VITRIFICATION OF THE SPERM OF EURASIAN PERCH *(PERCA FLUVIATILIS)*

ESZTER KÁSA[1*], GERGELY BERNÁTH[1], TÍMEA KOLLÁR[1], DANIEL ŻARSKI[1], JELENA LUJIĆ[1], ZORAN MARINOVIĆ[1], ZOLTÁN BOKOR[1], ÁRPÁD HEGYI[1], BÉLA URBÁNYI[1], ÁKOS HORVÁTH[1]

Department of Aquaculture, Szent István University, Páter Károly u. 1., H-2100 Gödöllő, Hungary

**Corresponding author: e-mail: kasa.eszter@mkk.szie.hu*

VITRIFIKACIJA MLEČA GRGEČA (*PERCA FLUVIATILIS*)

Apstrakt

Vitrifikacija je proces dovođenja vode ili rastvora u čvrsto stanje, odnosno u amorfno ili staklasto stanje koje može da se dostigne veoma brzom hlađenjem (10^6-10^{10} °C/s). Nedavno je objavljeno nekoliko istraživanja o vitrifikaciji mleča različitih vrsta riba, međutim nema dostupnih informacija o vitrifikaciji mleča grgeča *(Perca fluviatilis).*

Mužjaci grgeča su uzorkovani 6 dana posle hormonske injekcije (250 IU kg^{-1} hCG). Evaluirana je pokretljivost spermatozoida pomoću sistema kompjuterske analize sperme CASA. Za process vitrifikacije mleč je razblažen modifikovanim Tanaka ekstenderom na finalni odnos 1:5 (sa krioprotektantima). Posle preliminarnih testova sa kombinacijom metanola i propilen glikola (PG) u različitim koncentracijama, odlučeno je da se koristi 15% metanola i 15 % PG (ukupno 30% krioprotektanata). Suspenzija mleča je ubačena direktno u tečni azot bez prethodnog hlađenja u njegovoj pari. Za sve eksperimente vitrifikacije za hlađenje su korišćene cevčice Cryotop (Kitazato-Dibimed, za 2 µl rastvora).

Za fertilizacioni test su prikupljena jaja ženki grgeča. Vitrifikovane Cryotop cevčice otopljene su direktno u 10 µl rastvora za aktivaciju (50 mm NaCl) u petri šoljama koje su sadržale jaja. Svež mleč služio je za kontrolu. Oplođena jaja su inkubirana u plivajućem sistemu. Izvedena su 3 ogleda da bi se utvrdio odgovarajući broj cevčica Cryotop za svaku seriju jaja: 1, 6 i 18 cevčica Cryotop je isprobano za svaku seriju jajnih ćelija. U 2 µl rastvora mleča jedne Cryotop cevčice bilo je oko 0,33 µl mleča. Na osnovu stepena oplođenja u tri ogleda može se zaključiti da povećanje broja Cryotop cevčica pojačava stepen oplođenja. Dalja sitraživanja su neophodna da bi se razvio metod vitrifikacije sa većim preživljavanjem larvi posle oplođenja vitrifikovanim mlečom. Takođe je potrebno ispitati stepen izvaljenih embriona iz ogleda sa vitrifikovanim mlečom, kao i potencijalni uticaj vitrifikacije na larve, pre svega na deformitete i morfološke promene.

Ključne reči: vitrifikacija spermatozoida, veoma brzo hlađenje, krioprezervacija mleča, Perca fluviatilis, Cryotop
Keywords: spermatozoa vitrification, ultra-rapid cooling, fish sperm cryopreservation, Perca fluviatilis, Cryotop

INTRODUCTION

For preservation of cells and tissues without formation of ice crystals, two main methods have been developed: slow programmable freezing and vitrification. Programmable freezing requires expensive instrumentation and laboratory conditions during the cooling process. Accordingly, ultra-fast cooling or vitrification, which does not require special equipment, has attracted increasing interest in recent years. Vitrification is the solidification of a liquid into an amorphous or glassy state which can be attained at very fast cooling rates (10^6-10^{10} °C/s). The success of the ultra-rapid cooling relies principally on achieving ultra-fast cooling and thawing rate and determining an appropriate concentration of the cryoprotective agent combined with suitable cooling media, preventing ice formation during the process. Although high concentrations of cryoprotectants lower the ice formation temperature, they could be toxic to cells. Consequently, cryoprotectant 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 highly important for achieving fast heat transfer and to avoid creation of ice crystals (Tsai et al, 2015). Recently, several studies have been published on the vitrification of fish sperm (Cuevas-Uribe et al., 2011/a, b, Figueroa et al., 2013, 2015). These studies report results on different fish species, such as Channel catfish (*Ictalurus punctatus)*, Green Swordtail *(Xiphophorus hellerii)*, Rainbow trout *(Onchorynchus mykiss)*, Atlantic salmon *(Salmo salar)*, but no information is available on the vitrification of Eurasian perch *(Perca fluviatilis)* sperm.

MATERIALS AND METHODS

A broodstock of wild caught Eurasian perch males and females was maintained at the hatchery of the Department of Aquaculture, Szent Istvan University, Gödöllő, Hungary. We have collected sperm from Eurasian perch males. After drying the genital area with paper (to avoid contamination of samples with water or mucus), sperm was collected into 1,5 ml Eppendorf tubes by applying gentle abdominal pressure to anaesthetised (with MS-222) males (Fig. 1.). The fish were sampled 6 days after hormonal injection (250 IU kg^{-1} of hCG (human chorionic gonadotropin, Ferring, Saint Prex, Switzerland)). We have kept the sperm on ice during the preparation method. Progressive motility of fresh sperm was evaluated with computer assisted sperm analysis (CASA, Sperm VisionTM v. 3.7.4., Minitube of America, Venture Court Verona, USA). Modified Lahnsteiner's activating solution (75 mM NaCl, 2 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, pH 8 (Lahnsteiner et al, 2011)) in a mixture of 0.01 g/mL BSA (Bovine Serum Albumin) was used for CASA analysis.

For the vitrification, sperm was diluted with modified Tanaka extender (137 mM NaCl and 76.2 mM $NaHCO_3$ (Szabó et al, 2005)) to the final ratio of 1:5 (including cryoprotectants). Methanol (MeOH) and propylene glycol (PG) were used in three combinations: 10% MeOH

– 10% PG (20% total concentration), 15% MeOH – 15% PG (30%) and 20% MeOH – 20% PG (40%). Chemicals were purchased from Reanal (Budapest, Hungary) and Sigma-Aldrich (Budapest, Hungary). The sperm suspension was plunged directly into liquid nitrogen without pre-cooling in its vapour. For all vitrification experiments Cryotops (Fig. 2.) were used as cooling device (Kitazato-Dibimed, for 2 µl of solution). Motility of vitrified samples was determined using CASA following thawing of Cryotops directly into the activating solution under the microscope.

We have collected eggs from Eurasian perch females (Fig. 3.). After the use of single hormonal injection (hCG, 500 IU/g fish) the females were checked daily by ovarian biopsy, enable to predict the accurate time of the ovulation. One day before the planned ovulation the genital pore were sutured to avoid spontanous spawning into the tank. After removing the suture and drying the genital area the eggs were stripped into a dry dish.

For fertilization tests approximately 100 eggs were used for each sample. Sperm was vitrified in the presence of cryoprotectants at a final concentration of 30%. We thawed the vitrified Cryotops directly into 10 ml of activating solution (50 mm NaCl) in a petri dish containing the eggs. Fresh sperm was used for a control fertilisation test. Fertilized eggs were incubated in a floating system (styrofoam boards with filters on a tank, Fig.4.).

Three trials were realized to find the most appropriate number of cryotops per egg batch. During the experiments we have used 1, 6 and 18 cryotops for the fertilisation of one portion of eggs. The 2 µl diluted sperm on one cryotop contained approximately 0,33 µl sperm. Ferilization ratios were counted with a steromicroscope (Fig. 5.).

To analyze the results, statistical software GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, California, USA) was used.

Figure 1. *Sperm stripping*

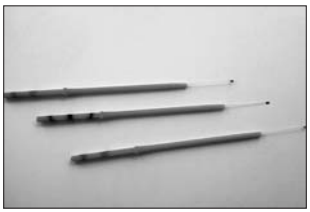

Figure 2. *Cryotops*

Figure 3. *Stripping the eggs*

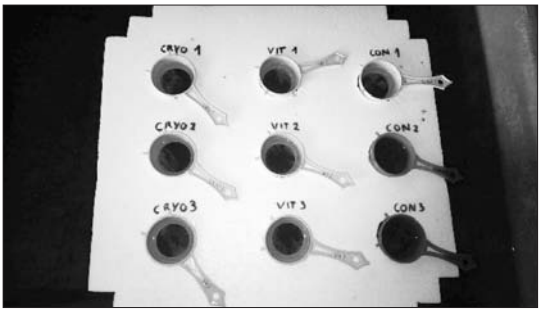

Figure 4. *Floating incubation*

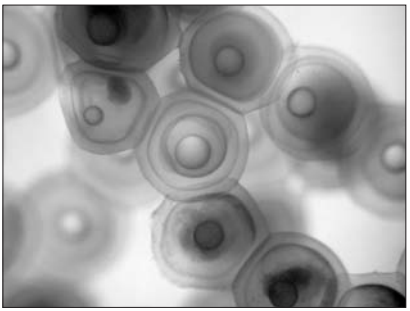

Figure 5. *Developing embryos*

RESULTS AND DISCUSSION

According to the motility data, the use of 20% cryoprotectant (10% methanol and 10% PG), the measured average progressive motility after thawing was 10,31% (±1,92). In the case of 30% cryoprotectant (15% methanol and 15% PG) this value was 13,95% (±1,67), and with using 40% total cryoprotective agent we reached 7,07% (±4,05). Several studies suggest that fish spermatozoa can tolerate high cryprotectant concentrations when the proportion of the chemicals is appropriate (Cuevas-Uribe et al., 2011a, b). During previous experiments with other fish species we have also found that the most appropriate cryoptotectant concentration is around 30% of the total solution, because with lower alcohol content the creation of harmful ice crystals is not fully inhibited, and higher concentrations of cryoprotectants are toxic for spermatozoa.

Thus we can conclude that best vitrification method was carried out with using the following protocol: 1:5 dilution ratio, Tanaka extender, 30% cryoprotectant (15% methanol + 15% propylene-glycol), cooling device: Cryotop, 2 µl droplets (Fig. 6.).

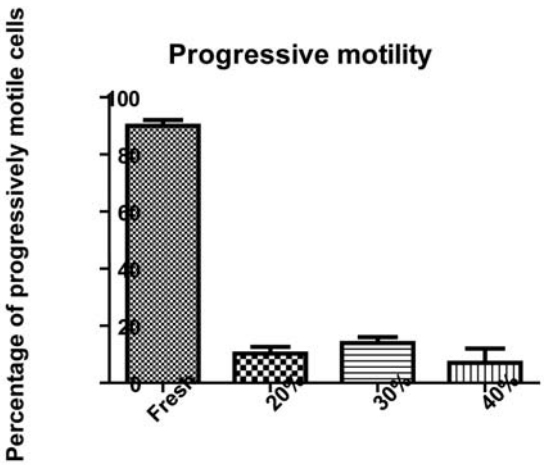

Figure 6. Progressive motility measured in fresh sperm, and sperm vitrified with 20%, 30% and 40% cryoprotectant. Columns represent the datas from 3 experiments, vitrified 3 samples in 4 replicates.

According to the fertilisation rates of the three trials, we can conclude that increasing the number of used Cryotops enhances the fertilisation ratio: fertilising with one Cryotop resulted 1,44% (±1,58) fertilisation, 6 Cryotops per egg batch resulted 4,9% ±4,77), and 18 Cryotops resulted 1,27% (±1,51). In the case of the third experiment (with 18 Cryotops), egg quality was moderate (under 70% fertilisation in the control group), thus further studies are needed to achieve more accurate results.

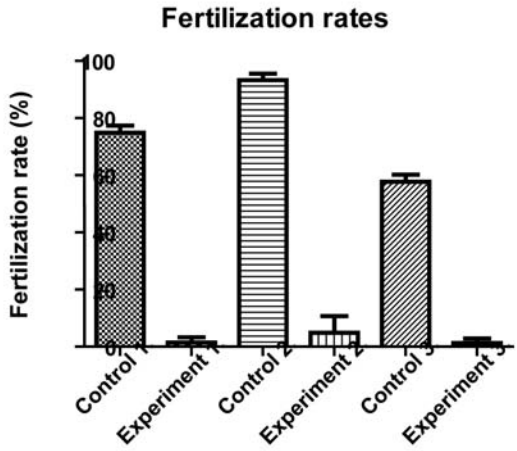

Figure 7. Fertilizaton rates measured in controlls, and vitrified sperm with 1/6/18 cryotops per egg batch. Columns represent the datas from 3 experiments.

CONCLUSION

Successful vitrification of Eurasian perch sperm was conducted for the first time. Motile spermatozoa were recovered following vitrification of sperm and fertilization of eggs with vitrified sperm resulted in developing embryos in this species. Thus, vitrification of sperm is feasible in the Eurasian perch, although futher studies are needed to improve this technique.

ACKNOWLEDGEMENTS

The work was funded by the OTKA grant number 109847. The work was supported by the project number 8526-5/2014/TUDPOL of the Ministry of Human Resources of Hungary and the project EUREKA_HU_12-1-2012-0056 (PERCAHATCH)

REFERENCES

Cuevas-Uribe, R., Leibo, S. P., Daly, J., Tiersch, T. R. (2011/a). Production of channel catfish with sperm cryopreserved by rapid non-equilibrium cooling. Cryobiology 63 (3): 186-197.

Cuevas-Uribe, R., Yang, H., Daly, J., Savage, M. G., Walter, R. B. Tiersch, T. R. (2011/b). Production of F1 Offspring with vitrified Sperm from a Live-Bearing Fish, the Green Swordtail (*Xiphophorus hellerii)*. Zebrafish 8 (4): 167-169.

Figueroa, E., Risopatrón, J., Sánchez, R., Isachenko, E., Merino, O., Isachenko, V., Valdebenito, I. (2013). Spermatozoa vitrification of sex-reserved rainbow trout *(Oncorhyncus mykiss):* Effect of seminal plasma on physiological parameters. Aquaculture 372-375: 119-126.

Figueroa, E., Merino, O., Risopatrón, J., Sánchez, R., Effer, B., Isachenko, E., Farias J. G., Valdebenito, I. (2015). Effect of seminal plasma on Atlantic salmon *(Salmo salar)* sperm vitrification. Theriogenology 83: 238-245.

F. Lahnsteiner, Spermatozoa of the teleost fish *Perca fluviatilis* (perch) have the ability to swim for more than two hours in saline solutions, Aquaculture 314 (2011) 221–224.

G. Szabó, T. Müller, M. Bercsényi, B. Urbányi, B. Kucska, Á. Horváth: Cryopreservation of European eel (Anguilla anguilla) sperm using different extenders and cryoprotectants, Acta Biologica Hungarica 56 (2005) 173–175.

Hsun-Heng Tsai, Chien-Hsiung Tasi, Wei-Te Wu, Fu-Zen Chen, Pei-Ju Chiang: Numerical investigation into thermal effect of pre-cooling zone in vitrification-based cryopreservation process. Cryobiology 70 (2015) 32-37.