Seminal plasma transferrin effects on cryopreserved common carp *Cyprinus carpio* sperm and comparison with bovine serum albumin and antifreeze proteins

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A B S T R A C T

In the current study, there was evaluation of the cryopreservation effectiveness of common carp *Cyprinus carpio* sperm when cryopreservation medium was supplemented with proteins. Semen was diluted with Kurokura's extender composing 180 mM NaCl, 2.68 mM KCl, 1.36 mM CaCl$_2$, 2.38 mM NaHCO$_3$, and 10% dimethylsulfoxide (DMSO). Cryopreservation medium was supplemented with purified seminal plasma transferrin (Tf), bovine serum albumin (BSA) or antifreeze protein (AFP) Types I and III. Concentration of proteins evaluated was 0.1 µg/ml, 1 µg/ml, and 10 µg/ml. Motility and curvilinear velocity of spermatozoa was evaluated by the Computer Assisted Semen Analyzer (CASA). The percent of motile cells and spermatozoa curvilinear velocity of frozen-thawed sperm with supplementation of Tf and AFP III at all concentrations were greater compared to samples with no added proteins. The protective effect of BSA and AFP I was less and dose-dependent. Thus, it is concluded that incorporation of Tf in the extender before freezing improves crypreservation of common carp spermatozoa whereas supplementation with AFP III in greater concentrations was more effective.

1. Introduction

Freezing semen has become an important component of assisted reproduction technology that permits preservation of genetic information across generations and facilitates artificial reproduction without regard to the natural spawning period (Del-Valle et al., 2017). Cryopreservation of common carp *Cyprinus carpio* sperm have been developed, and modifications of the methods for species-specific optimization have been proposed (Linhart et al., 2000; Horváth et al., 2003; Boryshpolets et al., 2017). For example, Babiak et al. (1997) reported that successful cryopreservation of common carp sperm was achieved by using complex saline and/or sugar extenders with dimethylacetamide (DMA). The first successful cryopreservation of common carp sperm based on the use of NaCl and sucrose with the addition of dimethyl sulfoxide (DMSO) has been reported by Irawan et al. (2010). Although there are many reports for sperm cryopreservation of the Cyprinidae, investigation of freeze-thaw procedures that do not impair carp milt quality have yet to be developed.

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The cryopreservation process produces irreversible injury to the structure and disruption of the physiology of spermatozoa. Cryostress starts with temperature decrease and brings about changes to the functional state of proteins such as those related to capacitation and acrosome reaction (Tabuchi et al., 2008), membrane and structure (Desrosiers et al., 2006), and apoptosis (Jeong et al., 2009). Variation in protein content can induce a decrease in the common macro-indices of spermatozoa and the capacity of these cells for fertilization. The addition of proteins to the storage medium has been recommended to improve spermatozoon integrity (Beirao et al., 2012; Shaliutina-Kolešová et al., 2014). In mammals, proteins such as bovine serum albumin (BSA) decrease lipid peroxidation in the plasma membrane caused by reactive oxygen species and protect the membrane during freezing and thawing (Sariozkan et al., 2013; Fu et al., 2017). Supplementation with seminal plasma proteins in extenders increases the fertilization rate of *Oncorhynchus mykiss* sperm and reduces DNA damage (Öğretmen et al., 2015). The use of antifreeze proteins (AFP) in the cryopreservation solution helps to maintain the lipid composition of the plasma membrane (Beirao et al., 2012) and stabilizes *Sparus aurata* sperm by reducing freezing-induced changes in the protein pattern (Zilli et al., 2014).

Seminal plasma proteins can affect sperm characteristics including motility, capacitation, survival and longevity, and protection against damage (Dietrich et al., 2014). Transferrin (Tf, 80 kDa) is an iron-binding protein which has been detected in a variety of biological fluids in all vertebrates (Lambert et al., 2005). In fish, especially common carp, Tf is the major protein of seminal plasma with a concentration of 330–340 µg/ml (Wojtczak et al., 2005). Besides binding of iron, transferrin is recognized as a multi-functional protein and may protect spermatozoa from bacterial actions (Stafford et al., 2001; Stafford and Belosevic, 2003; Jurecka et al., 2009) and oxidative damage (Saleh and Agarwal, 2002) as well as protect the reproductive tract against cadmium toxicity (Dietrich et al., 2011).

Information on the effect of adding transferrin derived from fish seminal plasma to freezing medium on quality of cryopreserved sperm is limited. Consequently, the current experiment was conducted to explore the potential benefits of applying the purified seminal plasma Tf at different concentrations to improve motility and velocity of common carp sperm following cryopreservation.

2. Materials and methods

All chemicals were reagent grade or greater in purity and, unless otherwise stated, were purchased from Sigma-Aldrich. Antifreeze proteins AFP I and AFP III were purchased from A/F Protein Inc.

2.1. Source of semen

Six sexually mature male common carp *Cyprinus carpio* weighing 4.2–4.5 kg were used as semen donors. Fish were obtained in Qingdao, Shandong Province, P.R. China. Before the experiment, males were maintained in 3 m^3 outdoor plastic tanks with a flow-through pond water at a temperature of 20–22 °C.

2.2. Sperm collection

Males were injected with carp pituitary extract at 1 mg/kg 24 h before striping according to common commercial practices in the Czech Republic (Rodina et al., 2010). Sperm were collected by gentle abdominal massage and collected directly into 10-mL plastic syringes, taking care to avoid contamination with feces, mucus, or urine. The fresh sperm was divided into two parts to be used as control and for cryopreservation. Samples were stored for no longer that 30 min on ice (0–4 °C) in closed assay tubes until assessment. For isolation of transferrin, sperm of the six males was pooled and centrifuged at 5000 × *g* for 30 min at 4 °C. The supernatant seminal plasma was collected and centrifuged a second time under the same conditions and stored at −80 °C until purification. The purification procedure of Tf was conducted using affinity chromatography according to Dietrich et al. (2011).

2.3. Experimental conditions

The percent of motile cells and spermatozoa curvilinear velocity (VCL) were analyzed in fresh sperm (control), sperm frozen in extender only, and sperm cryopreserved with the addition of 0.1 µg/ml, 1 µg/ml, and 10 µg/ml of Tf, BSA, AFPI or AFPIII. The concentration of spermatozoa in each sample was measured using hemocytometry and expressed as 10^9 spermatozoa/ml of sperm.

2.4. Cryopreservation procedure

Cryopreservation of sperm from individual males was conducted as follow: the sperm were diluted with Kurokura's extender containing cryoprotectant [180 mM NaCl, 2.68 mM KCl, 1.36 mM CaCl$_2$, 2.38 mM NaHCO$_3$, and 10% dimethylsulfoxide (DMSO)] (Kurokura et al., 1984) at 1:5 (v/v) dilution ratio (sperm: [extender + cryoprotectant]) with and without the supplemental proteins at the specified concentrations. The samples were mixed thoroughly and placed into 0.5 ml plastic straws. Dilution and mixing took about 40 s. The straws were subsequently placed on a 3 cm high Styrofoam frame floating on liquid nitrogen for 15 min before being plunged into liquid nitrogen. Straws were then thawed for 5 s in a water bath at 40 °C (Rodina et al., 2010; Boryshpolets et al., 2017).

2.5. Spermatozoa motility and velocity

Percent motility (%) and spermatozoa velocity (µm/s) were assessed at 10 s post-activation with 10 mM Tris with 0.2% Pluronic F-
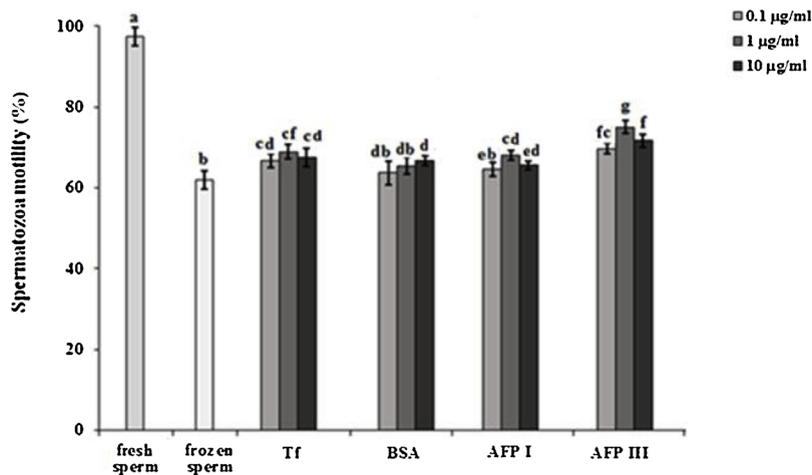
Fig. 1. Effect of transferrin (Tf), bovine serum albumin (BSA) and antifreeze proteins (AFP I and AFP III) supplementation at 0.1 μg/ml, 1 μg/ml, and 10 μg/ml on percentage of motile spermatozoa following freeze/thaw; Data are expressed as mean ± SD; Columns marked with the same superscript letter are not different (ANOVA, $P > 0.05$; $n = 6$).

127 (pH 8.0) medium (Linhart et al., 2000). Motility was assessed by using a computer-assisted sperm motion analysis system (CASAS-QH-III; Tsinghua Tongfang, Inc., Beijing, China). The software settings recommended by the manufacturer were adjusted to obtain a clear identification of the different spermatozoa. Light microscope, 10×20 magnification; temperature, room temperature ($18 \pm 1\,°C$) and video digitizing rate of 24 frames/s was used for determining of sperm motion characteristics. For each sample, the test time was no more than 30 s, and more than 100 spermatozoa were assessed. Assessment of spermatozoa motility variables was conducted in triplicate for each sample.

2.6. Statistical analysis

The statistical analysis was conducted using SPSS version 13.0 software (SPSS Inc. Chicago, Illinois, USA). Trials were conducted in triplicate. Normality and the homogeneity of variance of data were first tested with the Kolmogorov test and the Bartlett test, respectively. Data were expressed as mean ± standard deviation ($n = 6$) and compared using one-way analysis of variance (ANOVA) at the significance level of $P < 0.05$ followed by use of the Tukey's HSD test.

3. Results

The motility rate of fresh or cryopreserved sperm with or without Tf, BSA, AFP I, and AFP III varied (Figs. 1 and 2). The fresh sperm in the seminal plasma were immotile, and the flagella were straight and quivered slightly. After dilution with activation
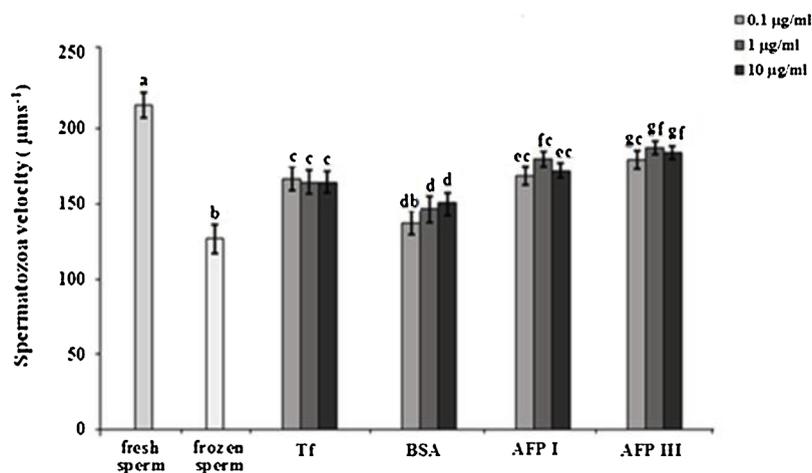
Fig. 2. Effect of transferrin (Tf), bovine serum albumin (BSA) and antifreeze proteins (AFP I and AFP III) supplementation at 0.1 μg/ml, 1 μg/ml, and 10 μg/ml on spermatozoa curvilinear velocity (VCL) following freeze/thaw; Data are expressed as mean ± SD; Columns marked with the same superscript letter are not different (ANOVA, $P > 0.05$; $n = 6$).

medium, the fresh sperm had 96%–100% motility and a curvilinear velocity of 216.30 ± 8.3 μm/sec. The cryopreservation procedure resulted in 62.2 ± 2.3% motility and VCL of 124.5 ± 9.3 μm/sec which was less than that of spermatozoa in fresh semen.

The addition of Tf to the cryopreservation medium was associated with mean motility of 66.5 ± 1.7% at a concentration of 0.1 μg/ml, 68.8 ± 2.1% at 1 μg/ml, and 67.4 ± 2.2% at 10 μg/ml. There were no differences (ANOVA; $P > 0.05$) in the percentage of motile spermatozoa in frozen/thawed samples with no supplementary protein from those with BSA at 0.1 and 1 μg/ml and AFP I of 0.1 μg/ml. The addition of AFP III at all concentrations that were assessed resulted in a greater (ANOVA; $P < 0.05$) motility rate than samples without protein and those containing BSA or AFPI, but there was no difference from Tf-cryopreserved samples at concentrations of 0.1 and 10 μg/ml (Fig. 1). For VCL, the value of transferrin-supplemented sperm was 166.3 ± 7.7 μm/s at 0.1 μg/ml, 163.6 ± 8.1 μm/s at 1 μg/ml, and 163.80 ± 7.23 μm/sec at 10 μg/ml which was greater compared to frozen-thawed samples without addition of proteins. Milt cryopreserved with BSA at 1 and 10 μg/ml had greater VCL values than milt not supplemented with BSA, but lesser values than samples containing Tf. The maximum VCL (185.20 ± 4.35 μm/s) was observed in spermatozoa cryopreserved with 1 μg/ml AFP III.

4. Discussion

In the present study, common carp spermatozoa could be 96%–100% activated after transfer to swimming medium. The freeze/thaw procedure impaired sperm functions [spermatozoa motility and curvilinear velocity (VCL)]. Because Tf is the major protein in common carp seminal plasma and has a protective effect on spermatozoa (Wojtczak et al., 2007), it was hypothesized that purified seminal plasma Tf could be supplemented to improve the spermatozoa motility rate and velocity after freezing-thawing. The results indicated that Tf is an effective additive to reduce damage caused by cryopreservation. The addition of BSA and AFP I also had a positive effect on the motility rate of post-thawed common carp sperm and 1 μg/ml of AFP III had the greatest effect on sperm motility and velocity. Nevertheless, compared to Tf, the results were highly dependent on the concentration of AFP III supplementation.

Spermatozoa motility and velocity is the basic standard for evaluation of cryopreserved sperm quality (Lahnsteiner et al., 2000). With use of cryopreservation, there is a decrease in values for spermatozoa motility variables (Boryshpolets et al., 2017). In the present study, addition of Tf resulted in an enhancement of motility rate of frozen-thawed sperm. The protective effect of Tf on frozen/thawed sperm may be attributed to several sources. Gomme and McCann (2005) reported that the biological actions of Tf include antioxidant effects as well as antibacterial activity. Supplementation of Tf in the extender may lead to prevention of damage by free radicals that are implicated in loss of motility during or immediately after cell freezing. It has been hypothesized that Tf induces adenosine triphosphate production, which is important for motility of spermatozoa (Macedo and de Sousa, 2008). Wojtczak et al. (2007) suggested that values for sperm motility variables are associated with transferrin polymorphism. Although Tf treatment had a beneficial effect on cryopreserved sperm quality, there was no obvious variation in motility and velocity as associated with the concentrations supplemented.

The use of BSA in cryopreservation medium had a concentrate-dependent action on the values for functional variables of common carp sperm during freezing (Figs. 1 and 2). The semen extender supplemented with BSA at 1 μg/ml and 10 μg/ml improved sperm velocity compared to that frozen only with DMSO, consistent with results from previous studies with mammals (Matsuoka et al., 2007; Hiwasa et al., 2009) and fish (Cabrita et al., 2001; Shaluei et al., 2017). The values were, however, less in frozen/thawed sperm containing Tf. This suggests that BSA may have a lesser stabilizing effect on the spermatozoon membrane during freezing than does Tf.

Antifreeze proteins are groups of proteins that bind to ice crystals and inhibit ice development through decreasing the freezing point to less than the equilibrium melting point. Antifreeze proteins also protect cell membranes from cryoinjury as a consequence of the inhibition of ice recrystallization (Knight et al., 1988; Garnham et al., 2008; Raymond et al., 2008). Recently, there have been reports of a marked capacity of AFPIII in the protection of sea bream (*Sparus aurata*) spermatozoa proteins and membrane lipid composition during the freezing-thawing procedure (Zilli et al., 2014). In contrast, Xin et al. (2018) reported that there were no significant differences in curvilinear velocity of spermatozoa between fresh and cryopreserved sperm with and without addition of AFPI or AFPIII. In the present study, there was assessment of effects of AFP I and AFP III as well. Samples cryopreserved in 1 μg/ml AFP III had a greater percentage of motile cells and spermatozoa VCL compared to sperm frozen in extender only and to that with supplementation of BSA and AFP I which is consistent with the findings of Beirao et al. (2012) in *S. aurata*. In this previous study and consistent with findings n the present study, the samples cryopreserved in extenders supplemented with AFP III had greater values for the variables that were assessed than did the transferrin-supplemented sperm. It can be speculated that AFPs have superior binding to the cell membrane compared to Tf or other proteins that were assessed in the present study and, as a result, stabilize the sperm membrane (Bagis et al., 2008). In addition, AFPs, especially AFP III, have an enhanced capacity to counteract the increase in saturated fatty acid proportions that occur during cryopreservation and interact preferentially with unsaturated fatty acids (Tomczak et al., 2002).

In conclusion, supplementation with different concentrations of Tf, BSA or AFPI/AFPIII in the extender had beneficial effects on motility and velocity of common carp sperm after freeze-thawing. Although supplementation with AFP III resulted in the greatest sperm motility rate and velocity, the effect of Tf was not directly concentration dependent in the way that occurs with the AFPs. Supplementation with Tf is, therefore, beneficial for preserving sperm quality during cryopreservation and values for functional variables can be improved for frozen/thawed spermatozoa even with Tf supplementation at low concentrations. With this preliminary study, there was identification of Tf as an additive to cryopreservation medium that can be supplemented at different concentrations for investigation of the mechanism of its protective role and applications in sperm cryopreservation in other species.

Conflict of interest statement

Acknowledgements

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