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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