sperm was diluted with species-specific isotonic cryomedia, than supplemented with cryoprotectants in different concentrations. The efficiency of vitrification techniques was tested with different devices, such as straw, inoculating loop and Cryotop methods. Devices containing the sperm suspension were plunged directly into liquid nitrogen without pre-cooling in its vapor. Cell suspensions vitrified on Cryotop and inoculating loop were thawed in activating media, straw was thawed in 40°C waterbath for 11 seconds. In parallel with the post-thaw motility values in case of zebrafish and eel sperm membrane integrity values had been studied. In case of species with available female broodstock, fertilization trials were carried out. In case of eel, morphometric studies were performed on the vitrified sperm.

2.2. Vitrification of fish spermatogonia

For the improvement of vitrification protocols for fish gonadal stem cells, zebrafish and carp were chosen as models. Gonads of the fish were surgically removed following overanasthesia in MS-222 solution. In case of zebrafish, gonads had been dissected under light microscope. Dissected gonads were placed into L-15 isotonic solution. Testes pieces had been pinned on acupuncture needles, and needles were placed into two different media (two-step cooling protocol). Numerous cryoprotectants had been tested in different concentrations and combinations. In the first step equilibration solution (E) was used for 5 minutes of incubation, and in the second step vitrification solution (V) was used for 30 seconds. All equilibration media had been tested in combination with all vitrification media. In addition to the intracellular cryoprotectants (methanol, propylene-glycol, dimethyl-sulfoxide), all media had been supplemented with trehalose (0.5 M), foetal bovine serum (FBS, 10%) and Hepes (25mM). Vitrified samples were thawed with a 3-step protocol with decreasing trehalose concentrations (10% FBS + 0.3M / 0.1M / 0M trehalose).

RESULTS

2.3. Vitrification of fish sperm

Sperm vitrification protocols were developed for 8 species belonging to 4 families. The optimal sperm vitrification methods varied among each other and among the species in numerous parameters, such as the cryoprotectant type and concentration, the cryomedia and the dilution ratio (table 1.) The optimal cooling volume was only effective below 10 microliters in all cases, and the most effective was the smallest (2.5 microliters on Cryotop) volume. The optimal cryoprotectant concentration was 30-40% in all cases. According to my results, below 30% total cryoprotectant concentration the creation of ice crystals is not entirely inhibited (in these cases the samples became whitish after plunging into the nitrogen), and above 40% cryoprotectant concentration the toxicity of the cryoprotectants is significant.

1. table: Vitrification protocols designed for different species. Prog. mot.=progressive motility after vitrification and thawing, fert.=fertilization rate, MOH=methanol, PG=propylene-glycol, MTX=methoxyethanol.

Family	Species	Prog. mot.	Fert.	Dilution ratio	Media	Cryo-protectant
Salmonids *(Salmonidae)*	Grayling *(Thymallus thymallus)*	8.75±6.25 %	13.1±11.7 %	1:1	Grayling extender	15% MOH + 15% PG
	Marble trout *(Salmo marmoratus)*	8.6±0.7%	-	1:1	Grayling extender	20% MOH + 20% PG
	Brown trout *(Salmo trutta)*	13.2±5.8 %	-	1:1	Grayling extender	20% MOH + 20% PG
	Zebrafish *(Danio rerio)*	10.8±5.2 %	0.7±0.3%	1:4	HBSS	15% MOH + 15% PG
Pontyfélék *(Cyprinidae)*	Common carp *(Cyprinus carpio)*	7.2±0.6%	-	1:100	Carp seminal plasma + grayling extender	10% MOH + 10% MTX +10% PG
	Tench *(Tinca tinca)*	3.1±0.1%		1:4	Grayling extender	10% MOH + 10% MTX +10% PG
Percids *(Percidae)*	Perch *(Perca fluviatilis)*	14.0±1.6 %	4.9±4.8%	1:5	Tanaka	15% MOH + 15% PG
Eel *(Anguillidae)*	European eel *(Anguilla anguilla)*	10.3±1.7 %	-	1:1	Tanaka + 0.2M trehalose	20% MOH + 20% PG

2.3.1. Salmonids

In case of grayling the post-thaw progressive motility was 8.75 ± 6.25 % (fresh control: 95.5 ± 0.5 %) with the following protocol: 30% cryoprotectant (15% MeOH + 15% PG), 1:1 dilution ratio (sperm:cryomedia), carp seminal plasma extender, vitrified on Cryotop. Trehalose supplementation of the cryomedia significantly decreased the post-thaw motility values. Following fertilization with vitrified grayling sperm, 14.3±12.7% of the embryos reached eyed stage (control: 77.1±9.0%), and 13.1±11.7% hatched (control: 73.9±10.4%). The fertilization parameters (eyed stage, hatching rate) were significantly lower in the experimental group compared to the control group, however, in the vitrified experimental group there was no significant decrease between the percentage of embryos developing to eyed stage and the hatching rate.

Progressive motility of marble trout sperm was 8.6 ± 0.7 % (fresh control: 78.4 ± 23.3 %) following vitrification. The vitrification protocol was as follows: 40% cryoprotectant (20% methanol + 20% propylene-glycol), 1:1 dilution ratio (sperm:cryomedia, w/w), grayling extender, vitrified on Cryotop.

The progressive motility of the brown trout sperm following vitrification was 13.2 ± 5.8% (fresh control: 84.4 ± 9.7 %). The vitrification protocol was as follows: 40% cryoprotectant (20% methanol + 20% propylene-glycol), 1:1 dilution ratio (sperm:cryomedia, w/w), grayling extender, vitrified on Cryotop. Fertilization trial was carried out with the vitridied-thawed sperm (5 Cryotop / 100 eggs), however, none of the

embryos from these test groups developed into eyed stage (control: 37.0 ± 5.2%).

2.3.2. Cyprinids

The progressive motility of the zebrafish sperm following vitrification was 10.8 ± 5.2% (fresh control: 84.5 ± 8 %). The vitrification protocol was as follows: 30% cryoprotectant (15% methanol + 15% propylene-glycol), 1:4 dilution ratio (sperm:cryomedia, w/w), HBSS extender, vitrified on Cryotop. Following this vitrification protocol the ratio of the membrane-intact cells (measured by SYBR/PI staining) was 91.4±2%, this value did not differ significantly from the control (96.0±1.4%). The supplementation of the cryomedia with 0.2 M trehalose had no significant effect on the post-thaw motility rates, however, with 0.4 M trehalose supplementation, the motility of vitrified sperm decreased significantly. The hatch rate of the eggs fertilized with vitrified sperm was 0.7±0.3% (control: 59.8±3%). The hatched embryos were not morphologically different from the individuals of the control group.

The progressive motility of carp sperm following vitrification was 7.2±5.8% (fresh control: : 88.5 ± 8.9%). The vitrification protocol was as follows: 30% cryoprotectant (10% methanol + 10% methyl-glycol + 10% propylene-glycol), 1:100 dilution ratio (sperm:cryomedia, w/w), carp seminal plasma extender supplemented with 0.4 M trehalose, vitrified on Cryotop. With trehalose supplementation in higher or lower concentrations (0.2 M, 0.8 M) had not positive effect on the post-thawing motility ratios.

The progressive motility of tench sperm following vitrification was 1.9 ± 1.2 % (fresh control: 81.0 ± 28 %). The vitrification protocol was as

follows: 30% cryoprotectant (10% methanol + 10% methyl-glycol + 10% propylene-glycol), 1:4 dilution ratio (sperm:cryomedia, w/w), carp seminal palsma supplemented with 0.4 M trehalose, vitrified on Cryotop. With the supplementation of the cryomedia with 0.4 M trehalose, the post-thaw motility was significantly higher than in the groups with lower or higher trehalose concentrations.

2.3.3. Perch

The progressive motility of perch sperm following vitrification was 14 ± 1.6 % (fresh control: 76 ± 17 %). The vitrification protocol was as follows: 30% cryoprotectant (20% methanol + 20% propylene-glycol), 1:5 dilution ratio (sperm:cryomedia, w/w), modified Tanaka extender, vitrified on Cryotop. There was no significant difference between the use of 20% and 30% total cryoprotectant concentration (methanol and propylene-glycol, mixed in 1:1 v/v), however, with 40% cryoprotectant concentration the post-thaw motility decreased significantly. The fertilization ratio with the vitrified sperm was 4.9 ± 4.8% (control: 76.0 ± 41.5%). There was no significant difference among fertilization rates with different numbers of Cryotops used for fertilization (1/6/18 Cryotop/100 eggs).

2.3.4. Eel

Firstly 4 different vitrification methods were investigated with fluorescent membrane staining (SYBR-14/PI). Above 50% cryoprotectant concentration in the cryomedia, the ratio of the membrane-intact cells decreased significantly, thus for further studies 40% cryoprotectant was used as a maximum level. With 30% and 40% total cryoprotectant concentrations the viability of the cells did not decrease significantly compared to the fresh

control. Comparing five different methods, there was no significant difference in the sperm head morphometry parameters (head perimeter and area, n=100/sample) between fresh and vitrified-thawed groups. Based on former studies we can conclude that sperm morphometry parameters correlate with viability, thus the vitrification methods described above are appropriate for European eel sperm vitrification.

Progressive motility of eel sperm following vitrification was 10.3 ± 1.7 % (fresh control: 88.3 ± 2.7 %). The vitrification protocol was as follows: 40% cryoprotectant (20% methanol + 20% propylene-glycol), 1:1 dilution ratio (sperm:cryomedia, w/w), carp seminal palsma supplemented with 0.2 M trehalose, vitrified on Cryotop. With higher and lower trehalose concentrations the motility values were significantly lower.

2.4. Vitrification of fish spermatogonia

All of the tested vitrification methods were effective for preserving zebrafish spermatogonia, however, with different efficiency. The highest viability (58.31 ± 13.79%) was observed with the use of E1 equilibration (1.5 M methanol + 1.5 M propylene-glycol) and V3 vitrification (3 M propylene-glycol + 3 M dimethyl sulfoxide) solutions.

Following carp testicular tissue vitrification the viability of spermatogonia was lower compared to the results of similar experiments in

zebrafish. The highest viability was observed with the use of E3 equlibration solution combined with V2 and V3 vitrification solutions (E3V2: 11.43 ± 5.2%, E3V3: 10.05 ± 4%).

2.5. New scientific results

1. I have proved that Cryotop is an appropriate device for the vitrification of sperm of different fish species.

2. Fort he first time I have developed sperm vitrification methods for the sperm of eight fish species: grayling, brown trout, marble trout, commpon carp, Eurasian perch, European eel. The effectiveness of the methods was proved with fertilization tests in 3 species (zebrafish, Eurasian perch and grayling).
3. I conclude that using the vitrification methods I have described, the morphometric parameters of sperm of European eel do not change significantly.
4. I developed testicular tissue vitrification methods for common carp and zebrafish.

3. CONCLUSIONS AND SUGGESTIONS

3.1. Vitrification of fish sperm

As a result of my experiments, it is possible to vitrify the sperm of a number of fish species for which previously no information was available. In general, it can be stated that from the tested devices (straws, inoculating loops, Cryotops) only two have been found suitable for the vitrification of fish sperm: inoculating loops and Cryotops. Both devices are suitable for fast freezing and thawing, and both can be marked individually, altough with the use of Cryotops the post-thaw motility was significantly higher compared to inoculating loops.

The post-thaw motility of vitrified sperm was lower in case of average motility values following conventional cryopreservation. This tendency has been described by other authors publishing in the topic of fish sperm vitrification, due to the fact the motility of sperm following vitrification is rather vibrating than progressive. The explanation for this phenomenon has not been clarified yet, but presumably the high viscosity of the solution causes the damage. In case of several fish species, successful fertilization tests were carried out with vitrified sperm, which also proves that the genetic material can be stored even with low motility rates.

The efficiency of the sperm vitrification methods is proved also by the eel sperm morphometry analysis, as the head area and perimeter of vitrified-thawed sperm did not decrease significantly compared to the control (fresh sperm) parameters.

The results of fluorescent membrane integrity staining showed that the viability of vitrified zebrafish and eel sperm did not decrease significantly following thawing (compared to the fresh control). Beside this the motolity values and in case of zebrafish the fertilization rates were significantly lower following vitrification compared to the control.

With trehalose supplementation of the cryomedia, post-thaw motility was significantly higher in case of carp, tench and eel sperm vitrification. Beside this, trehalose supplementation had no positive effect in case of zebrafish and grayling sperm vitrification.

Thawing is a crucial point of vitrification, as recrystallization can occur. In my experiments thawing was the most effective when vitrified sperm was directly thawed in activating media, immediately after removal from liquid nitrogen (within 1 second).

3.2. Vitrification of fish spermatogonia

The cryostorage of testicular tissue gives an opportunity to isolate spermatogonia after thawing of the samples, and later these cells can be implanted into sterile recipients. This way endangered can be restored, as females can also be reproduced.

The method of vitrification on acupuncture needle is suitable for this purpose in zebrafish and carp. Needles can be labeled individually and stored in cryotubes or goblets fixed on canes since thawing. After vitrification, spermatogonia had been isolated successfully in zebrafish and carp.

With the vitrification of spermatogonia the genom of both sexes can be stored, thus the cryopreservation of non-freezable fish embryos and eggs can be replaced. In recent years vitrification protocols had been described for gonadal stem cells in numerous fish species. Whole testicular tissue of medaka was succesfully vitrified in 2017. Previously the same research group cryopreserved a whole fish without cryoprotectants, and after thawing gonadal stem cells had been succesfully isolated from the gonads of the fish. The isolated cells had been injected to a sterile recipient salmon, cells integrated and strated their proliferation, thus the recipient fish produced the gametes of the donor fish. With eggs and sperm obtained with this method, donor-derived offsprings were produced, thus it was proven that this method can be successfully applied for gene preservation of endangered species.

3.3. Suggestions

- I suggest the use of Cryotops as cooling devices for fish sperm vitrification, as according to the results of my experiments, this is the most suitable device for fish sperm vitrification.
- I suggest the use of vitrification for zebrafish sperm preservation as due to the small body size of the species, other cryopreservation techniques are not well adaptable for sperm cryopreservation.
- I suggest the method of testis tissue vitrification for zebrafish and carp gene preservation, as the female sex can also be reproduced using this method, thus the cryopreservation of non-freezable fish embryos and eggs can be replaced.

- I suggest the use of trehalose as a cryoprotectant for sperm vitrification of the following species: common carp, tench, European eel as it had a positive effect on the post-thawing motility rates.

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