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Research paper

Improvement of common carp (*Cyprinus carpio*) sperm cryopreservation using a programable freezer

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ABSTRACT

The applicability of a programmable freezer for the increased-scale cryopreservation of common carp sperm was investigated. The effect of different equilibration times, cryopreservation methods, extenders, dilution ratios, activating solutions on the post-thaw motility of common carp sperm was investigated. The suitable post-thaw storage time-interval as well as fertilizing capacity of cryopreserved sperm was also examined. The motility, curvilinear velocity (VCL) and straightness (STR) values did not decrease significantly during 60 min of equilibration neither in equilibrated nor thawed groups. Motility parameters of thawed sperm were similar using a conventional cryopreservation technique using a polystyrene box [motility (33%), VCL (47 μm/s) and STR (88%)] and a programmable freezer: [motility (32%), VCL (54 μm/s) and STR (89%)]. The highest motility and VCL was measured with a sugar based extender (grayling extender) at a ratio 1:9 (motility: 52%, VCL: 76 μm/s) and 1:20 (motility: 49%, VCL: 76 μm/s). The activating solution for cyprinids (ASC) could prolong sperm movement up for 2 min. A storage time of six hours following thawing did not have a significant effect on the motility parameters of thawed carp sperm. Agglutination was observed during cryopreservation of an elevated volume of sperm whereas motility 47%, VCL 62 μm/s and STR 91% were measured after thawing. Fertilization rate with thawed sperm (32%) was significantly lower compared to the control group (73%). According to our results, the developed method using a programmable freezer is suitable for the cryopreservation of elevated number of straws. However, carp sperm agglutination during freezing may have a negative effect on the fertilizing capacity.

1. Introduction

Common carp (*Cyprinus carpio*) is one of the most important cultured freshwater fish in the world. The intensive hatchery propagation of carp has been developed several decades ago (Horvàth et al., 1992). However, there is an increasing interest in methodical and practical innovation in controlled reproduction of common carp (Kucharczyk et al., 2008; Żarski et al., 2015). Cryopreservation is an efficient assisted reproductive technology that can improve the effectiveness of farm production (gamete storage and transfer, gamete production synchronization etc.). Success of cryopreservation is dependent on numerous parameters (Cloud and Patton, 2009). Extenders are sugar- or ion-based solutions (or the combinations of the two) that reversibly immobilize spermatozoa and provide isotonic conditions and appropriate pH for their survival. Cryoprotectants are low molecular weight compounds that penetrate cells and lower the freezing points of solutions. Cryoprotectants in combination with an effective dilution ratio can also improve the cryo-resistance of spermatozoa (Cloud and Patton, 2009; Dziewulska and Domagala, 2013). Cryoprotectants need time to penetrate to the cells (equilibration), however, prolonged exposure before cryopreservation can be toxic for sperm (Cloud and Patton, 2009; Yang and Tiersch, 2009). The appropriate cooling rates facilitate cells to be frozen without the formation of lethal intracellular ice crystals or crucial dehydration of sperm (Cloud and Patton, 2009). Storage of cryopreserved sperm at −196 °C enables long-term preservation of vulnerable and valuable genetic resources (Cabrita et al., 2010).

Several studies have been reported on the cryopreservation of common carp sperm however, there is a lack of consensus on several parameters that could constitute a standardized protocol. First experiments on the cryopreservation of common carp sperm

Abbreviations: ASC, activating solution for cyprinids; VCL, curvilinear velocity; HBSS, Hank's balanced Salt Solution; STR, straightness.

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were reported by Moczarski (1977) and the topic has been studied extensively since then. For cryopreservation, various extenders in combination with different types of cryoprotectants were already used, such as egg yolk (Bozkurt et al., 2014, 94 ± 1% fertilization), methanol (Horváth et al., 2003, 74 ± 15% fertilization), dimethyl sulfoxide (Kurokura et al., 1984, 70% fertilization) or propolis (Ögretmen et al., 2014, 69 ± 2% fertilization). Also, the effect of a wide range of sperm dilution ratios, such as 1:1 (Boryshpolets et al., 2009, (25% motility), 1:9 (Horváth et al., 2003, (74 ± 15% fertilization), 1:7 (Lahnsteiner et al., 2000, 52 ± 7% fertilization) or 1:5 (Linhart et al., 2000, 56 ± 10% fertilization) as well as cooling profiles (Irawan et al., 2010; Warnecke and Pluta, 2003) on cryopreservation success was investigated. Irawan et al. (2010), tested different freezing rates by modifying the height (from 2 to 6 cm) above the surface of liquid nitrogen, where the straws were placed. The highest fertilization was observed when samples were frozen at 2 cm above the level of liquid nitrogen and 10 min freezing time (74 ± 7%). Warnecke and Pluta (2003) also compared various cooling profiles with a programmable freezer, which was found to be an efficient device for the cryopreservation of carp sperm. However, an aluminium dish fixed above the liquid nitrogen resulted in the highest fertilization rate (99%) using a 10 °C/min cooling rate (Warnecke and Pluta, 2003). The main advantages of these devices are the fully controlled environment around the samples and high repeatability of cooling dynamics (Babiak et al., 1999; Bernáth et al., 2015a; Butler and Pegg, 2012). Post-thaw motility duration in common carp sperm has been reported as a quality parameter [Linhart et al., 2000 (105 s), Ögretmen et al., 2014 (42 ± 1 s)] however, no standard protocol for the assessment of post-thaw motility duration exists, yet. Information regarding the post-thaw storage time of cryopreserved sperm as a practical parameter is limited in common carp [Boryshpolets et al., 2009-(up for 10 min without significant effect on hatching rate)]. This ability can prolong the time between thawing and the experimental application or artificial fertilization. Thus, carp sperm cryopreservation remains a problematic issue, mainly due to the low replicability of published protocols which limits the practical utilization of cryopreservation methods. (Tiersch, 2008). Success of a given method depends on laboratory conditions (protocols, handling process, and technician skills). The large number of various protocols make difficult to sort out the most efficient method (Martínez-Páramo et al., 2016).

The objective of this study was the technical standardization of a protocol for the cryopreservation of common carp sperm. In contrast with former studies (mentioned above), we systematically tested in one experiment a series of different parameters stepwise from the optimal extender to the applicable storage time following thawing. Our work focused on the evaluation of various extenders (with variable glucose-ion ratio) and activating solutions as well as optimization of dilution ratios and equilibration times. The duration of effective post-thaw storage, assuring the time necessary of using the cryopreserved sperm for commercial fertilization purposes was also measured. We adapted a formerly established cooling protocol in common carp to a programmable freezer. A fast cooling program (according to former studies) was tested in carp sperm cryopreservation. The programmable freezer was tested for increased-scale (high number of replicates) cryopreservation of common carp sperm whereas repeatability in motility and fertilizing capacity results was investigated following thawing.

2. Materials and methods

2.1. Broodstock and sperm collection

A pond-reared broodstock (16 males: body weight 0.77–1.9 kg, 12 females: body weight-2–3.5 kg) of common carp (*Cyprinus carpio*) were purchased from Aranyponty Zrt., (Százhalombatta, Hungary) and maintained at the hatchery of the Department of Aquaculture, Szent Istvan University, Gödöllő, Hungary. The spermiation of carp males was induced 24–48 h before sperm collection by an intraperitoneal injection of carp pituitary at a dose of 2.5 mg/kg body weight homogenized in 0.5 mL distilled water. Females, also stimulated with carp pituitary homogenate, were injected in two doses: 0.4 and 3.6 mg/kg body weight, 12 h apart. Water temperature during the spawning operation was 22–23 °C. Eggs were stripped approximately 26 h following the first injection. Their genital pore was closed with surgical suture after injection to prevent spontaneous release of eggs according to the standard hatchery procedure by Horvath et al. (2002). Before sampling, individuals were anesthetized using 2-phenoxyethanol (99.5%) at a dose of 0.4 mL/L. The genital apertures of males and females were wiped dry before the collection of gametes to prevent their activation. Sperm was hand-stripped into 10 mL test tubes. Eggs were stripped manually into dry plastic containers. Sperm samples were stored on crushed ice at 4 °C before the experiments (0–60 min and according to the Section 2.5). Eggs were stored in the hatchery at a temperature 22–24 °C for a maximum of 1 h.

2.2. Sperm analysis

Progressive motility (from here onwards referred to as motility, %), straightness (STR, %) and curvilinear velocity (VCL, μm/s) (Computer-aided Sperm Analysis, 2010) of carp sperm was analyzed both before (for control quality) and after cryopreservation using a CASA (computer-assisted sperm analysis) system (Sperm Vision™ v. 3.7.4., Minitube of America, Venture Court Verona, USA). For the analysis of fresh sperm motility, 1 μl of sperm was mixed with 99 μl extender. In contrast, 1 μl of diluted sperm was mixed with 20 μl of extender during thawed sperm motility measurements. In order to prevent sperm from adhering to the glass surface, 0.01 g/mL bovine serum albumin (BSA) was used.

2.3. Cryopreservation and thawing process

For cryopreservation, the following extenders were used according to the Section 2.5:

1. Grayling" extender (200 mM glucose, 40 mM KCl, 30 mM Tris, pH 8, Horváth et al., 2012) in *Experiment 1–7*.
2. Modified grayling extender: (100 mM glucose, 100 mM KCl, 30 mM Tris, pH 8) in *Experiment 4*.
3. Carp seminal fluid: (0.05–0.6 mM glucose: Gosh, 1985; Kruger et al., 1984) in *Experiment 3*.
4. Hanks' Balanced Salt Solution (HBSS, 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2 \times 2\ H_2O$, 0.4 mM $MgSO_4 \times 7\ H_2O$, 0.4 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, 6 mM $C_6H_{12}O_6$, pH 7.5, Sigma-Aldrich, H9269) in *Experiment 3*.

All chemicals were purchased from Reanal (Budapest, Hungary) and Sigma-Aldrich (Budapest, Hungary).

Grayling extender is a modification of the extender originally developed for common carp by Horváth et al. (2003) and later modified for salmonids and HBSS is standard solution that is used in a wide range of studies on fish sperm cryopreservation. To extract seminal fluid, carp sperm was centrifuged at 20,817*g* for 5 min at 4 °C (Eppendorf 5810R, Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany).

Diluted semen in all experiments was loaded into 0.5 mL straws (Minitube GmbH, Tiefenbach, Germany). Two different freezing methods were used for the cryopreservation of common carp sperm depending on the Section 2.5. As the first method, a