Detailed scientific report of the project OTKA K129127

Introduction

Cryopreservation of sperm has long been recognized as an effective tool in a variety of applications including assisted reproduction, livestock farming and conservation of genetic resources of both farmed and wild species. Sperm cryopreservation protocols have also been developed for marine and freshwater fish species. In spite of efforts to develop these protocols, very few have been applied to actual aquaculture practices. This is due to several factors, however, one of the most problematic of these is high variability of results as far as post-thaw sperm survival is concerned. Variation can be attributed to extrinsic problems (such as the lack of methodical standardization) but also to intrinsic ones such as sperm selection during the process of freezing and thawing.

An earlier study by Babiak et al. (2002) has shown that the sperm of rainbow trout individuals hatched from eggs fertilized with cryopreserved sperm had higher post-thaw fertilizing capacity (in other words had better cryoresistance) than that of fish hatched from eggs fertilized with fresh sperm. This means that during the process of cryopreservation spermatozoa undergo a process that causes changes in their external or internal structure and allow a selection process to take place that favors higher cryosurvival even in the progeny.

Thus, the objectives of the current project were to investigate cell selection during cryopreservation of fish sperm as well as the possibility of inherited cryoresistance by investigating whole genome methylation pattern of the sperm and embryos through several generations. We intended to achieve this by breeding several generations of full-sib families of zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*) originating from fertilization with either fresh or cryopreserved sperm. Changes through generations were intended to be monitored using sperm quality parameters (post-thaw motility, fertilizing capacity) as well as using molecular methods such as whole-genome bisulfite sequencing (WGBS) in investigate the potential epigenetic alterations caused by cryopreservation. In addition to this, development and standardization of existing cryopreservation methods was a secondary objective of our research as this contributes to the reduction of variation in cryopreservation outcomes.

Materials and Methods

Fish treatment and spawning

Common carp individuals were kept in a recirculating fish housing and breeding system (Sentimento Kft., Érd, Hungary) of the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences (Gödöllő, Hungary). Scaly and mirror carps (P: N = 66, age: 4+, body weight: 817-3000 g, F1: N=46 hatched from fertilization with cryopreserved sperm, N = 63 hatched from fertilization with fresh sperm, age: 2+, body weight: 100-520 g; F2: N = 63 hatched from fertilization with cryopreserved sperm, N = 68 hatched from fertilization with fresh sperm, age: 1+, weight: 28-640 g) were used for the experiments.

Fish were raised in 10-liter plastic tanks until the age of 3-4 months and moved and kept in a 3-m^3 plastic tank in cages, with constant water quality parameters (23 ± 2 °C, pH 7.0 ± 0.2, redox potential; 230 ± 2 mV, dissolved O2-level; 4 ± 1 mgL^{-1}). Carps were fed once a day with 10 g per kg body weight of Aqua Uni (Aqua Garant, Pöchlarn, Austria) pelleted feed.

The fingerligs were fed in the first month with artemia (*Artemia salina* nauplii; INVE Aquaculture NV, Belgium) 8 times a day. In the 2nd month the fingerlings were also fed with zebrafish food (> 100 μm – 600 μm < ; ZEBRAFEED®, Sparos Lda, Portugal). From the 3rd month artemia was not involved in the feeding and from the 4th month they were fed with larger food (1.00–4.5 mm; Aqua start; Aqua Uni, Aqua Garant, Pöchlarn, Austria). At the age of half year the fish were tagged abdominally (Agrident GmbH, Barsinghausen, Germany) and released in the system.

The zebrafish were kept in 3- and 8-L polycarbonate tanks at 25 ± 2 °C (pH 7.0 ± 0.2; conductivity 525 ± 50 μS; alkalinity: 0 mM OH^-, 0 mM CO_3^{2-}, 0.4 mM HCO_3^{2-}; hardness: < 0.5 odH; DOC: > 90%; from here onwards referred to as system water) in a recirculating zebrafish housing system (ZebTEC® (Tecniplast, Italy)) at the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences (Gödöllő, Hungary). The photoperiod was 14-h light and 10-h dark.

Fish were fed twice a day with commercial zebrafish feed (ZEBRAFEED® diet (Sparos Lda, Portugal) and with live artemia (*Artemia salina* nauplii) (TQ type; INVE Aquaculture NV, Belgium) every other day. The fingerlings were kept in incubator (25 ± 2 °C) until the age of 1.5 weeks and were fed once a day with artemia before the water changing.

Carps were induced hormonally for spermiation and ovulation. All treatments of fish were carried out in anesthesia using a solution of 0.04% 2-phenoxyethanol (Reanal, Hungary). After that male fish were placed on a wet towel and injected intraperitoneally with 1 pellet per kg body weight of Ovopel (Interfish Kft., Hungary) containing 12-20 μg per pellet of synthetic GnRH analogue (D-Ala6, Pro9Net-mGnRH) and 8-10 mg per pellet of dopamin receptor antagonist metoclopramide 48 h before the experiments. Females were aministered 10% of the total dose 24 hours before the planned ovulation and 12 hours later, the other 90%. The zebrafish were anesthetized with tricaine methane sulfonate (MS-222, Arlos Organics™ , Geel, Belgium, 168 mg/l). In case of zebrafish no hormonal treatment was used.

Formation of full-sib families and subsequent generations of fish

The *P* generation arrived in 2016 at the age of 1 year from Dinnyési Halgazdaság Kft. and Jászkiséri Halas Kft. to the recirculation system of the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences. From the 41 males 9 were randomly chosen. Fresh and thawed motility parameters of these fish were examined for 3 months 6 times with CASA. Between each spawning the fish rested for a minimum of 1 week. These tests were conducted in order to find the individuals with the lowest post-thaw progressive motility and the ones with more than 40% of post-thaw progressive motility. There was no significant difference found among the sperm motility parameters of the afore mentioned fish, thus 6 individuals was randomly chosen from the 9 for creating the *F1* generation.

From 74 females considering size and health one was randomly chosen. The female was injected with Ovopel 24 hours before fertilization. For fertilization, eggs were divided into 12 batches of 10 g, each. The batches were fertilized with 50 μl of fresh sperm from the males and 500 μl of cryopreserved sperm (due to the 1:9 dilution ratio) from the same males creating 12 groups.

Eggs were incubated in 10-L polycarbonate tanks in a recirculating system at 23 ± 2 °C. Motility parameters of the sperm were measured with CASA prior to cryopreservation. Sperm and subsequently 100 µl of system water were added to the eggs and mixed for 1 minute. After 1 minute Woynárovich solution (10 L system water, 40 g NaCl, 30 g urea) was added to eliminate the adhesiveness of eggs. More Woynárovich solution was added while mixing for approximately 1.5 hours during the process of egg swelling. After that the eggs were washed three times in 5% tannic acid for 10 s and incubated at 22-24 °C in 10 l tanks in a recirculating system. The fingerlings hatched in 3-5 days. In the first 3 days methylene blue was added to the water to avoid infections. After hatching, the egg shells were removed from the tanks. At the age of 3-4 months the fish were put to 3 m^3 polycarbonate tanks.

In order to create the *F2* generation, one male from 4 *F1* families and a single female were selected. All treatments were conducted identically to that of the *F1* generation. The eggs were divided into 8 batches of 10 g each for fertilization. The batches were fertilized with 50 μl of fresh sperm from *F1*-f males (hatched from fertilization with fresh sperm) and 500 μl of cryopreserved sperm (due to the 1:9 dilution ratio) from *F1*-c males (hatched from fertilization with cryopreserved sperm) creating 8 groups.

In zebrafish, the sperm of 50 males were measured with CASA every 2 weeks 3 times. 6 individuals were chosen whose sperm had progressive motility above 80%. 6 females were randomly chosen also for creating the *F1* generation. The 6 females' batches of eggs were individually separated in two and fertilized with the 6 males' thawed and fresh spem. 12 full-sib groups were created. 6 groups were hatched from fertilization with cryopreserved and 6 from fresh sperm. 3 males were chosen from each group for creating the *F2* generation in zebrafish.

To this end, sperm of the 3 *F1* individuals from each group were pooled. 6 females were randomly chosen and the eggs were also pooled and divided in 6. Half of the batches of eggs were fertilised with cryopreserved sperm from the ones hatched from fertilization with cryopreserved sperm and another half with the fresh sperm from males hatched from fertilization with cryopreserved sperm creating 12 groups. The pooling was necessary because the quantity of sperm and eggs was not enough for creating the next generation.

In order to create the *F3* generation of zebrafish, 3 *F2* males were chosen from each group. The sperm of the 3 individuals from each group were pooled. 6 females were randomly chosen for each male groups (fresh and cryopreserved full-sibs) and their eggs were also pooled. Half of the batches of eggs were fertilised with cryopreserved and another half with fresh sperm of the males hatched from fertilization with fresh and cryopreserved sperm of the males hatched from fertilization with cryopreserved sperm creating 12 groups.

Sperm quality and analysis and cryopreservation

Carp sperm was collected 24 hours after the hormonal injection, in anesthesia. Fresh and thawed sperm motility parameters were measured with CASA. The sperm was cryopreserved as follows. Samples were diluted at a ratio of 1:9 in an isotonic extender (200 mM glucose, 40 mM KCl, 30 mM Tris, pH: 8.0 ± 0.2) and methanol (10%, v/v final concentration). Diluted samples were loaded into 0.5 ml plastic straws (Minitube GmbH, Tiefenbach, Germany). Samples were cryopreserved in the vapor of liquid nitrogen 3 cm above its surface for 3 minutes. After cryopreservation, samples were plunged into liquid nitrogen. Samples were thawed for 13 sec at 40 ˚C (Thermo Haake P5, Thermo Electron Corp, Waltham, Massachusetts, USA).

Zebrafish were anesthetized with tricaine methane sulfonate (MS222 Arlos Organics™, Geel, Belgium, 168 mg/l). After anesthesia, sperm was collected by stripping into 10 μl glass capillaries by abdominal massage of the individuals using a slide forceps. Fresh and thawed sperm motility parameters were measured with CASA. The cryopreservation followed Caetano et al.'s (2019) protocol. Sperm was diluted with an extender (200 mM glucose, 40 mM KCl, 30 mM Tris, pH: 8.0 ± 0.2) and methanol was added to reach a final concentration of 8%. Samples were loaded into 0.25 ml straws. Programable freezer was used for cryopreservation (IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria) at cooling rate 10 °C/min. Samples were stored in liquid nitrogen and before use were thawed at 40 °C for 5 sec.

Whole-genome bisulfite sequencing of zebrafish gametes and embryos

In order to analyze the potential inheritance of a 'cryoresistance' phenotype, we have suggested studying the DNA methylation pattern in both fresh and cryopreserved sperm, oocytes, as well its transferability to the offspring. Even though we found that phenotypically 'cryoresistance' does not exist in zebrafish and common carp, we have decided to examine whether sperm cryopreservation induced changes in the global DNA methylome, and whether these changes (if they exist) can be transferred to the offspring. We have chosen to study this on zebrafish as several studies have confirmed that in this species offspring inherits the sperm methylome, contrary to what happens in mammals. However, the major challenge in testing this was the very small number of cells (both sperm and eggs) obtained from zebrafish, as most DNA methylome protocols necessitate large starting quantities of DNA. To overcome this challenge, we needed to find a way to isolate as much DNA as possible from the samples, but also to find an alternative approach to study the methylome. To isolate DNA, we have tried several commercial kits based on different technologies: salting out, column-based or magnetic bead-based kits. We observed that using magnetic bead-based kits yielded the highest amount of high-quality DNA. As for library preparation, we decided to use the post-bisulfite adaptor tagging (PBAT) approach which enables library preparation from very low quantities of starting DNA. PBAT methodology was efficient in library preparation, especially from egg samples where the starting material ranged from 30–50 eggs dominated by RNA. However, as we used a custom library preparation methodology which does not rely on predetermined library preparation kits, the subsequent problem was found in the sequencing itself, and establishing the sequencing procedure with the chosen sequencing company.

Assessment of carp sperm concentration and adjustment of concentration before cryopreservation

For further investigations in the common carp, a method was needed with which sperm concentration can be measured easily and fastly. For measuring concentration, a CASA system (Computer-assisted sperm analysis, Minitüb GmbH AndroVison) and a microplate reader (Thermo Scientific Varioskan Lux) were tested and compared with the Bürker-Türk type hemocytometric results.

For microplate reader analyses, semen was collected from 9 individuals. Firstly, 10 or 5 μl of each sperm sample was diluted in 990 or 995 μl extender (100- and 200-fold dilution; 200 mM glucose, 40 mM KCl, 30 mM Tris, pH: 8.0±0.2). Subsequently, 100 μl from the pre-diluted sample was diluted further in 900 μL extender (1000- and 2000-fold dilution) and spermatozoa were counted using a Bürker-Türk type hemocytometer (96-well plate, 200 μl/well) and absorbance was also measured at 505 nm by a

spectrophotometer (Hermo Scientific Varioskan LUX multimode microplate reader, SkanIt RE v.5.0). Each (1000- and 2000-fold dilution) dilution was repeated 3 times.

For concentration determination using CASA, samples were collected from 12 individuals. Computer-assisted sperm analysis (CASA) was conducted with an AndroVision (Minitüb, Tiefenbach, Germany) sytem equipped with a Motic BA310 microscope with a 20× negative phase contrast objective. The samples were diluted in extender to reach a 100× dilution ratio. Aliquots of 3 μl of diluted sperm were pipetted into a Makler-type cell counting chamber, covered with a dedicated coverglass and placed under the microscope. CASA was run on the immotile samples to determine cell concentration. Only cell concentration data were retained at this point. For measurement with Bürker-Türk type hemocytometer the dilution rate was 1000×. Each dilution was repeated 3 times.

In order to adjust sperm concentration prior to cryopreservation, sperm of 5 males was diluted in extender containing 10% methanol (v:v, final concentration) to reach a final concentration of 0.5; 1; 2; 4 × 10^9 spermatozoa per ml (3 straws per dilution) which resulted in dilution ratios of 25–43-fold for 0.5 × 10^9, 12–21-fold for 1 × 10^9, 6–11-fold for 2 × 10^9 and 3–5-fold for 4 × 10^9 spermatozoa per ml. The concentrations of samples were measured by the previously used CASA system. The standard, 1:9 (or else 10-fold) dilution ratio (sperm:extender) served as a positive control which is this case corresponded to a concentration of 1.2–2.1 × 10^9 spermatozoa per ml. The samples were loaded into 0.5 mL straws and frozen in a polystyrene box using polystyrene frames 3 cm above the surface in the vapor of liquid nitrogen for 3 minutes. Following freezing, the samples were plunged into liquid nitrogen. After storage, the straws were thawed in a 40 °C water bath for 13 s.

Results

Sperm quality and fertilizing capacity in the subsequent generations

During the establishment of the *F1* generation in the common carp, fertilization rate of fresh and cryopreserved sperm of *P* males was 67 ± 12% and 64 ± 6%, respectively. No significant difference was found between the mean fertilization rates ($p = 0.2925$).

In the *F1* generation, both the sampling date ($p < 0.001$) and the origin of males (fresh or cryopreserved sperm) had a significant effect ($p = 0.024$, $N = 46$ for cryopreserved, $N = 63$ for fresh) on the progressive motility of cryopreserved carp sperm, although, the family of fish had no effects on the results. The origin of the males did not affect other motility parameters (VCL, VAP, VSL, STR or LIN). No significant difference ($p = 0.86$) was found between the fertilizing capacity of cryopreserved (87 ± 5%) and fresh sperm (86 ± 13%) of *F1* males used to establish the *F2* generation.

In the second generation of carps no significant difference ($p > 0.05$) was found between the fertilizing capacity of thawed sperm in the two groups. On the progressive motility of cryopreserved carp sperm, neither the sampling date nor the origin of males (fresh or cryopreserved sperm) had a significant affect. The origin of the males did not affect other motility parameters (VCL, VAP, VSL, STR or LIN), either. The density of the sperm was not affected by its origin. However the concentration was higher in the group originated from cryopreserved sperm (1 × 10^{10} ± 2.5 × 10^9 spermatozoa/ml) than the group originated from fresh sperm (7 × 10^9 ± 3 × 10^9 spermatozoa/ ml).

In the zebrafish, there was no significant difference found ($p = 0.9$) between the fresh motility of the sperm of the *F1* groups originated from fresh (80 ± 11%) and cryopreserved (80 ± 14%) sperm. The progressive motility of fresh and thawed sperm was significantly different ($p < 0.001$) as expected. The progressive motility of thawed sperm was not significantly different ($p = 0.73$) between the cryopreserved (25 ± 8%) and the fresh (21 ± 12%) groups, nor were other motility parameters (VCL, VAP, VSL, STR and LIN). The concentration measurement with Bürker-Türk type hemocytometer showed no significant difference ($p = 0.56$) between the males originated from cryopreserved (5 ± 4 × 10^8 spermatozoa/ ml) and fresh sperm (4 ± 3 × 10^8 spermatozoa/ ml). For fertilization, 5000:1 sperm and egg ratio was used. As a control fresh sperm was utilized. The fertilization rate showed no significant difference ($p = 0.73$) between the fresh (1 ± 3%) and the cryopreserved (1 ± 1%) groups, however the fertilization rate was very low.

The *F2* generation was tested as the first except for the fertilization rate. Measuring with CASA, fresh motility rates were not significantly different ($p = 0.281$) between the ones hatched from fertilization with fresh (67 ± 12%) and with cryopreserved sperm (70 ± 9%). The progressive motility of the fresh and thawed

sperm was significantly different ($p < 0.001$) as in the first generation. In case of progressive motility of thawed sperm, there was no significant difference ($p = 0.54$) between the males originated from cryopreserved (17 ± 9%) and the ones originated from fresh sperm (21 ± 3%). The origin of the males did not affect other motility parameters (VCL, VAP, VSL, STR or LIN), either. In the concentration of the sperm there was no significant difference ($p = 0.073$) between the fish hatched from fertilization with cryopreserved ($1 \pm 2 \times 10^9$ spermatozoa/ ml) and fresh sperm ($2 \pm 1 \times 10^9$ spermatozoa/ ml).

In the *F3* generation, progressive motility of fresh sperm was not significantly different ($p > 0.05$) between the two groups. Neither was there a significant difference ($p = 0.781$) in the progressive motility of the thawed sperm between the males originated from cryopreserved (30 ± 16%) and from fresh sperm (15 ± 2%). The origin of the males did not affect other motility parameters (VCL, VAP, VSL, STR or LIN). There was no significant difference ($p = 0.44$) in sperm density, measuring the males hatched from fertilization with cryopreserved ($2 \pm 1 \times 10^9$ spermatozoa/ ml) and from fresh sperm (2×10^9 spermatozoa/ ml). The fertilization rate was not statistically different ($p > 0.05$) between the fish hatched from fertilization with cryopreserved (5 ± 7%) and from fresh (5 ± 9%) sperm. There was no significant difference between any of the sperm parameters in any of the generations in zebrafish.

Assessment of carp sperm concentration and adjustment of concentration before cryopreservation

Sperm concentration of 9 common carp males was $1.849 \times 10^{10} \pm 3.853 \times 10^9$ spermatozoa per ml. A significant positive linear relationship was detected between absorbance measured in the microplate reader and sperm concentration assessed using a hemocytometer ($p < 0.0001$, $r^2 = 0.8289$) resulting in the equation $y = 1.720 \times 10^{11}x + 3.851 \times 10^9$.

Sperm concentration of 12 common carp males measured with CASA was $1.853 \times 10^{10} \pm 7.854 \times 10^9$ spermatozoa per ml while that measured with a hemocytometer was $1.442 \times 10^{10} \pm 6.212 \times 10^9$ spermatozoa per ml. The concentration values measured with CASA were in a significant linear relationship ($^p < 0.0001$, $r^2 = 0.8559$, $y = 0.7317x + 8.555 \times 10^8$) with sperm concentration counted in a hemocytometer. For further studies, CASA was used to determine the concentration of sperm.

No significant main effect of sperm concentration was found on any of the parameters measured by CASA. The only exception was LIN ($p = 0.0112$) where the post-hoc test found a significant difference ($p = 0.0056$) between linearity value for the sperm concentration of 0.5×10^9 spermatozoa ml^{-1} (0.86 ± 0.03) and that for the dilution ratio of 1:9 (0.74 ± 0.08).

A significant main effect ($p = 0.0156$) of cell concentration on the fertilizing capacity of cryopreserved common carp sperm was found. The post-hoc test detected a significant difference ($p = 0.0121$), between the fertilization percentage of batches fertilized with sperm frozen at a cell concentration of 4×10^9 spermatozoa ml^{-1} (66 ± 6%) and the positive control (sperm diluted at a ratio of 1:9, 49 ± 5%). The control fertilization rate was 95±5% confirming satisfactory egg quality.

Deviations from the original research plan

In the original research plan, we were planning to create 4 or 5 subsequent generations of zebrafish. This was not feasible due to the methodical inadequacies of zebrafish cryopreservation protocols used in our laboratory. These resulted in very poor fertilization and hatch rates (see Results) and in a very low number of fish produced that was insufficient to produce further generations. Thus, the experiment was terminated with the *F3* generation of zebrafish.

In the original research plan, we planned to analyze the effect of cryopreservation on the methylation pattern of DNA using whole-genome bisulfite sequencing (WGBS) in both species. In the end, we decided to conduct this analysis only in zebrafish. Although the genome of the common carp has been described, the level of annotation is far lower than in the more commonly used zebrafish model.

Finally, due to time constraints and the lengthy public procurement procedures the results of WGBS are not included in this report. The sequencing process is being conducted as this report is written, thus, the results are expected to arrive within a month of the final conclusion of the project.

Discussion

In this project, we have found no conclusive evidence of inherited cryoresistance of zebrafish or common carp sperm. In general, the sperm of fish originating from fertilization with cryopreserved sperm did not

display higher post-thaw motility or fertilizing capacity than that of fish originating from fertilization with fresh sperm. This was evident even after breeding full-sib families of the same parent individuals for several generations. Although, a significant effect of the origin of fish (fresh or cryopreserved sperm) was observed in the *F1* generation of common carp on the post-thaw motility of sperm, this difference was not present in the *F2* generation. Thus, we cannot confirm the observation by Babiak et al (2002) in the rainbow trout using the models employed in this project.

Although the results of the WGBS are not available, yet, it can safely be predicted that some changes can be attributed to cryopreservation in the methylation pattern of sperm or the embryo. For instance, sperm aging has affected the methylation level at cytosine-phosphate-guanine sites in common carp spermatozoa as demonstrated by WGBS (Cheng et al., 2021). It is, however, difficult to ascertain that these changes in the methylome are indeed meaningful and result in any adverse or favorable alterations in the embryo and the progeny.

The lack of evidence of inherited cryoresistance, however, can also be attributed to the low resolution of methods and protocols currently employed in fish sperm cryopreservation. This is clearly evidenced in the case of zebrafish where the use of cryopreserved sperm resulted in very low fertilization. This problem has been recognized by the scientific community working on the cryopreservation of fish sperm. In order to improve the precision of currently used methods, steps have been made for the standardization of cryopreservation protocols. A typical example is the concentration of sperm per unit of cryopreservation which is common practice in dairy bull sperm freezing, however, in fish this is a relatively novel idea as for convenience dilution ratios were employed. Several studies conducted in salmonids demonstrated the advantages of standardizing spermatozoa concentration in fish sperm prior to cryopreservation (Nynca et al., 2017; Judycka et al., 2018). Thus, an additional objective of our research was to establish a quick and reliable method based either on absorbance measurement or CASA readings for the determination of sperm concentration as well as to test if sperm concentration in the common carp is a determining factor in cryopreservation success. We have established that CASA is a reliable method for the determination of sperm concentration in the common carp, however, the CASA software needs to be run on immotile samples in order to have accurate results. Also we have determined that sperm concentration does not offer a meaningful advantage over dilution ratios in the common carp. Pre-set sperm concentrations resulted in higher fertilizing capacity of cryopreserved sperm than 10-fold dilution of sperm, however, this difference was evident only in the highest investigated concentration.

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