Szent István University

INVESTIGATION OF DAMAGES ATTRIBUTED TO CRYOPRESERVATION IN THE SPERMATOZOA OF 3 FISH SPECIES

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1. SCIENTIFIC BACKGROUND, OBJECTIVES

Freshwater fish farming has centuries-old traditions in Hungary due to its favorable geographical and climatic conditions. Ecological conditions of the country and the available technological knowledge serve as an adequate background to the increasing role of this sector in Hungarian agriculture. As freshwater aquaculture is a priority sector in the European Union and due to the limited number of fish ponds induced spawning is required in the hatchery practice to increase the quantity and quality of production.

In most farmed animals artificial insemination and fertilization is an organic part of industrial-size farming, for example in case of dairy cattle artificial insemination is used almost exclusively. Sperm cryopreservation allows farmers to carry on the genetic material of only those bulls that were selected for the best traits, thus, achieving genetic progress.

Similarly to other farmed animals a long term cryopreservation of at least one of the gametes of fish not only in laboratories but also in economic scales with the elimination of damages of genetic material would provide great economic advantages. This would offer a solution not only for the preservation of bred stocks with great value but with this method desired properties could also be reproduced after an accidental epidemic (eg. koi herpes virus) or an environmental catastrophy (eg. cianide pollution in Tisza river).

In most species of fish the appropriate protocol of cryopreservation is already available for researchers but its application in practice is significant only in a few species of fish. It is expected within a short period of time that fish farmers will use cryopreserved gametes for propagation in a growing number of species. It is the task of researchers to develop methods for advancing it. After the application of cryopreserved gametes researchers have reported about a high ratio of deformed larvae which could be the result of damaging effects on spermatozoa that appeared during the freezing process. By the elimination of factors causing the appearance of deformed larvae and the application of bigger straws great steps could be taken towards the spreading of cryopreservation in the daily practice of fish propagation.

On the basis of the above mentioned I aimed to compare extent and appearance of damages in 3 fish species (African catfish /*Clarias gariepinus*/, carp /*Cyprinus carpio/,* rosy barb /*Barbus conchonius*/). Rosy barb is a widespread model species of laboratories, it has a short generation interval, can easily be propagated and handled and results can quickly be

evaluated. African catfish is a farmed species with tropical origin and increasing economic importance. Due to its simple propagation, low demands towards place compared to its size and short incubation time of eggs it is an ideal laboratory animal, too. Carp is a major economical fish species of Hungary, adaptation of breeding and improvement technologies (including the cryopreservation of gametes) in fish farming could first be expected at this species.

In all three fish species listed above the reason for hatching of deformed embryos observed after propagation with cryopreserved sperm should be cleared.

Aims of my work were the following:

1. I examined the effect of different cryoprotectants on the propagation ability of cryopreserved sperm and the development and malformation of hatched larvae when cryopreserved sperm was applied.
2. I tested the engagement of larger straws in case of carp and African catfish as a further development of cryopreservation technology with the intention to convert the method into a readily usable one in the practice of hatching, too.
3. I attempted to cryopreserve the sperm of rosy barb, in which no other researcher was engaged before. I planned to adapt cryopreservation methods of carp with some modifications if necessary.
4. I wished to examine negative effects of cryopreserved sperm on hatching larvae with direct chromosome tests. After the determination of chromosome number of hatched individuals I tried to find connections between malformations and freezing technology (applied cryoprotectant, extender and size of straw).
5. With the application of gel electrophoresis (Comet assay) I wanted to find an answer for the impairment rate of spermatozoa DNA during the process of cryopreservation which could provide an indirect proof for the explanation of malformations.

2. MATERIALS AND METHODS

2.1. Experiments on African catfish

I performed chromosome tests on African catfish (*Clarius gariepinus*) after the application of cryopreserved sperm to optimize different extenders, cryoprotectants and straw sizes.

African catfish used for the experiments was ensured by the Research Institute for Fisheries, Aquaculture and Irrigation of Szarvas and TEHAG Warm-Water Fish Hatchery Ltd. Groups of males and females with different genetic background were kept separately. Hormonal induction was followed by taking out and pressing the testicle then sperm was graded. Cryopreservation happened in straws with different sizes in the vapor of liquid nitrogen with adding 6 % fructose extender and 10 % methanol or dimetil-sulfoxid (DMSO) cryoprotectants. When using DMSO cryoprotectant 10 minutes of equilibration time was applied. Eggs were gained from females by stripping after 12 hours of hormonal injection then propagation was performed by native and thawed sperm. Different groups were hatched in similar conditions and chromosome numbers were determined in non-feeding larval stage.

50-100 larvae/group were incubated in 0,05 % kolchicin solution for 3 hours then they were hipotonised in 0,075M KCl solution for 25 minutes. Larvae were fixed in a mixture of methanol:acetous acid of 3:1 ratio then suspended in 50 % acetous acid. Cell suspensions were dropped onto preheated slides and dried. Coloring happened in 4 % Giemsa solution for 8 minutes. Results were analyzed under a 1200 scale of magnification. In properly extended metaphases chromosome number was defined by 755 pieces of larvae preparations.

2.2. Experiments on carp

2.2.1. Application of large straws

Effect of the application of larger straws (1, 2 and 4 ml) was studied on malformations of carp (*Cyprinus carpio*) and the effect of 5 types of extenders with 2 types of cryoprotectants was tested in favor of the optimization of propagation and hatching results.

Animals and hatching system used for the experiment was assured by the Dinnyés Fish Harchery of the Hungarian Association of Fish Farmers.

In the first set of experiments sperm was stripped from males after a hormonal induction, it was graded then samples were cryopreserved in large straws after adding glucose extender and methanol cryoprotectant to them. Eggs were gained from females after a hormonal treatment, too, corresponding to hatchery practice.

Compared to propagation with 10 g egg/0,5 ml sperm regarded as standard I propagated half, the same and one and a half times the regular amounts. After stickiness was abolished hatching happened in Zuger-bottles.

Following hatching chromosome examination was made as described at African catfish.

2.2.2. Application of different extenders

In the second set of experiments an optimal combination of extenders and cryoprotectants was determined on carp by the following method.

Gametes were gained from both sexes as described in the previous experiment. The following extenders were given to sperm before cryopreservation: glucose, fructose, sucrose, KCl and carp extender. All kinds of extenders were combined with methanol or DMSO cryoprotectant, too. To one dose of sperm 1 dose of cryoprotectant and 8 doses of extender were added so the final ratio was 1:9. After freezing and thawing eggs were propagated and later hatching results as an effect of the application of different extenders were examined.

2.3. Experiments on rosy barb

I successfully managed to cryopreserve male gametes of rosy barb (*Barbus conchinius*), made experiments on hatching and detected genome destructions of thawed sperm by Comet method.

Stripping of males happened by a pipette then gametes were taken into tubes containing glucose or carp extender. After the motility test cryopreservation was performed with methanol and DMSO cryoprotectants in straws of 0,25 ml then finally after thawing eggs were propagated. There was no significant difference in motility test after thawing in either of the extenders applied together with any of the cryoprotectants. Considering fertilization best results were obtained when carp extender with DMSO cryoprotectant was used.

Genome destructions of thawed sperm were detected by the Comet method.

After motility test thawed and native sperm samples were diluted by PBS and mixed with low melting point agarose. Prepared samples were dropped onto slides formerly coated with normal melting point agarose. After placing coverslips on them they were placed onto a metal tray thus the solidification of the 2nd gel layer was fastered. When removing the coverslip a 3rd gel layer was placed on the slides. They were placed on cooled metal tray for 5 minutes then coverslips were removed. During cooling time lysis buffer was prepared. In a darkened room slides were reposed into a glass tank then lysis buffer was poured on them. One hour later slides were removed from the tank, they were drained off then placed on a vertical gel electrophoresis tank. After the tank was filled with an electrophoresis buffer 40 minutes had passed before starting up the device. This time period was required for the unwinding of the DNA chain. Potential was set to 20 volt and current intensity to 300 mA in the device. Electrophoresis took for 24 minutes. Next slides were removed from the tank, drained off, placed on a metal screen then poured neutralizing solution on their surface two times with five minute intervals.

0,05 ml of ethidium-bromide solution was pipeted on the drained slides and covered with coverslips. Proceeded pictures were examined in fluorescent light under a microscope.

3. RESULTS

3.1. Results of experiments on African catfish

Fertilization of egg doses propagated with cryopreserved sperm varied between 62 and 83 % with a control group of 83 % fertilization rate. Regarding fertilization there was no statistical difference between individual treatments either when used cryoprotectants or when applied straw sizes were considered. Results of hatching varied between 43 and 53 % with 59 % of control hatching. No significant difference was found between treatments either in this case.

Results of hatching varied between 43 and 53 % with 59 % of control hatching. Neither in this case were there any differences between treatments.

Ratio of deformed larvae compared to hatched ones was between 33 and 44 %. In the control group ratio of deformed larvae was 28 %. No statistical difference was found between individual treatments.

Depending on the chromosome number of samples if it was larger or smaller than 84 the presence of triploid or tetraploid individuals was detected. It is important to note that these individuals could also be mosaic ones which means that they could have cells with diploid, triploid and tetraploid chromosome sets at the same time. Moreover, in the 0,25 M group a haploid-diploid mosaic individual was found, too.

Poorly strained preparations or the ones not containing fissions were listed in the group of "Uncertain".

Till this time 755 larval preparations were examined. Haploid larvae (28 chromosomes) were found in four treatments when 0,25 and 0,5 ml straws were applied.

3.2. Results of experiment on carp

3.2.1.Results of the application of large straws

Hatching of eggs propagated with cryopreserved sperm varied between 20 and 86 % with a fertilization rate of 82 % of the control. Highest hatching results (86±12 %) were gained by the application of 1,2 ml straws and half of the egg portion optimized for 0,5 ml straws (0,5 ml thawed sperm/10 g eggs). When 4 ml straws were used best

results were obtained by the propagation of a whole portion of eggs (65±18 %).

In general it can be stated that both the size of used straws (p=0,0186) and the amount of eggs (p=0,0093) had a statistically significant effect on hatching results.

Comparing different egg amounts a varying quantity of hatched and deformed larvae was found. Surprisingly the highest amount of deformed larvae (15±9 %) was found in the control group. In groups propagated with cryopreserved sperm the highest amount of deformed larvae (13±7 %) was observed when 4 ml straws were applied with a whole portion of eggs. Only by the combination of 4 ml straws and a double portion of eggs were no deformed larvae found after hatching though in this group hatching results were already weak.

The amount of used eggs had a significant effect on the ratio of malformations (P=0.0032) but in case of larvae hatched from eggs propagated with cryopreserved sperm the size of applied straws had no significant effect on it.

Finally the chromosome number of deformed larvae was examined and altogether more than 260 larvae were worked up.

3.2.2. Results of the application of different extenders

Every time after inoculation all of the five males gave an appropriate amount of sperm (5-15 ml). Females also responded for hormonal induction without exception.

Motility of fresh sperm was 84±7 % in the control group at n=15 repetition number. After thawing highest motility (63±9 % n=9) was gained with glucose extender and methanol cryoprotectant. There was no statistical difference between control and methanol-glucose combination. Application of other extenders with methanol did not result in such high values of motility. Nevertheless, it can be stated that in general samples with methanol proved to provide better results than when DMSO was used as cryoprotectant.

Best fertilization rates, 74±15 %, in 4-8 cell stages at n=12 repetition number (value of control: 84±13 %) were obtained with methanol cryoprotectant and glucose extender. No statistically significant difference was found between samples of control, fructose and glucose when methanol was used as a cryoprotectant. However, it

can be declared that results were better when methanol was used as cryoprotectant instead of DMSO.

Best results (67±17 %, n=9) were obtained again when glucose was used as an extender combined with methanol as cryoprotectant. At this time the result of control was 69±14 %. After a statistical evaluation similar issues were gained than in case of propagation results. Neither in this case was there any difference between control and combinations of fructose-methanol and glucose-methanol. Results of methanol samples exceed outcomes of that of DMSO in this part, too.

No deformities were found in hatched larvae.

3.3. Results of experiments on rosy barb

Quantity of sperm gained by the stripping of males was sufficient, on the average 0,01 ml was gained from each individuals. Density of the sperm was $1{,}928 \pm 0{,}223 \times 10^{10}$ bits/ml (n=9). Stripped sperm was diluted by two different types of extenders: glucose and carp extender. Considering certain extenders motility of freshly stripped male gametes didn't issued in significant difference: motility of fresh sperm in glucose extender was 53±27 % while in carp extender results of 58±24 % were gained.

In searches done after the thawing of sperm no remarkable fluctuations were observed between motility values of starting and post-thawing. There were some samples in which starting motility didn't exceed 20 % but this value was retained also during cryopreservation so after thawing the same results were obtained as at starting.

In motility investigations made after thawing it was observed that motility values of male gametes were highest when they were treated with carp extender. When certain extenders were compared against added cryoprotectants it was perceived that after thawing maximal motility (39±18 %, n=26) was obtained when carp extender was used with DMSO as cryoprotectant. Lowest value for motility was attained when carp extender and methanol cryoprotectant was used (27±13 %, n=30). Samples treated with glucose extender could be considered as more balanced: in case of glucose extender and DMSO cryoprotectant 36±16 % motility was observed at a sample number of n=26 while with the application of glucose extender and methanol cryoprotectant a motility value of 32±14 % was obtained at a sample number of n=30.

Statistically significant difference with a significance level of $p<0,05$ could not be detected with the application of either of the extenders against the two types of cryoprotectants.

Best fertilization results gained after the thawing of cryopreserved sperm examined in a 4-8 cell stage with the application of carp extender and DMSO cryoprotectant was 38±5 % with a repetition of n=3 against a 79 % fertilization rate of the control group.

Highest hatching result was 10±7 % (n=3) while 77 % of the control group was hatched.

With the application of Comet method when fresh sperm was examined in the untreated group no cells of V. level - meaning completely disintegrated - were observed while in groups treated with hidrogen-peroxid mainly cells of IV. and V. level were detected. When methanol cryoprotectant was applied a high ratio (47 %) of entirely normal, I. level cells were present but when DMSO was employed such cells could only be observed in 5,2 %. With the examination of thawed sperm samples by the application of methanol 27,5 % was the ratio of cells showing complete disintegration while after DMSO employment this ratio had a multiple value (69,2%).

4. NEW SCIENTIFIC RESULTS

- Development of a new method for the application of cryopreserved/thawed sperm in economic scales with the employment of large straws in case of African catfish and carp.

- Registration of hatching of haploid individuals in minor amount from eggs propagated with cryopreserved sperm in case of African catfish and carp.

- Successful cryopreservation of rosy barb sperm.

- By the help of Comet assay it was detected in rosy barb that the genome of cryopreserved sperm was less injured when methanol was used as cryoprotectant than in the presence of DMSO.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

In case of **African catfish** by the help of 755 larval preparations it was established that neither in fertilization nor in the ratio of hatching could a significant difference be detected when different cryoprotectants, extenders or straws were used.

It was established that the reason for irregular growth of larvae in most cases was not of genetic origin and it couldn't be attributed to cryopreservation.

It was observed that there was a close connection between morphological and cytogenetic malformations since all examined individuals with abnormal chromosome set also suffered from genotypic distortions.

It was observed in **carp** that the majority of the 260 larvae preparations were diploid so most of the malformed larvae had a normal chromosome set.

It was observed that both the size of applied straws and the amount of eggs affected hatching results.

It was observed that the amount of used eggs had an effect on the ratio of deformed larvae but the size of employed straws had not.

With the application of methanol formerly omitted in carp higher and more balanced results of motility, fertilization and hatching were obtained than with the employment of the more popular DMSO.

Among extenders sugar based ones, mainly glucose proved to be adequate for this species of fish. Agglutination observed at sugar based extenders did not affect results of hatching.

It was observed that sperm cryopreservation of **rosy barb** was successful both with the application of methanol and DMSO cryoprotectants.

It was observed that both glucose and carp extender can be used as an extender.

When fertilization was examined it was observed that highest results were gained by the combination of carp extender and DMSO cryoprotectant.

It was observed by the application of Comet method that the ratio of non-deformed cells was higher when methanol was used as cryoprotectant than when DMSO was used.

5.2. Recommendations

On the basis if results gained by my experiments for future research and industrial-size tests I have the following recommendations:

In case of African catfish I recommend the optimization of hatching conditions. This way the high number of fenotipically deformed larvae could be eliminated in the control group which would help to filter out genotypic malformations.

I recommend the application of cryopreserved sperm of African catfish and carp for propagation.

I recommend the application of sugar based extenders combined with methanol cryoprotectant in case of carp.

In case of rosy barb I recommend the execution of widespread research on cryopreservation by the application of other types of extenders.

I recommend the optimization of hatching conditions in case of rosy barb for the realization of higher hatching ratio.

I recommend the application of Comet method for the sperm qualification of other fish species, too, to find an optimal freezing diluent by the help of it.

6. PUBLICATIONS

6.1 Publications connected to present thesis

Publications in international papers:

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Presentations:

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E. Miskolczi, B. Urbányi Á. Horváth, 2005. Cryopreservation of common carp sperm, Aquaculture America 2005, New Orleans, Louisiana, USA. Összefoglaló: 275.

6.2 Other publications

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