Effects of semen extender supplemented with L-methionine and packaging methods (straws and pellets) on post-thaw goldfish (*Carassius auratus*) sperm quality

Running Tittle: Effects of semen extender supplemented with L-methionine

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Abstract

The aim of the present work was to analyse the effect of extender supplementation with L-methionine on post-thawed sperm motility and viability and also it was tested the feasibility of using straws and pellets for the cryopreservation of goldfish (*Carassius auratus*) sperm. Extenders were supplemented with different L-methionine concentrations of 1 mM; 1.5 mM; 3 mM; 6 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 and pellets, the straws and pellets were placed on the tray, frozen in nitrogen vapor and plunged into liquid nitrogen. Our results indicated that an increase in the concentration of L-methionine caused a significant increase in the motility rate and duration of sperm in goldfish (*C. auratus*) ($p<0.05$). In addition, duration and percentage of motility in pellets were higher than in straws. Comparing all concentrations of L-methionine, the best concentration of L-methionine was 1.5 mM. Higher post-thaw motility (45.00±7.07%) and duration of motility (17.00±0.71s) were obtained with the extender at concentration 1.5 mM in pellets. Consequently, pellets could be use for goldfish sperm cryopreservation and the tested amino acid affected the motility parameters and semen extenders could be supplement with L-methionine.

Key words: Goldfish, *Carassius auratus*, L-methionine, amino acid.

1. Introduction

Long-term storage of gametes is important for fish farming and conservation of genetic diversity [1, 2]. Moreover, cryopreservation technology provides ease of global germplasm shipping and supply [3, 4], selective breeding and hybridization with desirable characteristics [4-6], protection of endangered species [7, 8]. In addition, later usage of sperm in hatchery seed production or laboratory experimentation is maintained continuously [4, 9]. Success of cryopreservation protocol depends on several factors such as extenders, dilution ratios, and freezing and thawing methods [10]. Storage container is one of these factors and generally used small-volume storage containers such as French straws (i.e. 0.25–0.50 mL), pellets, ampoule, capillar, cryotube, aluminium disc and vials [11-13] and storage containers affect post-thaw sperm quality, fertilization and hatching rate [10, 14-17]. Therefore, determination of species-specific sperm freezing method is essential for commercial aquaculture applications.

Amino acids protect spermatozoa against cell damage during cryopreservation due to have antioxidant property and found in seminal plasma at high concentration [18-21]. Lahnsteiner [22] stated that in the seminal plasma of *O. mykiss*, the main free amino acids (FAAs) were arginine, glutamic acid, isoleucine, leucine, methionine and proline, in spermatozoa cysteine, arginine and methionine. In the seminal plasma of *C. carpio*, the main FAAs were alanine, arginine, cysteine, glutamic acid, histidine, leucine, lysine, methionine and proline, in spermatozoa arginine, glutamic acid, histidine, leucine and lysine. Due to these reasons, amino acids have been used in sperm cryopreservation as a non-permeating cryoprotectant of many mammalian species to preventing against cold shock [23] and freezing stress [24-29]. Thus far, performed studies in mammalians and different fish species (e.g. *Dicentrarchus labrax*, *Sparus aurata*, *Oncorhynchus mykiss*, *Cyprinus carpio*) have demonstrated that supplementation of amino acids (e.g. taurine, hypotaurine, proline,

glutamine, glycine, histidin, cysteine, methionine) to extenders reduced sperm damage and DNA fragmentation and improved post-thaw motility [30-31, 18-21].

Methionine is one of essential amino acids and a glutathione precursor. Therefore, it reduces reactive oxygen species (ROS) and thus protects cells from oxidative stress during cryopreservation. Additionally, methionine provides the synthesis of polyamines (spermine and spermidine) [32]. As concerns fish, the knowledge about the use of extenders containing methionine is limited. Recently, studies about antioxidant property and supplementation to extenders of methionine have been performed in different fish species (*Oncorhynchus mykiss*, *Cyprinus carpio*, *Salvelinus fontinalis*) [7, 22 33]. In these studies, it was determined that addition of methionine to extenders was improved sperm quality.

Goldfish (*Carassius auratus*) is a Cyprinid fish species living in fresh and brackish water and has received great attention as ornamental fish and remain a core of inland fish production in many countries throughout the world. They inhabit naturally lakes, ponds and slow-moving rivers throughout Europe and Asia [34]. The species is benthopelagic, non-migratory and omnivorous fish [35]. To date, there are few reports regarding cryopreservation of goldfish (*Carassius auratus*) semen. The use of dimethyl sulfoxide (Me_2SO) was proven to be effective in the cryopreservation of goldfish semen [36]. Nathanailides and colleagues [37] studied DNA fragmentation, sperm quality and fertilizing ability of cryopreserved goldfish sperm using different cryoprotectants. To our knowledge, any study has not been performed about supplementation with amino acid to extenders. In this context, the major aim of the study was to examine effect of supplementation of extender with different L-methionine concentrations (1 mM; 1.5 mM; 3 mM; 6 mM) on goldfish (*C. auratus*) sperm cryopreservation. The specific objectives were to: (1) assess sperm quality; (2) compare the packaging methods (straws and pellets).

2. Material and Methods

2.1. Collection of sperm

Experiments were performed in Muğla Sıtkı Koçman University (Muğla, Turkey). Six mature goldfish males (2.77±0.52 kg, 42.5±3.3 cm as mean±SD) were randomly selected from the stock aquarium for sperm collection. Water temperature and oxygen were 22±1ºC to 8.1±0.4 mg l^{-1} respectively. Males 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 stripping. Caution was exercised to prevent contamination of the semen with urine, feces, blood, mucus or water. The sperm was collected by a gentle abdominal massage, collected into glass vials and stored on ice (2-4 °C) until use.

2.2. Sperm cryopreservation

Sperm from 6 goldfish males were selected and used to cryopreservation individually with the following cryomedia. Control group were diluted (1:9) in an modified Kurokura (MK) extender composed of the following: 432 mg NaCl, 1200 mg KCl, 35 mg $CaCl_2$, 10 mg $MgCl_2$, 24 mg $NaHCO_3$ for 100 ml pure water, pH 8.2, Osmolarity 365 mOsm, 12.5% Me_2SO as permeating cryoprotectant, 10% egg yolk used non-permeating cryoprotectant [8].

L-methionine was separately added to the extender (one per experimental group): (a) 1 mM, (b) 1.5 mM, (c) 3 mM, (d) 6 mM. After dilution the sperm was aspirated into 0.25 ml straws and pellets, and sealed with polyvinyl alcohol and equilibrated at temperature 2-4 ºC for 5 minutes, vaporized at a height of 3 cm above liquid nirogen surface for 10 min and plunged into liquid nitrogen. At least five straws and pellets per sperm sample were frozen. After 7 days of storage in liquid nitrogen, the samples were thawed in a water bath 20 ºC for a period of 30 s. After thawing, each sample was evaluated for the motility parameters using a light microscope with a digital image processing software connected to the computer (Zeiss Axio Scope with AxioVision) to evaluate the percentage of spermatozoa motility and

viability. 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.

3. Results

In fresh semen, the percentage and duration of motile spermatozoa was 90±5.0% and 35±7.0 s, respectively. Means of post-thaw motility for all concentrations with goldfish (*C. auratus*) sperm were presented in Figure 1. The findings of the present study showed that an increase in the concentration of L-methionine in extender caused a significant increase the motility rate of sperm in goldfish (*C. auratus*) ($p<0.05$). Higher post-thaw motility (45.00±7.07%) was obtained with the extender at concentration 1.5 mM in pellets. However, higher post-thaw motility (35.00±7.07%) was at concentration 6 mM in straws.

The addition of L-methionine to the extender was increased the post-thaw motility duration. Differences in the motility duration of frozen-thawed sperm were significant among the treatments ($p<0.05$). Duration of motility (17.00±0.71s) in straws and pellets was obtained with the extender at concentrations 1 mM and 1.5 mM.

4. Discussion

In the study, we compared the effects of extender supplemented with L-methionine on post-thaw motility and duration of sperm of cryopreserved goldfish (*C. auratus*) sperm in straws and pellets. Overall, we demonstrated that the addition of the extender with L-methionine was increased the post-thaw motility rate and duration significantly. The highest protective effect of L-methionine was determined at a concentration of 1.5 mM. However, increasing doses of L-methionine (3 mM and 6 mM) decreased the post-thaw sperm motility. This may be due to destroye the functional integrity of the axosome and mitochondria of the sperm cells of high doses of antioxidant additives or its toxic effect [28, 38].

Packaging methods in cryopreservation process are important for commercial aquaculture application. Therefore, small-volume storage containers such as French straws

(i.e. 0.25–0.50 mL), pellets, ampoule, capillar, cryotube, aluminium disc and vials are used in sperm cryopreservation [11-13]. Especially, straws are generally used because of the geometrical structure and equilibrate with the temperature of its environment very quickly of the extended semen, commercially available, be stored efficiently and easily labeled. In addition, the semen in the straws could be freeze and thaw readily [39]. Bozkurt and colleagues [10] determined that the straw method for freezing rainbow trout sperm yielded considerably better fertilization rates than the pellet method. In contrast, Altunok et al. [40] stated that packaging with pellets was the more time efficient method, although packaging with straws was eased due to the handling of small amounts of sperm and allowed tagging and recognition of individual samples. Similarly, in the study, highest post-thaw duration and percentage of motility were obtained from pellets. This may be due to fish species, sperm quality, extenders, dilution ratios, and freezing and thawing methods [41].

Methionine is one of two sulfur-containing proteinogenic essential amino acids. It has antioxidant properties because of being a glutathione precursor, a tripeptide that reduces reactive oxygen species (ROS) and thus protects cells from oxidative stress. In addition, methionine is required for the synthesis of polyamines (spermine and spermidine), which take part in nucleus and cell division events and the most important methyl group donor for methylation reactions of DNA and other molecules [32]. Due to these properties of methionine, studies about effects of methionine on improvement of post-thaw sperm quality have been performed in fish species. Lahnsteiner (2009) suggested that methionine had a positive effect on the sperm viability in rainbow trout *O. mykiss* and carp *C. carpio*. Lahnsteiner et al. [33] determined that methionine only slightly increased post-thaw motility the brook trout (*Salvelinus fontinalis*). In contrast with this result, they suggest that these effects were not detectable in rainbow trout. Kutluyer et al. [7] found that addition of methionine to extenders increased the post-thaw sperm motility and duration in comparison to

the standard extender. Similarly, in this study, supplementation of L-methionine to extenders was increased post-thaw sperm motility and duration.

In conclusion, the present study indicates that L-methionine is the important amino acid in sperm of goldfish for increase the quality of sperm. The effective concentration was 1.5 mM. Additionally, pellets would be commercially practical for cryopreservation. Further research is required in order to select the best concentration of L-methionine.

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List of Figures

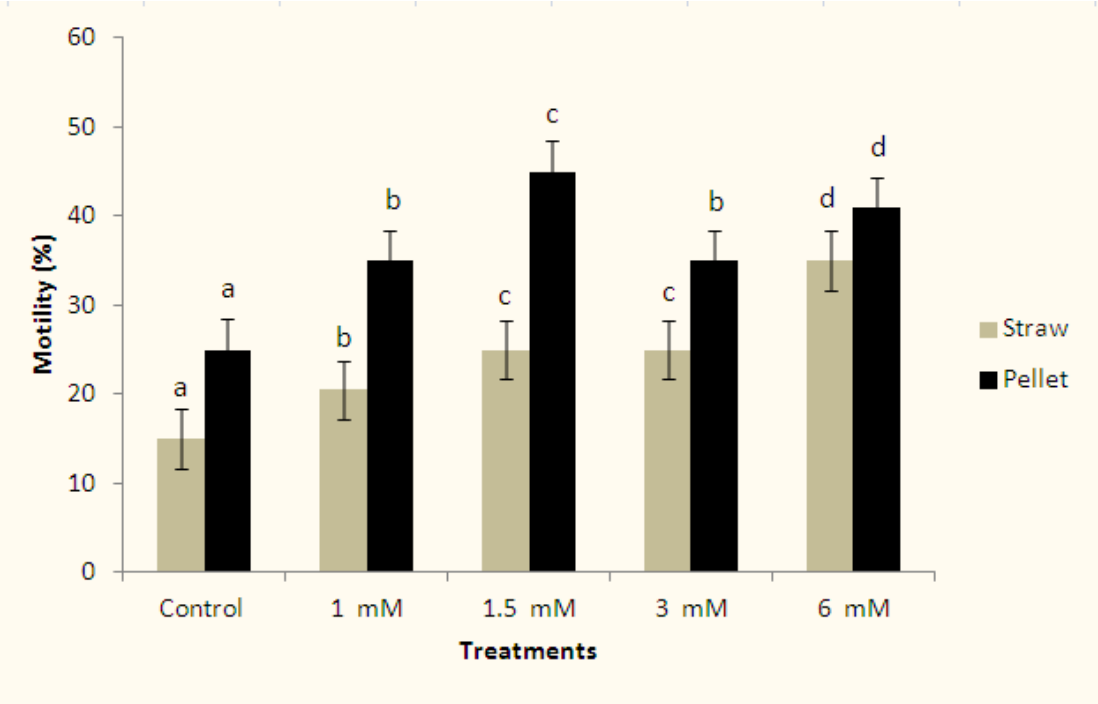

Figure 1. Effect of L-methionine on the motility rate of frozen-thawed goldfish (*C. auratus*) sperm (n=3) in straws and pellets. Different letters show differences between treatments ($p<0.05$).

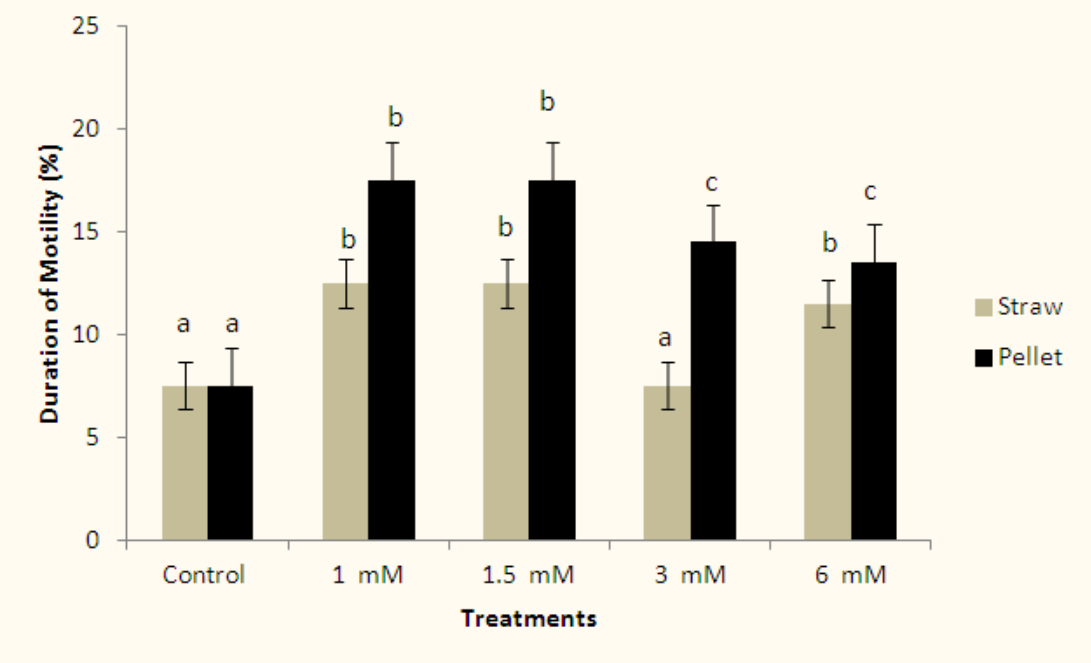

Figure 2. Effect of L-methionine on the motility duration of frozen-thawed goldfish (*C. auratus*) sperm (n=3) in straws and pellets. Different letters show differences between treatments ($p<0.05$).