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CRYOPRESERVATION OF GOLDFISH (*Carassius auratus*) SPERMATOZOA: EFFECTS OF EXTENDER SUPPLEMENTED WITH TAURINE ON SPERM MOTILITY AND DNA DAMAGE

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Abstract

BACKGROUND: Amino acids, present in seminal plasma at high concentration, protect spermatozoa against cell damage during cryopreservation. **OBJECTIVE:** Experiments were designed to analyze the effect of semen extender supplemented with taurine on post-thawed sperm motility and duration, as well as DNA damage. **MATERIALS AND METHODS:** Extenders were supplemented with 1, 2 or 4 mM taurine. Semen samples were diluted at the ratio of 1:9 with the extenders. Diluted samples were aspirated into 0.25 ml French straws and 0.1 ml pellets. DNA damage was assessed with the comet assay after cryopreservation. **RESULTS:** The percentage and duration of sperm motility were significantly increased by taurine. Additionally, sperm motility and the motility period in pellets were higher than in straws. The best concentration of taurine was 4 mM, and the highest post-thaw motility rate (72.50±3.54%) and duration (17.50±0.71 s) were obtained from the extender with 4 mM in pellets. DNA damage was decreased after taurine supplementation. **CONCLUSION:** Pellets could be used for goldfish sperm cryopreservation. The addition of 4 mM taurine increases the post-thaw motility and decreases DNA damage on goldfish semen.

Keywords: Amino acid, *Carassius auratus*, goldfish, taurine, sperm quality.

INTRODUCTION

Long-term semen storage is important for preserving valuable genetic resources through sperm banks, being the guarantee of a constant commercial supply of semen (6), maintaining hatchery seed production with cryopreserved sperm (25), selective breeding and hybridization with desirable characteristics (18, 21, 25), as well as protecting endangered species (22, 31). The optimal diluent composition, cryoprotectant concentration, sperm packaging method, dilution ratio, equilibrium time, freezing/thawing rate, and storage vessel are important for the the succeess of cryopreservation (7, 40). Sperm packaging units (i.e., straws, pellets, ampoule, capillary, cryotube, aluminum disc and vial) affect post-thaw sperm quality, fertilization and hatching rate (6, 10, 19). The assessment of the best sperm packaging unit is also important for successful cryopreservation for a given species.

The freezing/thawing process causes the generation of reactive oxygen substances (ROS). Sperm cells are susceptible to lipid peroxidation (LPO) due to the high concentration of poly-unsaturated fatty acids (PUFA) (1, 11, 34). Hence, post-thaw motility, viability, intracellular enzymatic activity, fertility and sperm functions, DNA integrity are impaired by cold shock during sperm cryopreservation (1). Due to their antioxidant property, amino acids could reduce LPO during freeze/thaw. In recent years, amino acids, such as taurine, hypotaurine, proline, glutamine, glycine, histidine, cysteine, and methionine, have been used as antioxidants for sperm cryopreservation of different fish species (e.g. *Dicentrarchus labrax*, *Sparus aurata*, *Oncorhynchus mykiss*, *Cyprinus carpio*) (8, 15, 20, 32, 43).

Taurine (2-aminoethanesulfonic acid) is a sulphonated beta amino acid (37) and has many physiological and pharmacological functions, including membrane stabilization, antioxidation, osmoregulation, modulation of ion flux, and control of Ca^{2+} homeostasis (35). ROS is reduced by taurine, reducing membrane damages

during cryopreservation (16). To date, studies about addition of taurine to extenders have been performed in different fish species (*O. mykiss*, *D. labrax*, *S. aurata*, *Pagrus major*) (8, 15, 25, 28). These studies show taurine improved sperm quality and decreased DNA damage.

Goldfish (*C. auratus*) has received great attention in the world ornamental aquarium industry due to its varieties with attractive body shape and skin color (26, 45). Although several studies have investigated the effects of amino acids (taurine, hypotaurine, proline, glutamine, glycine, histidine, cysteine, methionine) in different fish species (e.g. *D. labrax*, *S. aurata*, *O. mykiss*, *C. carpio*) (8, 15, 20, 32, 43), there is no report on supplementation with amino acid to extenders for goldfish sperm cryopreservation. Thus far, conducted studies in goldfish are generally about the determination of the best extender, sperm quality, DNA damage, fertilizing ability of cryopreserved goldfish sperm using different cryoprotectants (30, 42). The main goal of the study was to investigate the effect of the supplementation of extender with different taurine concentrations on goldfish (*C. auratus*) sperm cryopreservationm, including the assessment of sperm quality, packaging methods (straws and pellets) and DNA fragmentation.

MATERIALS AND METHODS

Sperm collection

Six mature goldfish males (10.77±0.72 cm, 32.92±4.33 g, as mean±SD) were selected from stock aquarium in Muğla Sıtkı Koçman University (Muğla, Turkey). Temperature and oxygen values in aquariums were 18±1ºC and 8.5±0.4 mg l^{-1}, respectively. The males were anesthetized with 2-phenoxyethanol (1:3000 aqueous solution) before stripping. Males with detectable running sperm in response to soft abdominal pressure were given a single injection of ovopel (1 pellet/kg body weight). Twenty four hours after treatment, the urogenital papilla was carefully dried, and sperm was hand-stripped directly into glass vials and stored on ice (2-4°C) until use. Caution was exercised to prevent contamination of the semen with urine, feces, blood, mucus or water.

Sperm cryopreservation and motility assessment

Freezing protocol was performed according to the method described by Magyary et al. (27) for semen cryopreservation. Semen samples were diluted in a modified Kurokura (MK) (360mg NaCl, 1000mg KCl, 22mg $CaCl_2$, 8mg $MgCl_2$, 20mg $NaHCO_3$ for 100ml pure water, pH 8.2, Osmolarity 365 mOsm, 12.5% Me_2SO as permeating cryoprotectant, 10% egg yolk used non-permeating cryoprotectant) in a ratio of 1:9 [semen: modified Kurokura (MK) medium]. Taurine was separately added to the extender (one per experimental group) at 1, 2 or 4 mM. After dilution, the sperm was aspirated into 0.25 ml straws and sealed with polyvinyl alcohol. For freezing pellets, an aluminum tray was placed on 3 cm above the vapor of liquid nitrogen; 0.1 ml pellets were dropped on the aluminum tray and kept for 10 min, then plunged into liquid nitrogen. Samples were equilibrated at temperature 2-4ºC for 5 min, vaporized at a height of 3 cm above liquid nitrogen surface for 10 min and plunged into liquid nitrogen. At least five straws and pellets per sperm sample were frozen. The samples were thawed in a water bath 40ºC for a period of 5 s. After thawing, each sample was evaluated for the percentage and duration of spermatozoa motility and DNA integrity. The percent of motile spermatozoa and motility duration was immediately recorded for 1 min post-activation using a CCD video camera mounted on a phase-contrast microscope (Zeiss Axio Scope with AxioVision) at room temperature (20°C). The obtained video records were scanned to determine the percentages of the progressive motility (%) and the durations of progressive motility (s). The percentage of sperm motility was estimated as the cell performing progressive forward movement, while 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%. An activating solution composed of 45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8.2 was used to freshly collected and cryopreserved samples.

Assessment of sperm DNA damage

The comet assay was used to examine DNA fragmentation of cryopreserved samples, and performed according to the method as described in Öğretmen et al. (31). After electrophoresis, the slides were photographed using PI to stain DNA and observe the comets. Comet images were analyzed with Comet Score 1.5 software to obtain parameters such as comet length, tail length, and % DNA in the tail. The extent of DNA damage was assessed from the length of DNA migration derived by subtracting the

diameter of the nucleus from the total length of the image. Grading was as follows: Grade I: tail length/diameter of the nucleus <1; grade II: tail length/diameter of the nucleus <2; grade III: tail length/diameter of the nucleus =2. Grades I and II indicate generic rupture of the DNA chain. Comet rates were calculated as: comet rate = (comet cell number / total cell number) ×100%.

Statistical analysis

Results were expressed as mean±S.D. Sperm motility duration, and Comet rate data were analyzed by analysis of variance, followed by Duncan *post-hoc* test to determine significant differences between the groups. Differences with P value < 0.05 were considered to be statistically significant. Statistical analyses were performed using the SPSS 14.0 package programme.

RESULTS

In fresh semen, the percentage and duration of motile spermatozoa were 90 ± 5% and 35 ±7 s, respectively. The means of post-thaw motility of goldfish (*C. auratus*) sperm for all concentrations were presented in Figure 1. An increase of taurine concentration led to a significant increase on the motility rate of sperm ($p < 0.05$). The highest post-thaw motility (72.5 ± 3.5%) was obtained from the extender at 4 mM in pellets. However, the highest post-thaw motility (35±7%) was at 4 mM in straws.

The post-thaw motility duration increased with the addition of taurine (Figure 2). Differences in motility duration of thawed sperm were significant among the treatments ($p<0.05$). Highest motility duration (17.5 ±0.7 s) in pellets was obtained from the extender at 4 mM.

DNA damage after cryopreservation was compared in (Table 1). There were significant differences among treatments ($P <0.05$). Cells in the absence of taurine have the largest tails and the highest percentage of DNA damage. DNA damage decreased with supplementation of taurine compared to control group.

DISCUSSION

This study examined a cryoprotective effect of the natural antioxidant, taurine. In comparison with fresh goldfish sperm, taurine improved the post-thaw motility and duration, and reduced DNA damage.

Sperm packaging affects cryopreservation. Therefore, small containers such as 0.25–0.50 mL French straws, pellets, ampoule, capillary,

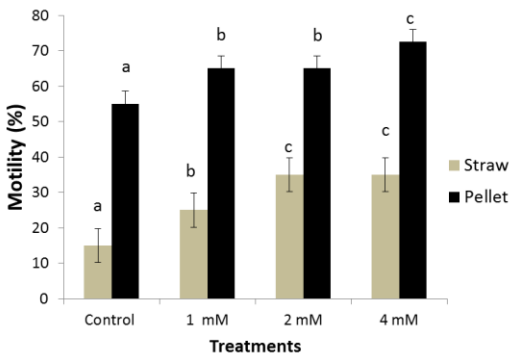

Figure 1. Effect of taurine on the motility rate of frozen/thawed goldfish (*C. auratus*) sperm in straws and pellets. Different letters show differences between treatments (*p<0.05*).

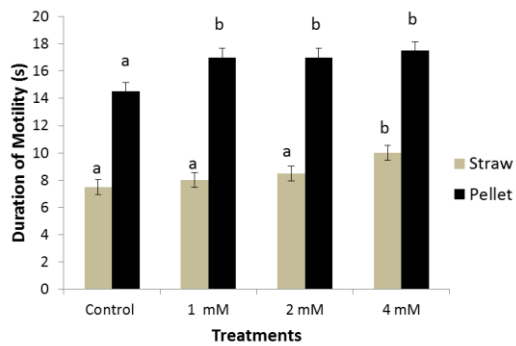

Figure 2. Effect of taurine on the motility duration of frozen/thawed goldfish (*C. auratus*) sperm in straws and pellets. Different letters show differences between treatments (*p<0.05*).

Table 1. Effect of taurine on the DNA integrity of frozen-thawed goldfish (*C. auratus*) sperm. Classification of DNA damage detection with comet rate.

	Grade 0 (%)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Total Comet rate (%)
Control	29.10±2.05^{a}	26.26±1.06^{a}	28.31±2.59^{a}	16.33±1.10^{a}	70.90±2.05^{a}
1 mM	40.97±1.63^{c}	43.57±1.10^{c}	15.47±1.79^{b}	0.00±0.00^{c}	59.03±1.63^{c}
2 mM	44.27±0.91^{b}	17.03±2.57^{b}	29.70±2.27^{a}	9.00±0.95^{b}	55.73±0.91^{b}
4 mM	44.80±1.68^{b}	34.23±3.93^{d}	20.57±1.68^{c}	0.40±0.69^{c}	55.20±1.68^{b}
F	61.219	62.952	30.038	280.236	61.219
P	0.000	0.000	0.000	0.000	0.000

Control: Extender without taurine.

$^{a, b, c}$: Different superscripts within the same column demonstrate significant differences (*p<0.05).*

cryotube, aluminum disc and vials are used in sperm cryopreservation (6). Straws are used because of the geometrical structure, equilibrate with environment temperature very quickly, and are commercially available, can be easily labeled and stored efficiently. Additionally, the semen in the straws could be frozen and thawed readily (9). Sperm quality parameters frozen in straws were significantly better than frozen sperm in pellets (14). The straw method for rainbow trout sperm yielded considerably better fertilization rates than the pellet method (7). However, Altunok and colleagues (3) demonstrated that pellet method was a more efficient method, although the straw method was eased due to the handling of small amounts of sperm and allowed tagging and recognition of individual samples. This study, in agreement with these results, shows the highest post-thaw duration and the percentage of motility from pellets. This may be due to fish species, sperm quality, extenders, dilution ratios, and freeze/thaw methods (2). The thermal conductivity can affect cryopreservation success in consequence of a thawing process of straws due to the diameter of the package and the temperature of water bath (41).

Taurine (2-aminoethanesulfonic acid) is a sulphur-containing free amino acid (26, 28) and has physiological roles such as osmoregulation, calcium modulation and antioxidation (29). Taurine can act as a direct antioxidant by scavenging ROS (17) and/or as an indirect antioxidant by preventing changes in membrane permeability due to oxidant injury (16). Ekici et al. (15) demonstrated that motility, fertilization rate and eyed embryo percentages were not affected by taurine; but Cabrita et al. (8) found that taurine (1 mM) slightly increased the percentage of motile cells in *S. aurata* but DNA fragmentation parameters were significantly reduced both by protecting DNA against strand breaks. Martínez-Páramo et al. (28) stated that some parameters of European sea bass sperm quality improved with addition of 1 mM taurine to extenders after thawing. Liu et al. (25) determined that 50 mM taurine provided the most pronounced effect in improving post-thaw quality of red seabream sperm. In agreement with these results, was the post-thaw motility and duration on goldfish semen increased with the addition of taurine to extenders and the best concentration of taurine was 4 mM.

The integrity of cell membrane, damage to cellular organelles, sperm quality and viability change after cryopreservation due to temperature reduction, cellular dehydration, impairment of lipid-protein interactions, and modifications of DNA and proteins, freezing, and thawing (11, 24, 44). DNA integrity is important due to preserving genetic material. DNA damage leads to reduced growth, abnormal development and reduced survival of embryos, larvae and adults (23). Furthermore, the skeletal abnormality occurrence in hatched larvae (13) and abnormal embryogenesis had been observed (33). In the literature, different species-specific results were reported about DNA damage. Song et al. (36) suggested that mechanical injury on sperm DNA stability was insignificant. Suquet et al. (39) detected that there were no genome alterations in turbot *Psetta maxima* sperm after thawing. Gwo et al. (17) stated that cryopreservation process was not affected the nucleus of Atlantic croaker *Micropogonias undulatus* sperm. On the other hand, Rani et al. (32) reported that the toxicity of cryoprotectant was the main reason of DNA damage. In some studies, it has been reported that supplementation of amino acids to extenders improved motility and viability of spermatozoa by reducing lipid peroxidation and DNA damage from ROS during cryopreservation in fish (12, 38). The effect of extender supplemented with taurine on DNA damage have been performed in fish sperm. Cabrita et al. (8) determined that DNA fragmentation of *S. aurata* sperm decreased significantly with the addition of 1 mM taurine to extenders. Martínez-Páramo et al. (28) reported that DNA fragmentation of European sea bass (*D. labrax*) sperm also reduced with the presence of 1 mM taurine. In agreement with these studies, we showed DNA damage after cryopreservation and a significant decrease in DNA damage of goldfish sperm with increased taurine concentration. This may be due to the affinity of the amine group with nucleic acids that neutralize the generation of free radicals, thus reducing DNA damage (20).

In conclusion, sperm motility improved with the presence of taurine and decreased DNA damage. Additionally, motility and duration significantly increased with the usage of pellets as sperm packaging unit compared to straws. In this regard, oxidative damage could be reduced by addition of the semen extender with taurine. The cryoresistance to ice crystal formation and recrystallisation during freezing-thawing procedure could be provided to addition of taurine to basic media.

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