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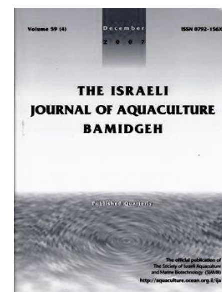

Vitrification of Common Carp (*Cyprinus Carpio*) Spermatozoa, Post-Thaw Sperm Quality, and Fertility

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Abstract

The aim of this investigation was to test a new technology, vitrification, or ultra-rapid freezing of the spermatozoa of common carp, and to study the ability of glucose, BSA, and other cryoprotectants to protect these cells from cryo-injuries. Spermatozoa were isolated and vitrified using 10 different cryoprotectant solutions: (1) Glucose based medium (GBM) + 1% bovine serum albumin (BSA); (2) GBM + 1% BSA + 10% DMSO; (3) GBM + 1% BSA + 20% DMSO; (4) GBM + 1% BSA + 30% DMSO; (5) GBM + 1% BSA + 10% DMA; (6) GBM + 1% BSA + 20% DMA; (7) GBM + 1% BSA + 30% DMA; (8) GBM + 1% BSA + 10% methanol; (9) GBM + 1% BSA + 20% methanol; (10) GBM + 1% BSA + 30% methanol. Fertilization rates for vitrification experiments were low and the use of low concentrations of cryoprotectants yielded lower fertilization rates than the vitrification solutions containing high cryoprotectant concentrations. In conclusion, this study reported successful vitrification of common carp spermatozoa by direct transfer into liquid nitrogen.

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Introduction

Research on cryopreservation of fish sperm, with a focus on cryopreservation protocols, has progressed significantly since the first successful cryopreservation of herring sperm 60 years ago. Cryopreservation enables ease of global shipping and supply of germplasm, selective breeding, and hybridization of desirable characteristics, as well as conservation of genetic diversity (Ohta et al., 2001; Bozkurt and Yavas, 2013). Furthermore, a sperm bank of frozen sperm could maintain a continuous and stable supply of gametes for hatchery seed production or laboratory experimentation (Lubzens et al., 1997).

According to freezing rate, sperm cryopreservation techniques can be divided into two main categories: slow freezing (conventional freezing), and ultrarapid freezing (vitrification). During conventional freezing, sperm cells undergo stress such as changes in osmotic balance and temperatures during cooling, freezing, and rewarming. These changes lead to ice crystal formation, which is a major biophysical factor causing sperm death (Holt, 2000; Bozkurt et al., 2005). Cryoprotective agents (CPAs) that permeate the cell membrane are needed to increase membrane fluidity and partially dehydrate the cell, lowering the freezing point, and thus reducing the number and size of intracellular ice crystals which form. However paradoxically, CPAs themselves can have a toxic effect on sperm and this effect is related directly to the concentration used, and duration of cell exposure (Swain and Smith, 2010).

Alternatively, vitrification is a cryopreservation technique used mainly to freeze embryos and tissues. The vitrification process solidifies the sample into a glass-like state, avoiding the formation of both intra and extracellular ice (Vajta and Kuwayama, 2006). Vitrification has been reported to be the most promising option for cryopreservation, and direct plunging of oocytes and embryos of several mammals including humans, into liquid nitrogen has proved successful (Bagchi et al., 2008; Liebermann and Tucker, 2002).

In fish, only a few studies have been published on spermatozoa vitrification in rainbow trout, *Oncorhynchus mykiss*, (Merino et al., 2012) and channel catfish, *Ictalurus punctatus*, (Cuevas-Uribe et al., 2011). Successful freezing of fish sperm by vitrification could be aided by more research not only into quick freezing but also into the benefits for aquaculture biotechnology (Merino et al., 2012). To our knowledge, there are no published records on the vitrification of spermatozoa of common carp *(Cyprinus carpio).*The aim of this study was to find an efficient method for vitrification by determining the effect of glucose based solution containing three different cryoprotectants at different concentrations, on post-vitrification sperm quality, and fertility of common carp.

Materials and Methods

Reagents. Three cryoprotectants, dimethyl sulfoxide (DMSO), dimethyl acetamide (DMA), and methanol (MeOH) were used at final concentrations of 10%, 20% and 30% (v/v) (Table 1).

Cryoprotectant	*Percent (v/v)*	*Molarity (mol/L)*	*Osmolality (mOsmol/kg)*
	10	1.41	2015
DMSO	20	2.82	3349
	30	4.22	5118
	10	1.08	828
DMA	20	2.15	1413
	30	3.23	1507
	10	2.47	271
Methanol	20	4.94	261
	30	7.41	234

Table 1. Cryoprotectant concentrations expressed as percent (v/v), molarity, and osmolality. The cryoprotectants were diluted in Hanks' balanced salt solution at 300 mOsmol/kg for osmolality measurement (Cuevas-Uribe et al., 2011).

Cryoprotectant solutions were prepared in a glucose based medium (GBM) which was 300mM glucose in 100 ml distilled water, modified from Tekin et al. (2003) at double the final concentration and kept cold (4°C) before being added at that temperature to the sperm suspension at a ratio of 1:3. The samples were held on ice during the experiment.

Broodstock management. Common carp (*Cyprinus carpio*) 4-year-old, sexually mature broodstock males (n=10), were supplied by State Hydraulic Works (SHW) Fish Production Station in Adana, Turkey. The broodstock was collected from wintering ponds by seining, and transported to the hatchery 48 h prior to gamete collection. During the experimental period, the broodstock was kept in shaded tanks (V=1000 l) supplied with continuously well-aerated water (2.5 l/min) at 24°C under a natural photoperiod. State of maturity was monitored from about 2 weeks before the expected date of spawning until the fish were in a running stage. The broodstock was fed ad libitum twice a day.

Gamete collection. The broodstock were anaesthetized in 100 ppm of MS 222 and semen was collected from males by manual abdominal stripping 12 h after a single injection of 0.5 pellet (kg/body weight) ovopel (Interfish, Hungary) at 24°C water temperature. The ovopel pellets were pulverized in a mortar and dissolved in 0.7% NaCl. The first injection, 0.5 pellet (kg/body weight), was given 12 h before the second injection which was 1.0 pellet (kg/body weight). (Horvath et al., 1997). Eggs were collected by hand stripping 12 h after a double injection of 1.5 ovopel pellet (kg/body weight). Their abdomens and urogenital papilla were dried before stripping. Samples contaminated with fecal material or urine were discarded. Only transparent, well rounded, unwrinkled eggs were used for fertilization.

Semen quality. Motility was estimated subjectively using a light microscope (Olympus, Japan) with 400x magnification. Samples were activated by mixing 1µl of sperm with 20µl activation solution (0.3% NaCl) on a glass slide. The motility percentage was defined as the percentage of spermatozoa moving in a forward motion. Spermatozoa density was determined according to the hemacytometric method (Akcay et al., 2004). Sperm was diluted at a ratio of 1:1000 with Hayem solution (HS) (5g Na2SO4, 1g NaCl, 0.5g HgCl2, 200 mL bicine) and density was determined using a 100µm deep Thoma hemocytometer (TH-100, Hecht-Assistent, Sondheim, Germany) at 400x magnification with an Olympus BX50 phase contrast microscope (Olympus, Japan). Counting chambers were kept humid for at least 10 min before cell counting. Spermatocrit was defined as the ratio of the total volume of white package material to the total volume of semen x100 (Rurangwa et al., 2004). Sperm pH was measured using indicator papers (Merck, 5.5-9) within 30 min of sampling. To assess viability of sperm (live/dead sperm percentage), eosin-nigrosin preparations were made according to the method noted by Björndahl et al. (2003). A total of 300 sperm cells were counted on each slide at 1000x magnification.

Spermatozoa preparation and semen dilution. Sperm with good initial motility (total motility > 80%) were pooled and chosen for vitrification experiments. Sperm was centrifuged at 300 g for 10 min at 4°C. GBM was used as the basic medium for handling the sperm samples and preparing the vitrification solutions. The semen was diluted gently at a ratio of 1:3 (v/v) with the GBM. These sperm suspensions were divided into ten equal aliquots and placed in ten different sterile tubes. All experiments were performed in triplicate.

Vitrification experiments. The following 10 experimental groups were formed for the spermatozoa vitrification experiments.
(1): GBM+1%BSA
(2): GBM+1%BSA10%DMSO
(3): GBM+1%BSA+20%DMSO
(4): GBM+1%BSA+30%DMSO
(5): GBM+1%BSA+10%DMA
(6): GBM+1%BSA+20%DMA
(7): GBM+1%BSA+30%DMA
(8): GBM+1%BSA+10%Methanol
(9): GBM+1%BSA+20%Methanol
(10): GBM+1%BSA+30%Methanol

The pooled sperm was adjusted to $5x10^6$ sperm/ml with GBM+1%BSA containing 10%, 20% and 30% DMSO, DMA and methanol separately. Cryoprotectant solutions were diluted with sperm suspension at a ratio of 1:3. Samples were loaded within 15 s

into 0.25 ml straws with a micropipette. Then the straws were submerged in liquid nitrogen (-196°C) for about 5 min. after which, straws were placed in goblets attached to a canister and stored in liquid nitrogen until thawing.

Thawing process. After storage for at least 48 hours in liquid nitrogen, the vitrified samples were warmed by plunging the straws into a water bath at 35°C for 25 s. The thawed sperm suspensions were then placed in 10 ml tubes containing 5 ml GBM+1%BSA at 10°C for 10 min prior to centrifuging at 360 g for 5 min. Motility percentage of spermatozoa was performed using phase contrast microscope.

Fertility assessment. Fertilization and incubation were conducted at room temperature (20±1°C). Eggs from 5 females were collected. Fertility assessment was performed according to the procedure described by Valdebenito et al. (2010). A sample of pooled eggs from five females was divided into batches of 100 eggs each in sterile plastic containers and their quality was assessed microscopically. The sperm suspensions (post-vitrification) were poured evenly over the eggs and stirred gently. The sperm density used was 1×10^5 spermatozoa/egg for all treatments. The spermatozoa were activated by addition of 10 ml of 0.3% NaCl solution. After 5 min of incubation, 30 ml of fertilization solution (0.3g of urea, 0.4g of NaCl in 10ml distilled water) was added in order to prevent stickiness of eggs (dry fertilization). All fertilization experiments were performed in triplicate and eggs were incubated in Zuger-type hatching jars at 24°C. The fertilization ratios were counted at the 4-cell stage under a stereomicroscope at 20x magnification.

Statistical analysis. All data are expressed as mean±standard deviation. Relative quantities were transformed by angular transformation and metric data were tested for normality. Data were analyzed one-way multifactorial variance (ANOVA) with subsequent Tukey's-b test and the results are presented as mean±SE. Significance level was $P<0.05$. Statistical analyses were performed with SPSS 10 for Windows statistical software package.

Results

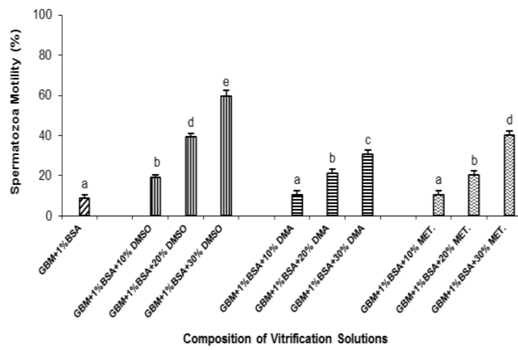
Evaluation of spermatozoa quality and vitrification. Mean motility of fresh sperm collected from five male broodstock was determined as 92.0±2.0% and motility period as 54.8±1.6s (Table 2).

	Minimum	*Maximum*	*Mean*	*SD*
Volume (ml)	2.4	6.8	5.1	0.7
Motility (%)	85	95	92.0	2.0
Motility period (s)	39	72	54.8	1.6
Density ($\times10^9$/ml)	15.2	23.6	19.4	1.4
Total density ($\times10^9$)	36.4	139.2	69.3	18.1
Spermatocrit (%)	68.2	79.5	72.6	1.2
pH	7.2	7.6	7.4	0.01

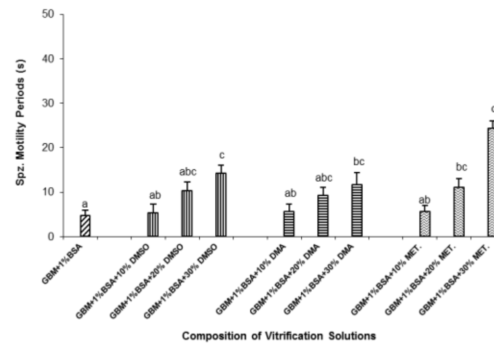
Table 2. Sperm quality variables (%) recorded in freshly collected common carp (*Cyprinus carpio*) semen (mean±SD; n=5).

Mean motility and motility period of pooled sperm was determined as 80.7±1.5% and 32.6±1.4s. respectively. It should be noted that motility, motility period, and viability rates of vitrified sperm decreased sharply compared to fresh sperm ($p<0.05$). Mean post-vitrification sperm motility, motility period, and viability results are presented in Figures 1(a), (b), (c). The highest post-vitrification motility was determined in 30% DMSO group. On the other hand, higher motility periods and viability rates were found in the methanol treatments as compared to other tested groups. In other words, supplementation of methanol to the GBM had a better cryoprotective effect on sperm motility and viability against freezing damage than other tested groups ($p<0.05$) (Figure 1).

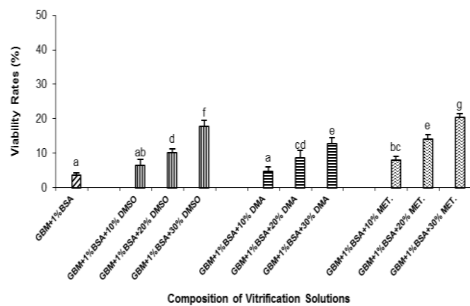
Fertilization experiments. The highest fertility rate was determined as 48.0±1.15% by spermatozoa vitrified in GBM medium+1%BSA+30% methanol (Figure 2). There were statistically significant differences between tested groups containing DMSO, DMA and methanol ($p<0.05$). The spermatozoa in the three groups supplemented with different cryoprotectants showed significantly lower fertilization rates than fresh sperm (84.6±2.4%) ($p<0.05$).



(a)



(b)



(c)

Figure 1. (a) Motility, (b) motility period and (c) viability of vitrified common carp spermatozoa.
Different lowercase letters on bars indicate significant differences ($p<0.05$).

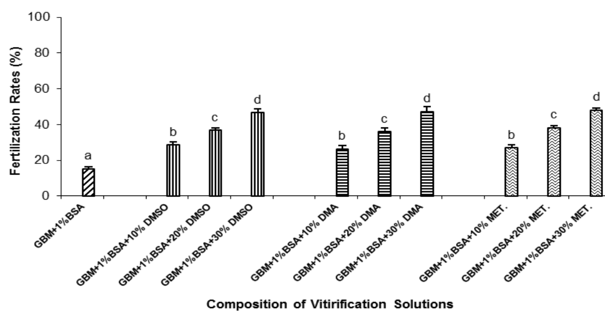


Figure 2. Fertility of vitrified common carp spermatozoa. Different lowercase letters on bars indicate significant differences ($p<0.05$).

Discussion

Conventional slow freezing techniques have been well investigated and it has been shown that these techniques cause extensive physicochemical damage to extra- and intracellular membranes of the sperm due to changes in lipid-phase transition or increase in lipid peroxidation during saturation with cryoprotectants, freezing, or after thawing (Gao et al., 1997). On the other hand, vitrification is a simple and less expensive alternative to conventional freezing. Vitrification is the process of forming a glass-like solid without the formation of ice-crystals. These cooled solutions allow the direct plunging of prepared cell samples into LN_2. The purpose of vitrification is to reach the glass transition temperature as quickly as possible through rapid cooling by increasing the concentration of cryoprotectants (Merino et al., 2012). In general, permeable cryoprotectants are used to protect spermatozoa from the negative effect of low temperatures caused by conventional freezing. This process may offer some practical benefits in cryopreservation, such as time saving, and eliminating the need for expensive programmable freezing equipment.

Previous studies to cryopreserve aquatic species sperm by plunging them into liquid nitrogen have produced inconsistent results. While one study using sperm from the

Pacific herring (*Clupea pallasi*) yielded fertilization rates as high as 95% (Pillai and Yanagimachi, 1994), most studies have been unsuccessful. The low success rates reported in most other studies where samples were submerged in liquid nitrogen is probably due to a combination of insufficient cryoprotectant concentration, long pre-freeze exposure times, large sample sizes, and use of containers that inhibit heat transfer, and slow cooling rates. A high cryoprotectant concentration (40% ethylene glycol) which had the potential to achieve glass formation was used, but exposure time was long (2 h), and the sperm most likely experienced damage due to cryoprotectant toxicity before the onset of cooling (Trus-Cott et al., 1968).

In the present study, the use of low concentrations of cryoprotectants yielded low post-thaw motility, viability, and fertilization, while vitrified solutions containing high cryoprotectant concentrations increased fertilization rates. High fertilization in the present study was probably due to partial intracellular vitrification and chemotaxis where the sperm became activated when coming in contact with egg chorion (Morisawa, 2008). Chemotaxis is an important factor in egg fertilization because carp eggs contain proteins that facilitate the union of the gametes. The high fertilization in the present study could be explained by the unique fertilization strategy employing these proteins that guide the sperm into the micropyle.

In our study common carp spermatozoa were able to survive the CPA-free vitrification protocol by using only GBM and 1%BSA. The most suitable cryoprotectants (DMSO, DMA and methanol) were used. Methanol has a high rate of permeability and relatively low toxicity, while DMSO and DMA are considered to form good glass and may be useful in vitrification solutions. In addition to these cryoprotectants, BSA was added to inhibit ice crystal formation (Figueroa et al., 2013) and enhance glass formation at lower concentrations of cryoprotectants. However this fertilization percentage is still lower than the rates observed for fresh semen (99%) or conventionally frozen sperm (around 70%) (Cabrita et al., 2005; Bozkurt et al., 2012). Our preliminary results are therefore promising, especially considering the role of the BSA during the process.

Data regarding the application of vitrification for fish spermatozoa are limited. Our study examines the protective effect of three permeable CPA concentrations on vitrification of common carp sperm. The results of this study for spermatozoa vitrification from common carp indicate that fish spermatozoa can be vitrified in GBM+1%BSA containing high concentrations (30%) of DMSO, DMA, and methanol, without significant loss of important physiological characteristics however more testing is required to improve these results.

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