

The applicability of 10 ml cryotubes for sperm cryopreservation in a Hungarian carp landrace (*Cyprinus carpio carpio morpha accuminatus*)

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SUMMARY

In our study, the comparison of 5 ml straw and 10 ml cryotube during sperm cryopreservation in a Hungarian carp landrace (*Cyprinus carpio carpio morpha accuminatus*) was carried out. Three different dilution ratios (1:1, 1:4 and 1:9) were also tested using the cryotube. A significantly higher *p*MOT was recorded using the cryotube in comparison with the straw. VCL and STR were similar in both groups. Cryopreservation had a negative effect on *p*MOT and VCL using the cryotube and also the straw where, STR was not reduced significantly. An increasing tendency was observed using higher dilution of sperm during cryopreservation; however, significant difference was not recorded between the three groups. VCL and STR were similar in all groups. Cryotube was applicable for the sperm cryopreservation of the Hungarian carp landrace. However, the standardization for the freezing method specified for this volume is recommended. The different dilution ratios needed be tested also during fertilization.

Keywords: Hungarian carp landrace, sperm, motility, cryopreservation, CASA

INTRODUCTION

Cryopreservation of fish sperm is an assisted reproduction method which allows the storage of sperm cells on the same quality for numerous years (Ashwood-Smith 1980, Whittingham 1980, Stoss 1983). During deep freezing, the sperm diluted in specific extenders (contains regularly ions and sugars) and cryoprotectants (e.g. methanol (Horváth et al. 2003) can survive the lethal cryo-injuries (Cloud and Patton 2009). Cryopreservation can solve the problems originated from the asynchronous gamete production in males and females (Cabrita et al. 2010). Long-term preservation can allow enough sperm for whole year propagation in different fish species (also out of the reproduction season) which is an important key factor because of the market demand (Migaud et al. 2002, Bernáth et al. 2016a). Cryopreservation also allows the transportation of sperm for long distances. Furthermore, long-term of storage of sperm (the genetically background of male individuals) can have an important role also in conservation management (Cabrita et al. 2010).

The cryopreservation in common carp started to develop since 40 years ago (Moczarski 1977). Key parameters in freezing success, such as extender composition, type of cryoprotectants, straw volume, freezing methods were intensively investigated in laboratory conditions. However, the practical utilization (in farm production) of common carp sperm cryopreservation procedure was not established yet. A possible reason for the mentioned statement is the high costs of the developed methods. Namely, sperm cryopreservation is an expensive method. The costs can be reduced with the standardization (repeatability of the results) of the protocols (Caffey and Tiersch 2000, Cabrita et al. 2010). Carp sperm was successfully frozen using high volume of containers (5 ml straw and 2 ml cryotube) (Linhart et al. 2000,

Horváth et al. 2007). Simultaneous cryopreservation of higher amount of sperm can reduce the costs via commercialization (high volume sperm deep freezing) of the method (Horváth et al. 2007, Bernáth et al. 2016a). Common carp is in the largest quantity produced fish species in Hungary. Hungarian angling associations take care privately of the continuous support of carp populations in their managed lakes. The *Cyprinus carpio carpio morpha accuminatus* is a state-approved landrace. The Balaton Fish Management Non-Profit Ltd. is the maintenance breeder and owner of the variety (Udvari 2017). The aim of our study was to adapt our formerly established method to 10 ml cryotubes where 5 ml straw was also tested. Three different dilution ratios were investigated during *Cyprinus carpio carpio morpha accuminatus* sperm cryopreservation using 10 ml cryotubes.

MATERIALS AND METHODS

A broodstock of carp males (N=12, body weight: 944–1654 g, standard length: 32–42 cm) was transported from Irmapuszta Fish farm (Balaton Fish Management Non-Profit Ltd.) to the recirculating system of the Department of Aquaculture. Fish were kept in a 3 m³ plastic tank at an average temperature in July 21 °C and in August 23 °C. For gentle handling of individuals, males were anesthetized using 2-phenoxyethanol in a dose 0.4 ml per l of hatchery water. Prior to our experiments (approximately 24 hours), spermiation was hormonally induced using 2.5 mg body weight kg⁻¹ carp pituitary extract. The genital pore was whipped dry before sampling to avoid milt contamination with urine, feces or blood. Sperm was stripped using abdominal massage of the fish and collected with 2 or 5 ml syringes. Samples were stored at 4 °C approximately 1 hour prior to our experiments.

Sperm motility assessment

Motility parameters (WHO 2010) [progressive motility – pMOT (%), straightness – STR (%), curvilinear velocity – VCL ($\mu\text{m}/\text{sec}$)] were recorded following activation using a saline solution designed for cyprinids (45 mM NaCl, 5 mM KCl, 30 mM Tris, pH 8.0 \pm 0.2) (Saad et al. 1988) in a mixture with approximately 0.01 g BSA (Bovine Serum Albumin). Motility was analyzed with a CASA system (Computer-assisted Sperm Analysis, Sperm VisionTM v. 3.7.4., Minitube of America, Venture Court Verona, USA) both following stripping and thawing. Measurements were carried out at least in duplicates.

Cryopreservation and thawing process

Prior to cryopreservation milt was diluted at various ratio (according to the experimental design) with a glucose based extender (200 mM glucose, 40 mM KCl, 30 mM Tris, pH 8 \pm 0.2) (Horváth et al. 2012) and 10% of methanol (as intracellular cryoprotectant). Diluted sperm (4 ml and 8 ml) was loaded into 5 ml straws or 10 ml cryotubes (detailed in the experimental design). To set up a cooling program for the controlled-rate freezer (CRF, IceCube 14s, IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria), the cooling rate was measured 3 cm above the liquid nitrogen for 7 minutes (Bokor et al. 2010) using a thermocouple attached to a digital thermometer (Digi-Sense DualLogR₂, Eutech Instruments Pte Ltd, Singapore). Cryotubes were frozen using CRF with a cooling program (from 4 °C to -160 °C, cooling rate: 15 °C/min), where straws were cryopreserved in a polystyrene box 3 cm above the surface of liquid nitrogen on a floating polystyrene frame for 7 minutes. Frozen samples were stored in canister tanks (Statebourne Cryogenics, UK and VWR XSS 48/10, VWR International Kft., Debrecen, Hungary). Cryotubes were thawed at 35–40 °C for approximately 4 minutes, where straws were thawed at 40 °C for 40 seconds in the same water bath (Thermo Haake P5, Thermo Electron Corp, Waltham, Massachusetts, USA). All chemicals were purchased from Reanal (Budapest, Hungary) and Sigma-Aldrich (Budapest, Hungary).

Experimental design

Experiment 1 – The comparison of 5 ml straw and 10 ml cryotube

Freshly stripped sperm from 6 males was diluted in the above mentioned extender at a ratio 1:9 and were loaded into 5 ml straws and 10 ml cryotubes. Motility was analysed in both groups.

Experiment 2 – The comparison of 3 different dilution ratios

Native carp sperm from 3 males was diluted in the sugar based extender at a ratio 1:1, 1:4 and 1:9. Based on the results of Experiment 1, samples were loaded into 10 ml cryotubes. Sperm motility parameters were recorded in the 3 groups.

Statistical analysis

Statistical software packages SPSS 14.0 (SPSS Inc., Chicago, USA) and GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, California, USA) were used for the analysis of results obtained from motility assessments of the cryopreservation experiments. Normal distribution was tested using Kolmogorov-Smirnov test at the significance level of $P < 0.05$. Data not showing a normal distribution were transformed using arcsine square root function (STR, %). Different groups were compared with one-way ANOVA (Experiment 1) or Kruskal-Wallis (Experiment 2) test followed by Tukey's, Dunnett's T3 (Experiment 1) and Dunn's (Experiment 2) multiple comparisons post hoc tests.

RESULTS

Experiment 1 – The comparison of 5 ml straw and 10 ml cryotube

A significantly higher pMOT (25 \pm 8%) was recorded with cryotubes in comparison with the straw (5 \pm 2%). No significant difference was measured in VCL (cryotube: 49 \pm 4 $\mu\text{m}/\text{sec}$, straw: 39 \pm 7 $\mu\text{m}/\text{sec}$) and STR (cryotube: 87 \pm 4%, straw: 60 \pm 22%). pMOT and VCL decreased significantly following thawing compare to the fresh control (pMOT: 94 \pm 4% VCL: 135 \pm 14 $\mu\text{m}/\text{sec}$, STR: 80 \pm 2%) where STR did not reduce significantly (*Figure 1*).

Experiment 2 – The comparison of 3 different dilution ratios

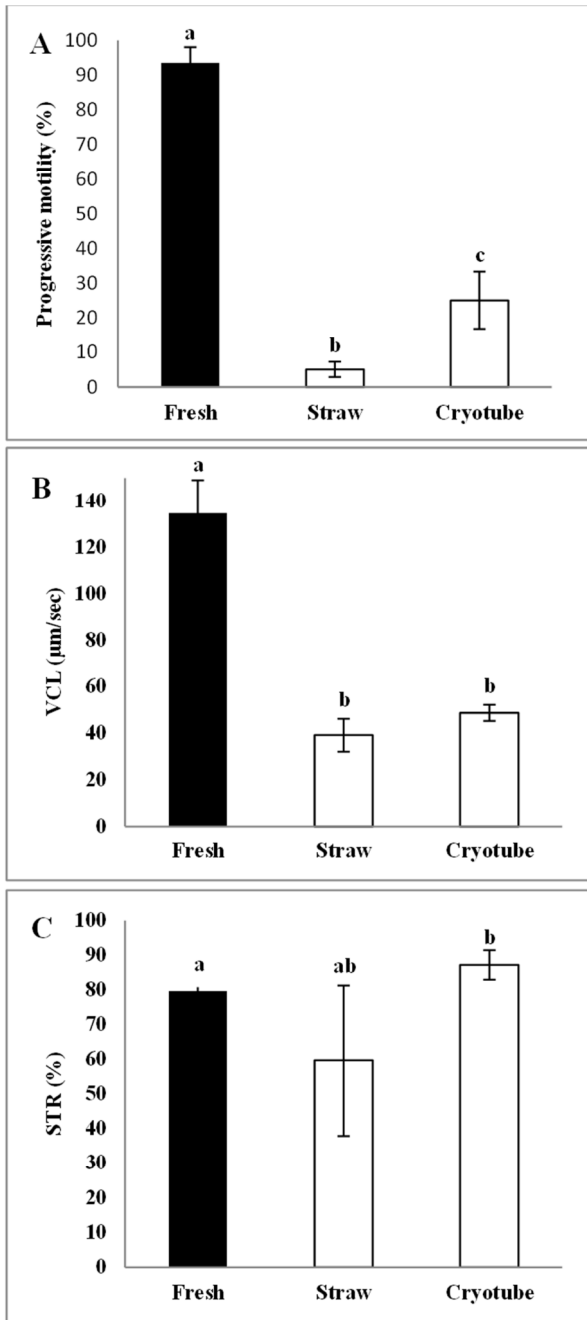
Increasing tendency was observed in pMOT (1:1: 8 \pm 4%, 1:4: 15 \pm 9%, 1:9: 19 \pm 9%) with higher dilution of sperm following thawing although, no significant difference was measured between the 3 cryopreserved groups. No significant difference was recorded in VCL (1:1: 35 \pm 6 $\mu\text{m}/\text{sec}$, 1:4: 39 \pm 6 $\mu\text{m}/\text{sec}$, 1:9: 45 \pm 2 $\mu\text{m}/\text{sec}$) and STR (1:1: 89 \pm 2%, 1:4: 87 \pm 3%, 1:9: 90 \pm 3%) between the 3 dilution ratios (*Figure 2*).

DISCUSSION

In our experiments, approximately 50% of the thawed substance was agglutinated and the other amount showed a regular cell suspension. The phenomenon was already presented in common carp and goldfish as well (Horváth et al. 2003, Irawan et al. 2010, Bernáth et al. 2016b, 2017).

In our study, 10 ml cryotube was successfully applied for the cryopreservation of Hungarian carp landrace sperm. However, progressive motility reduced drastically following thawing in the case of 10 ml cryotubes and 5 ml straws as well compare to the fresh control. Contrary, Horváth et al. (2007) achieved a high hatching rate using the same volume straw and a similar glucose based extender (350 mM glucose, 30 mM Tris, pH 8.0). However, their results showed the highest hatching rate with 5 minutes of freezing time. In our study, 7 minutes could lead a too slow freezing rate for common carp which resulted drastic intracellular cryo-injuries (Cloud and Patton 2009).

Figure 1: pMOT (A), VCL (B) and STR (C) during the comparison of 5 ml straw (white column) and 10 ml cryotube (white column) (N=6)

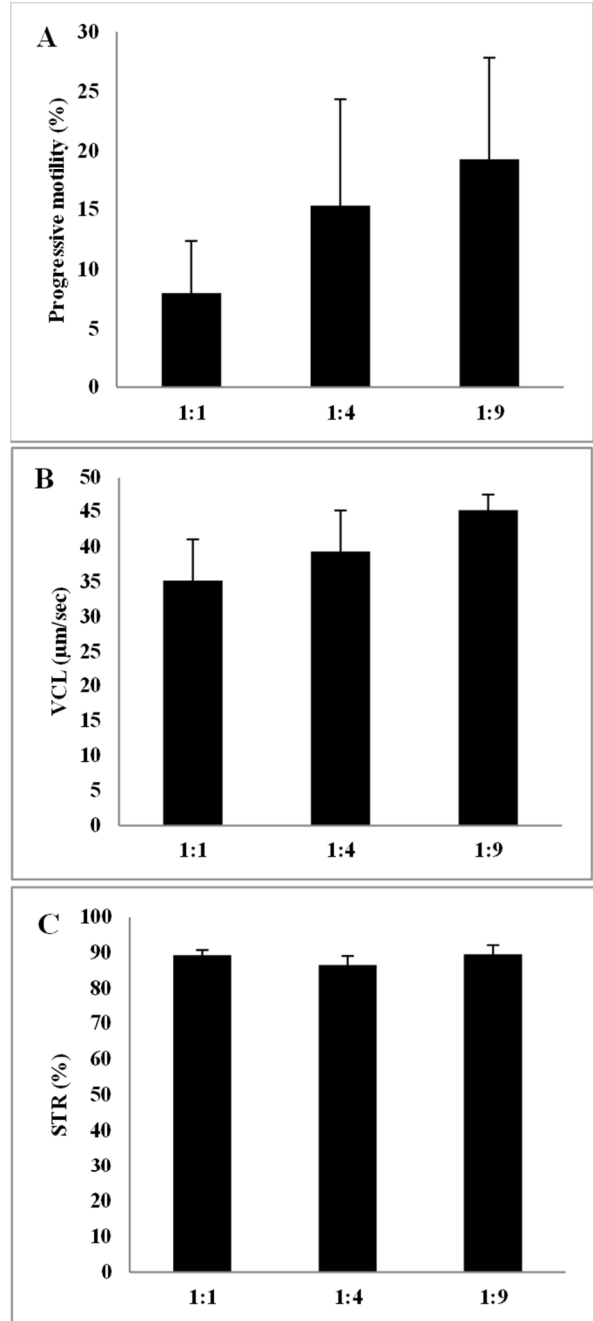


Note: different letters indicate statistically significant difference at the significance level of $P \leq 0.05$. Graph represents Mean and SD

Besides of the above mentioned formerly published result, large amount of carp sperm was also successfully cryopreserved using another strategy of packaging diluted sperm by Bernáth et al. (2016b). Increased scale cryopreservation was carried out using also a controlled-rate freezer whereas diluted sperm was loaded into numerous 0.5 ml straws (104 pcs). However, higher post-thaw motility was recorded in 10 randomly chosen thawed straws ($47 \pm 5\%$) compare to the 10 ml cryotubes ($25 \pm 8\%$). Linhart et al. (2000) applied 2 ml tubes also for carp sperm

cryopreservation. The results showed a high percentage of motility following thawing (69 ± 14). Our results indicate that the standardization of different parameters (cooling rate, extender composition, equilibration time etc.) during carp sperm cryopreservation is needed to be optimized specifically using 10 ml cryotubes as well.

Figure 2: pMOT (A), VCL (B) and STR (C) during the comparison of 3 different dilution ratios (N=3)



Note: graph represents Mean and SD

Higher dilution ratios were more effective for carp sperm cryopreservation in 10 ml cryotubes however, significant difference was not observed. A similar tendency was observed by Bernáth et al. (2016b) where high post thaw motility was recorded at a

dilution 1:5–1:20 in the case of carp sperm cryopreserved using 0.5 ml straws. In contrast, Lahnsteiner et al. (2003) found a negative effect of dilution ratios higher than 1:7 on the hatching rate in Danube bleak (*Chalcalburnus chalcoides*) sperm. Our results, and former studies suggest, that dilution ratios is recommended to be standardized precisely also for 10 ml cryotubes, thereby to enhance its applicability. Bernáth et al. (2016a) found the dilution ratio 1:10 suitable for the increased-scale (3 ml, 67 straws) cryopreservation in Eurasian perch (*Perca fluviatilis*). The results showed a high fertilization rate using thawed sperm (72±14%). For fertilization, the ratio 1:9 with more concentrated sperm is recommended in carp also using 10 ml cryotubes (Bernáth et al. 2016b). However, the different dilution ratios using 10 ml cryotube needed be tested also during fertilization.

In conclusion, 10 ml cryotubes are suitable for carp sperm cryopreservation however its capability can be enhanced with standardization of the cryopreservation process. Higher dilution ratios can be applied during higher amount of carp sperm cryopreservation using 10 ml cryotubes however,

fertilization tests can verify more precisely our hypothesis. Nevertheless, the application of 10 ml cryotube during cryopreservation could provide a suitable amount of good quality carp sperm during or out-of-the spawning season for the hatchery practice. Furthermore, the possible decrement of the number of spawners (using cryopreserved sperm) could result a reduction of costs in production of this Hungarian carp landrace (Caffey and Tiersch 2000, Cabrita et al. 2010, Bernáth et al. 2016a).

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