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Effect of semen extender supplementation with cysteine on postthaw sperm quality, DNA damage, and fertilizing ability in the common carp (*Cyprinus carpio*)

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ABSTRACT

Amino acids have an important biological role for prevention of cell damage during cryopreservation. The objective of this study is to determine the effects of cysteine on postthaw sperm motility, duration of sperm motility, DNA damage, and fertility in the common carp (*Cyprinus carpio*). Sperm collected from 10 individuals was cryopreserved in extenders containing different cysteine concentrations (2.5, 5, 10, and 20 mM). Semen samples diluted at the ratio of 1:9 by the extenders were subjected to cryopreservation. After dilution, the semen was aspirated into 0.25-mL straws; the straws were placed on the tray, frozen in nitrogen vapor, and plunged into liquid nitrogen. DNA damage was evaluated by comet assay after cryopreservation. Our results indicated that an increase in the concentration of cysteine caused a significant increase in the motility rate and duration of sperm in the common carp (*C carpio*; $P < 0.05$). Comparing all concentrations of cysteine, the best concentration of cysteine was 20 mM. Higher postthaw motility (76.00 ± 1.00%) and fertilization (97.00 ± 1.73%) rates were obtained with the extender at the concentration of 20 mM. Supplementation of the extender with cysteine was increased the fertilization and hatching rate and decreased DNA damage. Consequently, cysteine affected the motility, fertilization, and DNA damage positively, and extenders could be supplemented with cysteine.

1. Introduction

The cryopreservation of fish sperm is a valuable technique because it plays an important role in transporting genetic material between facilities, optimal use in aquaculture, reducing the risk of spreading infections, performing hybridization studies, protecting gene pool, conserving biodiversity, selective breeding activities, and protecting endangered species [1–4].

The sperm is protected against oxidative stress with seminal plasma. Dilution during cryopreservation reduces the seminal plasma components having cells more sensitive to oxidative stress [5]. Amino acids have antioxidant properties and found in seminal plasma at high concentrations. Therefore, amino acids have an important biological role for prevention of cell damage during cryopreservation [5–8]. Thus far, conducted studies in mammalians have reported that supplementation of amino acids (e.g., taurine, hypotaurine, proline, glutamine, glycine, histidin, cysteine) to extenders reduced sperm damage and DNA fragmentation and improved postthaw motility [9–12]. Recently, studies about benefit from antioxidant property and addition to extenders of amino acids have been performed in different fish species

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(e.g., *Dicentrarchus labrax*, *Sparus aurata*, *Oncorhynchus mykiss*, *Cyprinus carpio*) [5–8]. Cysteine, which is a sulfur-containing amino acid, scavenges free radicals through direct chemical interactions with them [13,14]. As concerns fish, the knowledge about the use of extenders containing cysteine is limited. Stejskal et al. [15] determined the effect of cysteine in the sperm of the *Acipenser baerii* and *Acipenser ruthenus*, *Perca fluviatilis*, and *Sander lucioperca*. Kledmanee et al. [16] stated that the percentage of sperm motility, duration of sperm motility, and the percentage of sperm viability of the common carp (*C carpio*) semen stored with L-cysteine were positively affected. In this framework, the present study was conducted to obtain more information about the effect of supplementation of extenders with cysteine on motility and fertility of sperm in the common carp (*C carpio*).

DNA damage is one of the important indicators because it is a measure of decreasing sperm quality and fertilization [7,17]. Unsaturated fatty acids in plasma membranes of spermatozoa are very sensitive to free radical attack [18–20] and reactive oxygen species (ROS), which can cause DNA damage in spermatozoa [21–24]. Studies about DNA damage in spermatozoa were determined using a comet assay technique, which measures DNA breakage in individual cells [25], combining electrophoresis with fluorescence microscopy to visualize DNA migration from the individual cells in an agarose microgel [26], in the rainbow trout [27], sea bass [28], gilthead sea bream [29], Pacific oyster [30], loach [31], and freshwater catfish [7]. The aim of the study was to investigate the effect of supplementation of extenders with different cysteine concentrations (2.5, 5, 10, 20 mM) on the common carp (*C carpio*) sperm cryopreservation. The specific objectives were to (1) assess sperm quality, (2) evaluate the DNA damage of cryopreserved sperm, (3) evaluate the best concentration of cysteine, and (4) evaluate the fertilization and hatching rates of eggs using cryopreserved sperm.

2. Materials and methods

2.1. Collection of sperm

This study was performed in accordance with the ethical guidelines stipulated by the ethical committee of the University of Muğla. This study was conducted at the hatchery of General Directorate of State Hydraulic Works, Fish Production Station (Adana, Turkey) during May. Common carp males used in this study were 3 to 4 years of age. Ten mature common carp males (2.77 ± 0.52 kg, 42.5 ± 3.3 cm as mean ± standard deviation) were randomly selected from the stock pond for sperm collection. Water temperature and oxygen were 22 ± 1 °C to 8.1 ± 0.4 mg L^{-1}, respectively. Males and females were anesthetized in 1:3000 aqueous solution of 2-phenoxyethanol and given a single injection of 1 pellet kg^{-1} of Ovopel before 24 hours of stripping. The males were removed from the tanks and dried with a cloth towel. The initial male ejaculate was discarded, and the external urogenital pore was wiped dry with a paper towel to avoid water, urine, and feces contamination. The sperm was collected by a gentle abdominal massage, collected separately into glass vials and stored on ice (2 °C–4 °C) until use. Sperm from 3 of 10 carp males were selected and used for cryopreservation individually with the following cryomedia.

2.2. Sperm cryopreservation

The control group was diluted (1:9) in a modified Kurokura extender composed of the following: 360-mg NaCl, 1000-mg KCl, 22-mg $CaCl_2$, 8-mg $MgCl_2$, 20-mg $NaHCO_3$ for 100-mL distilled water, pH 8.2, osmolarity 365 mOsm, 12.5% Me_2SO as a permeating cryoprotectant, 10% hen's egg yolk used as a nonpermeating cryoprotectant [32].

Cysteine was separately added to the extender (one per experimental group) at the following concentrations: 2.5, 5, 10, and 20 mM. After dilution, the sperm was aspirated into 0.25-mL straws, sealed with polyvinyl alcohol, equilibrated at a temperature of 2 °C to 4 °C for 5 minutes, vaporized at a height of 3 cm above liquid nitrogen surface for 10 minutes, and plunged into liquid nitrogen. At least 10 straws per sperm sample were frozen. After 7 days of storage in liquid nitrogen, the samples were thawed in a water bath at 20 °C for a period of 30 seconds. After thawing, each sample was evaluated the percentage of spermatozoa motility, viability, and DNA integrity. The percent of motile spermatozoa and motility duration were immediately recorded for 1 minute after activation using a CCD video camera mounted on a phase-contrast microscope (Zeiss Axio Scope with AxioVision) at room temperature (20 °C). The percentage of sperm motility was estimated as the cell performing progressive forward movement, whereas the duration of motility was determined as the time until forward movement stops. The percentage of sperm motility was assessed using an arbitrary scale with 10% interval increments in which nonmotile represents 0% [32]. Three aliquots of each milt sample were inspected to calculate an average motility. An activating solution, composed of 45-mM NaCl, 5-mM KCl, 30-mM Tris-HCl, pH 8.2, was used for freshly collected and cryopreserved samples.

2.3. Fertilization and hatching assays

For fertilization assay, three females were stripped and mixed eggs were used for fertilization within 1 hour after collection. After egg stripping, 3 g of eggs (approximately 2500–3000 eggs) was used to fertilize either fresh or thawed sperm in a 500-mL round-bottomed dish. Eggs were fertilized at a ratio of approximately 1×10^5 spermatozoa/egg with an activating solution [33]. Dishes were placed on an experimental stand that supplied with hatchery water flow rates, 10 L/h for each experimental group. Fertilization ratios were evaluated from triplicates and recorded 3 hours after insemination, and dead eggs were counted and removed in each dish during incubation. Eyed embryos were counted after the third day and hatched fry the fifth day of incubation at 22 ± 0.5 °C.

2.4. Assessment of sperm DNA damage by comet assay

Sperm DNA damage was investigated using the single-cell gel electrophoresis (comet) assay that was generally performed at high alkaline conditions. Firstly, the sperm was

embedding in the agarose gel. Each microscope slide was precoated with a layer of 1% normal melting point agarose in PBS (20-g NaCl, 0.5-g KCl, 0.5-g KH_2PO_4, 2.9-g Na_2HPO_4, and 8-g Tris-HCl) and dried at room temperature for 15 minutes. Next, 0.5% low melting point agarose at 37 °C was mixed with the cell suspension at a ratio of 1:9 and dripped onto the first layer. Slides were allowed to solidify for 20 minutes at 4 °C in a moist box. Then, the coverslips were removed, and the slides were immersed in freshly prepared cold lysis buffer containing 36.75-g NaCl, 9.3-g EDTA, 0.3-g Trizma base, 1-mL Triton X-100 (pH 10) for 50 minutes at 4 °C. After 50 minutes, the slides were removed from the lysis buffer, drained, and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, which contained 6-g NaOH, 0.18-g EDTA with pH greater than 13 (for 500 mL). The slides remained in the unit for 25 minutes to allow the DNA to unwind. Electrophoresis was conducted at 40 V/300 A for 25 minutes. After electrophoresis, the slides were neutralized in neutralization buffer (250 mL; 1.82-g Tris-HCl, 0.125-g Trizma base and pH 7.5) and fixed for 20 minutes. The slides were stained with 70-μL diluted ethidium bromide, covered with a coverslip, and analyzed using a fluorescence microscope. Comet images were analyzed with the Comet Score 1.5 software to obtain parameters such as comet length, tail length, and % DNA in the tail. The comet rate and damage coefficient were calculated as follows: comet rate = (comet cell number/total cell number) × 100%.

2.5. Statistical analysis

Results are expressed as mean ± standard deviation. Sperm motility, motility characteristics, DNA damage, fertilization rate data were analyzed by ANOVA, followed by the Duncan *post hoc* test to determine significant differences between the groups. Differences with values of $P < 0.05$ were considered to be statistically significant. Statistical analyses were performed using the SPSS 14.0 package program.

3. Results

In fresh semen, the percentage and duration of motile spermatozoa were 93 ± 5.0% and 59 ± 7.0 seconds, respectively. Means of postthaw motility, fertilization, hatching, and DNA damage rates for all the concentrations with the common carp (*C carpio*) sperm were presented in Table 1. The findings of the present study showed that an increase in the concentration of cysteine in extenders caused a significant increase in the motility rate of sperm in the common carp (*C carpio*; $P < 0.05$).

The addition of cysteine to the extender increased the postthaw motility duration. Differences in the motility duration of frozen-thawed sperm were significant among the treatments ($P < 0.05$), although fertilization was not affected by cysteine significantly.

A postthaw fertilization rate of 94.33 ± 1.15% was obtained with the control group. Supplementation of the extender with cysteine increased the fertilization and hatching rate. A higher postthaw fertilization rate (97.00 ± 1.73%) was obtained with the extender at the 20-mM concentration. Differences in the hatching rate were significant among the treatments ($P < 0.05$).

Damage of DNA after cryopreservation, using the aforementioned mentioned four different concentrations, was compared in samples. DNA damage was divided into five grades according to the method of Goerin [34]. Among the cryopreservation treatments, there were significant differences ($P < 0.05$). Cells cryopreserved in the absence of cysteine showed the largest tails and the highest percentage of DNA damage. Supplementation of the extender with cysteine decreased the percentage of DNA damage.

4. Discussion

Overall, we found that the addition of cysteine to the extender significantly increased the postthaw motility rate and duration, fertilization success and decreased DNA damage.

Antioxidants are useful for inhibition of ROS generation [35]. Cysteine is a naturally occurring sulfur-containing nonessential amino acid. It has antioxidant properties because of being an important precursor in the production of antioxidant glutathione, which protects cells from free radicals [36]. Recently, studies about the addition of amino acids, having antioxidant properties, to extenders were performed in different fish species [5–8]. Especially, studies about the use of extenders containing cysteine is limited in fish species [15]. In studies about supplementation of the extender with amino acids, it has been reported that amino acids reduced both DNA fragmentation parameters and protecting DNA against strand breaks, although they were not significantly affected the postthawing motility

Table 1
Mean ± standard deviation postthaw motility, fertilization, hatching, and DNA damage rates for all concentrations with the common carp (*Cyprinus carpio*) sperm (n = 3).

	Motility (%)	Duration (s)	Fertilization (%)	Hatching (%)	DNA damage (%)
Control	46.67 ± 2.52^{a}	56.00 ± 1.00	96.00 ± 1.00	17.33 ± 2.08^{a}	75.00 ± 25.00^{a}
2.5 mM	50.00 ± 1.00^{b}	55.00 ± 2.00	96.00 ± 1.00	34.00 ± 4.58^{b}	33.33 ± 14.43^{b}
5 mM	64.67 ± 1.53^{c}	54.00 ± 0.00	96.33 ± 2.89	40.33 ± 3.51^{b}	8.33 ± 14.43^{bc}
10 mM	73.00 ± 2.00^{d}	56.33 ± 1.53	95.33 ± 1.53	60.33 ± 4.51^{c}	8.33 ± 14.43^{bc}
20 mM	76.00 ± 1.00^{e}	56.00 ± 1.00	96.00 ± 2.65	55.00 ± 6.00^{c}	0.00 ± 0.00^{c}
P	f	g	g	f	f

Different superscript letters (a, b, c, d and e) show differences between treatments ($P < 0.05$).
[f] The same column shows significant differences among proportions ($P < 0.05$).
[g] The same column shows no significant differences among proportions ($P > 0.05$).

percentages, motility duration, and sperm motility parameters (the percentage of motile cells [TM], motile cells with progressive movement [PM], curvilinear velocity [VCL], straight line velocity [VSL]) of sperm. In contrast, Kledmanee et al. [16] determined that L-cysteine increased the percentage of sperm motility, duration of sperm motility, the percentage of sperm viability, and fertilization capacity. Similarly, in the present study, the addition of cysteine to the extender increased the postthaw percentage of sperm motility, duration of sperm motility, and fertilization and hatching rates. This may be due to the fact that cysteine is involved in electron transfer reactions, assists an enzyme catalyzing reaction, and also involves in the production of antioxidant glutathione in the intracellular manufacture [36].

Sperm quality and viability are reduced because of changes in membrane fluidity and integrity, impairment of lipid–protein interactions, and modifications of DNA and proteins after cryopreservation [37,38]. Motility, membrane stability, spermatozoa functionality, and DNA integrity are also affected by oxidative stress because of generation of ROS during dilution in the extender media, cryoprotectant exposure, and cooling process [5,39–41]. Moreover, DNA damage could be a result of free radical–induced damage because of ice crystal formation and recrystallization during the freezing–thawing procedure [7,42,43]. DNA integrity is one of the indicators of cryopreservation success because of preserving genetic material and can be used to select the best treatment for fertilization trials [44]. DNA damage caused reduced growth, abnormal development, and reduced survival of embryos, larvae, and adults because of DNA strand break [45,46]. In addition, the skeletal abnormality occurrence in hatched larvae [47] and abnormal embryogenesis of the progeny leading to death, before hatching may occur, because of DNA damage [48]. Studies about DNA damage after cryopreservation were performed in several species using the comet assay [27,28,49,50]. In some studies, it has been reported that the cryopreservation process affected DNA stability by reason of DNA fragmentation [27,28,50]. Rani et al. [7] suggested that the main reason of DNA damage is the toxicity of cryoprotectant. In contrast, Song et al. [51] stated that mechanical injury on sperm DNA stability was negligible. Suquet et al. [52] detected that there were no genome alterations in the turbot, *Psetta maxima*, sperm after cryopreservation. Gwo et al. [53] determined that the nucleus of the Atlantic croaker, *Micropogonias undulatus*, sperm was not affected from the freeze-thaw process. In this study, it was detected damage of DNA after cryopreservation and also an increase in the concentration of cysteine in the extender caused a significant decrease in DNA damage of sperm in the common carp. Similarly, Cabrita et al. [5] reported that the amino acids, taurine and hypotaurine, significantly reduced DNA fragmentation in the gilthead seabream (*S aurata*) and European seabass (*Dicentrarchus labrax*). In addition, previous studies showed that L-cysteine improved viability of spermatozoa by reducing lipid peroxidation of sperm plasma membrane and preventing DNA damage of spermatozoa from ROS during cryopreservation in fish [15,54].

Determination of the best concentration and combination of amino acids is important for cryopreservation success. In previous studies, it has been reported that too high amino acid concentration negatively affected sperm quality because of osmotic toxicity and hypertonicity [55–59]. In a study on the effect of L-cysteine on chilled carp (*C carpio*) semen qualities, Kledmanee et al. [16] used different concentrations of L-cysteine (0, 0.5, 1, 1.5, and 2 mM), and they suggested that an increase in the concentration of L-cysteine caused low semen qualities because of its toxic effect. In contrast with this result, higher postthaw percentage and duration of motility, fertilization, and hatching rate were obtained with the extender at the 20-mM concentration. Additionally, the increase of concentration decreased DNA damage of common carp (*C carpio*) sperm.

In conclusion, preserving genetic material in the cryopreservation process is very important for the development of embryo, larvae, and adults. The results from the present study indicated that addition of cysteine to extenders significantly increased sperm motility and duration, and fertilization in the common carp (*C carpio*). Supplementation of the extender with cysteine decreased DNA damage. Further research could be performed to obtain better information in terms of the DNA integrity and fertilizing capacity and to select the best concentration of cysteine.

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