Detailed report of the project NKFI–K-109847: Vitrification of fish sperm

1. Introduction

We have demonstrated that vitrification is a feasible alternative method for sperm cryopreservation in both marine- or freshwater fish species. Sperm vitrification protocols were developed by our research group for 8 teleost species: the Adriatic form of the European grayling (*Thymallus thymallus,* referred from here onwards as the Adriatic grayling), brown trout (*Salmo trutta m. fario*), marble trout (*Salmo marmoratus*), carp (*Cyprinus carpio*), tench (*Tinca tinca*), zebrafish (*Danio rerio*), Eurasian perch (*Perca fluviatilis*), European eel (*Anguilla anguilla*). In case of three species, we were able to carry out successful fertilization tests with the use of vitrified sperm (zebrafish, Adriatic grayling and Eurasian perch) for the first time.

Ultra-fast cooling or vitrification, which does not require any special equipment or conditions, has attracted increasing interest in the recent years (Vajta and Nagy, 2006). Vitrification is the solidification of a liquid into an amorphous or glassy state which can only be attained at very fast cooling rates (10^6-10^{10} °C/s; (Franks, 1982)). The success of vitrification principally relies on achieving ultra-fast cooling and thawing rates and on the determination of appropriate (usually high) cryoprotectant (CP) concentrations in the cooling media, in order to prevent ice formation during the process. Although high concentrations of CP-s lower the temperature of ice formation, they can be toxic to cells. Consequently, CP concentration has to be reduced and the cooling rate has to be enhanced. For this reason, the material and capacity of the cooling device is very important to achieve fast heat transfer and avoid creation of ice crystals (Tsai et al., 2015).

Recently, several studies have been published on sperm vitrification of different fish species: channel catfish (*Ictalurus punctatus*; (Cuevas-Uribe et al., 2011a)), green swordtail (*Xiphophorus hellerii*; (Cuevas-Uribe et al., 2011b)), rainbow trout (*Onchorynchus mykiss*; (Figueroa et al., 2013; Merino et al., 2012)), Russian sturgeon (*Acipenser gueldenstaedtii*; (Andreev et al., 2009)), spotted seatrout (*Cynoscion nebulosus*), red snapper (*Lutjanus campechanus*), red drum (*Sciaenops ocellatus*; (Cuevas-Uribe et al., 2013)), Atlantic salmon (*Salmo salar*; (Figueroa et al., 2015)) and tambaqui (*Colossoma macropomum*; (Varela Junior et al., 2015)). A potential further application for fish sperm vitrification is the cryopreservation of the spermatozoa of small laboratory model fish species, such as the zebrafish. One male individual of this species can produce approximately 1 microliter of sperm (obtained with stripping), which is ideal for vitrification, contrary to slow freezing in straws.

In general, sperm of marine fish retains a higher quality after cryopreservation compared to that of freshwater species (Drokin et al., 1998; Suquet et al., 2000). Better adaptation of the sperm of marine fish to higher osmotic pressures can explain their survival following exposure to high cryoprotectant concentrations during vitrification (Cuevas-Uribe et al., 2013).

2. Materials and methods

Sperm from all experimental species was collected in anesthesia, by applying gentle abdominal pressure. In case of zebrafish this procedure was carried out below a light-microscope, sperm was collected by glass capillary.

Progressive motility of fresh sperm samples was evaluated with computer assisted sperm analysis (CASA). Live-dead fluorescent staining using SYBR Green and Propidium Iodide was used to determine membrane integrity of vitrified spermatozoa in the zebrafish.

Following dilution with an extender and cryoprotectants, sperm was applied to the vitrification device and immediately plunged into liquid nitrogen. For vitrification, several devices had been tested, however, only Cryotops and inoculating loops resulted in motile spermatozoa following vitrification. With 0.25 ml straws the cells were not motile in any of the tested species or protocols. We have also tested the microdrop method, however the handling during thawing was a problem in this case. Due to the slow thawing process, this method resulted in devitrification and re-crystallization of fish sperm.

3. Results

3.1. Vitrification of fish sperm in presence of cryoprotectants

It has previously been suggested that vitrification can only be attained by the presence of cryoprotectants in very high concentrations. However, in recent decades several published protocols demonstrated that vitrification can be achieved with the application of lower cryoprotectant concentrations, using a combination of alcohols, which can successfully inhibit ice crystal formation.

Successful sperm vitrification protocols were developed for 8 fish species (table 1.).

3.1.1. Adriatic grayling

The progressive motility of the Adriatic grayling sperm following vitrification was 8.75 ± 6.25 % (fresh control: 95.5 ± 0.5 %). The vitrification protocol was as follows: 30% cryoprotectant (15% methanol + 15% propylene-glycol), 1:1 dilution ratio (sperm:cryomedia, w/w), grayling seminal plasm extender, vitrified on Cryotop. 14.3±12.7% of the fertilized eggs reached the eyed stage (control: 77.1±9%), while the hatching rate of the eggs fertilized with vitrified sperm was 13.1±11.7% (control: 73.9±10.4%). There was no significant difference between fertilization rates at the eyed stage and hatching rates compared in vitrified or control groups. The values of the control groups were significantly higher than the experimental groups. Trehalose supplementation has not enhanced the progressive motility values measured following vitrification and thawing in case of grayling sperm (7.5±6.5%, control: 92.4±3%).

3.1.2. Marble trout

The progressive motility of the marble trout sperm following vitrification was 13.2 ± 5.8 % (fresh control: 97 ± 0.6 %). 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.

3.1.3. Brown trout

The progressive motility of the brown trout sperm following vitrification was 8.6 ± 0.7% (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.

3.1.4. Zebrafish

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 membrane-intact cells (measured by Sybr/PI staining) was 91.4±2%, this value did not differ significantly from the control (96±1.4%). As a result of the fertilization the hatching rate of the eggs fertilized with vitrified sperm was 0.7±0.3% (control: 59.8±3%). The hatched embryos were not different morphologically from the individuals of the control group.

1. Table. Summary of the efficient sperm vitrification protocols in case of the investigated fish species. Prog. mot. = Progressive motility, MeOH=methanol, PG=propylene-glycol, MG=methyl-glycol

Family	Species	Results	Cooling device	Dilution ratio (w/w)	Cryo-media	Cryo-protectant
Salmonids *(Salmonidae)*	Adriatic grayling *(Thymallus thymallus)*	8.75±6.25% prog. mot, 13.1±11.7% fertilization (hatching)	Cryotop	1:1	Grayling extender	15% MeOH + 15% PG
	Marble trout *(Salmo marmoratus)*	8.6±0.7% prog. mot	Cryotop	1:1	Grayling extender	20% MeOH + 20% PG
	Brown trout *(Salmo trutta)*	13.2±5.8% prog. mot	Cryotop	1:1	Grayling extender	20% MeOH + 20% PG
Cyprinids *(Cyprinidae)*	Zebrafish *(Danio rerio)*	10.8±5.2% prog. mot., 0.7±0.3% fertilization (hatching)	Cryotop	1:4	HBSS	15% MeOH + 15% PG
	Carp *(Cyprinus carpio)*	7.2±5.8% prog. mot.	Cryotop	1:100	Carp seminal plasma + 0.5M trehalose	10% MeOH + 10% MG +10% PG
	Tench *(Tinca tinca)*	1.6±1.2% prog. mot.	Cryotop	1:4	Grayling extender	10% MeOH + 10% MG +10% PG
Percids *(Percidae)*	Eurasian perch *(Perca fluviatilis)*	14±1.6% prog. mot., 4.9±4.8% fertilization	Cryotop	1:5	Tanaka	15% MeOH + 15% PG
Eels *(Anguillidae)*	European eel *(Anguilla anguilla)*	10.3±1.7% prog. mot.	Cryotop	1:1	Tanaka + 0.2M trehalose	20% MeOH + 20% PG

3.1.5. Common carp

The progressive motility of carp sperm following vitrification was 1.9 ± 0.4 % (fresh control: 87.6 ± 7.7 %). 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 0.5M trehalose, vitrified on Cryotop.

3.1.6. Tench

The progressive motility of tench sperm following vitrification was 1.9 ± 1.2 % (fresh control: 81 ± 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 0.5M trehalose, vitrified on Cryotop.

3.1.7. Eurasian 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. The fertilization ratio with the vitrified sperm was 4.9 ± 4.8% (control: 76 ± 41.5%).

3.1.8. European eel

The 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 0.2M trehalose, vitrified on Cryotop.

3.2. Cryoprotectant-free vitrification of fish sperm

Vitrification experiments in the absence of intracellular cryoprotectants have not resulted in motile spermatozoa in any species. Previous studies reported successful cryoprotectant-free vitrification in rainbow trout (Merino et al., 2012, 2011), however, with the protocol described by the authors, we were not able to obtain similar results. In fact, all attempts to recreate the original protocols invariably resulted in the crystallization of sperm samples immediately upon immersion into liquid nitrogen.

3.3. Effect of hydrostatic pressure on the survival of spermatozoa following vitrification

The positive effect of hydrostatic pressure on the cryosurvival of spermatozoa had been demonstrated in mammalian species (Pribenszky et al., 2011). Similar methods were attempted to be developed in order to increase the success of fish sperm vitrification, however, only straws allowed successful protection to spermatozoa from exposure and subsequent activation by water during the application of hydrostatic shock. Experiments were carried out with straws placed into the pressure chamber with zebrafish sperm, however the motility following vitrification was similarly zero in the pressure-treated and the control group. Vitrification of fish sperm was only feasible with devices for small volumes of sperm, such as Cryotops and inoculating loops, which has a capacity lower than 10 microliters.

4. Discussion

Our results demonstrate that for fish sperm vitrification, devices for low volumes of sperm (in the range of microliters) are needed. The efficiency of vitrification with inoculating loops was less successful compared to Cryotops, and the use of 250 µl straws was not suitable for vitrification in any of our experimental species. In our experiments, the equilibration time was below 1 minute in every case, and for the most successful protocols 2 or 3 different cryoprotectants were used. According to our results, cryoprotectant concentrations above 40% are too harmful to the cells, while cryoprotectant concentrations below 30% do not inhibit the formation of ice crystals entirely.

Generally, we have demonstrated that vitrification is a feasible alternative sperm cryopreservation method in both marine- or freshwater fish species. The motility rates in case of all investigated species were low compared to the average motility rates following conventional cryopreservation, however, these motility values are similar to the results reported by other authors (Cuevas-Uribe et al., 2011a, 2011b; Figueroa et al., 2013). It is a general observation that the sperm in vitrification solutions has a tendency toward local motility (vibration) rather than progressive motility (Cuevas-Uribe et al., 2011b). The reason of this vibration is not clear, it could be caused by cellular damage or the high viscosity of the solution. Nevertheless, fertilization of eggs with vitrified sperm resulted in developing embryos in case of Eurasian perch, zebrafish and grayling, thus vitrified sperm with low progressive motility rates are able to preserve their genetic material.

In addition to the motility results, the feasibility of the vitrification of eel sperm was evidenced through the absence of decreasing in head area and perimeter of vitrified European eel spermatozoa when compared to fresh spermatozoa. In this species, several studies concentrated on the head morphometry of spermatozoa (Asturiano et al., 2006; Marco-Jiménez et al., 2006) and the morphological damage caused by cryopreservation protocols (Peñaranda et al., 2009). As a consequence of osmotic stress, significant morphometric alterations could be observed in eel spermatozoa (Asturiano et al., 2007). The head regions of dead spermatozoa suffer a greater decrease than that of living cells(Peñaranda et al., 2009), thus our vitrification protocols were feasible for eel sperm. In this experiment, total CP concentrations below 40% were tested, based on the results of our preliminary experiments showing that membrane integrity (viability) of European eel spermatozoa decreases significantly when 50% total CP is used for vitrification. Contrarily, after thawing the samples vitrified with 30% and 40% of total CP, no significant decrease was observed in viability parameters.

Our results demonstrate that for fish sperm vitrification, devices for low volumes of sperm (in the range of microliters) are needed. The efficiency of vitrification with inoculating loops was less successful compared to Cryotops, and the use of 250 µl straws was not suitable for vitrification in any of our experimental species. When the thickness of the solution layer in these devices is around 0.07 cm, the calculated cooling rate of approximately 720,000 K/min (Isachenko et al., 2003) is fast enough to prevent ice formation.

Cryoprotectants used at concentrations high enough for successful vitrification have a toxic and hypertonic effect on spermatozoa (Yavin & Arav, 2007). Fish spermatozoa can tolerate high CP concentrations when the proportion of the chemicals is appropriate (Cuevas-Uribe et al., 2011a, 2011b), and it is possible to decrease the toxicity by combination of at least two different CPs and reducing the equilibration time to the minimum (below 1 minute). In combination, the effectiveness for vitrifying the solution is higher and the toxicity is lower than the use of a single CP (Ali and Shelton, 2007). In our experiments, the equilibration

time was below 1 minute in every case, and for the most feasible protocols 2 or 3 different CPs were used. According to our results, CP concentrations above 40% are too harmful for the cells, while CP concentrations below 30% do not inhibit the formation of ice crystals entirely.

The potential osmoprotectant role of trehalose was not observed in case of zebrafish or grayling. Contrary, according to our results, with trehalose supplementation the progressive motility following vitrification could be enhanced in case of carp, tench and European eel. Thus we can conclude that the reaction of the sperm cells to the trehalose has high species-specificity. The reason of this phenomenon could be the different tolerance of the species for the changing of the osmotic parameters of the environment.

Another crucial point of vitrification is the recrystallization occurring during thawing. In our experiments, samples were thawed directly into the activating solution without paying attention to its temperature. A previous study on vitrified green swordtail sperm showed that there were no significant differences between thawing in solutions at room temperature (24 °C) or 37 °C, therefore both methods were fast enough to prevent ice formation (devitrification or recrystallization) during thawing (Cuevas-Uribe et al., 2011b). However, another study on vitrification of mouse oocytes described that survival could be enhanced using laser-induced ultra-rapid thawing (Jin et al., 2014).

Regarding the findings in this project, one must be critical not to overemphasize the importance of vitrificaton in fish sperm cryopreservation. Vitrification will not replace conventional freezing as far as *en masse* storage of fish sperm is concerned. Thus, for most aquaculture applications cryopreservation by means of freezing will remain the most convenient and obvious method. Vitrification, however, can contribute to the conservation of genetic resources of wild or cultured fish species when minute volumes need to be stored for later use such as the case of zebrafish. It can also improve conservation protocols in species with fragmented populations that have a very low effective population size and where even minimal volumes of sperm count (such as the case of the marble trout in Slovenia).

5. Conclusions

In summary, the present study demonstrated that vitrification is a feasible alternative sperm cryopreservation method in either marine- or freshwater species. Successful sperm vitrification of 8 species was conducted for the first time. Motile spermatozoa were recovered following vitrification in case of 8 species, and fertilization of eggs with vitrified sperm resulted in developing embryos in case of 3 species.

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