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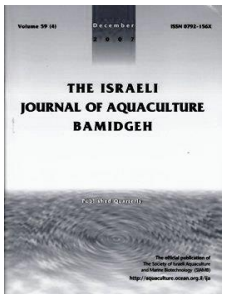

Effect of Extender Supplemented with Different Sugar Types on Post-thaw Motility, Viability and Fertilizing Ability of Cryopreserved Common Carp (*Cyprinus carpio*) Spermatozoa

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Keywords: cryopreservation; semen; extender; sugar; *Cyprinus carpio*

Abstract

The influence of various sugar types supplemented to the extender on post-thaw motility, viability, and fertilizing capacity of cryopreserved common carp (*Cyprinus carpio*) semen were investigated. The results indicated that types of sugar significantly influenced motility, motility duration, and viability rates ($P<0.05$). Glucose, maltose, sucrose, and trehalose provided higher motility compared to the sugar-free control in post-thaw samples. Trehalose provided highest progressive motility duration, and higher viable sperm rates were obtained with all sugar types except xylose. Xylose exhibited the lowest post-thaw progressive motility duration (35.2±1.4s). The mean highest fertilization (78.2±1.4%) and eyed egg rates (94.3±1.5%) were determined using trehalose in the extender, and differences between the treatments in the fertilization and eyeing rates were significant ($P<0.05$). Finally, the present study showed that sugars, especially maltose and trehalose (disaccharide), improved post-thaw spermatozoa motility and fertility in common carp semen.

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Introduction

Cryopreservation plays an important role in transportion of genetic material between facilities, optimal use of genetic material in aquaculture, reducing the spreading risk of infections, performing hybridization studies, protecting the gene pool, conserving biodiversity, selective breeding activities, and protection of endangered species. Thus, effective sperm cryopreservation protocol is an important part of breeding programs in aquaculture (Kopeika et al., 2007).

Cryopreservation techniques involve addition of cryoprotectants, freezing and thawing sperm samples, which may result in some damage to the spermatozoa and may decrease egg fertilization rates (Bozkurt et al., 2014). Therefore, before undertaking cryopreservation of spermatozoa it is essential to evaluate different extender solutions, cryoprotectants, cooling and thawing rates, in order to develop optimum cryopreservation protocols for various species (Routray et al., 2008; Cheng et al., 2016). It is still unclear which part of this process primarily affects post-thaw quality of sperm.

The composition of the freezing extender plays an important role in determining viability of thawed frozen sperm. Extenders maintain inactivity of spermatozoa when semen is diluted before freezing due to stabilization of physicochemical properties. For this purpose, extenders have been developed using saline- and sugar-based diluents (Urbanyi et al., 1999). Sugars are frequently supplemented into freezing extenders where they have several functions. They provide energy substrate for sperm cells during incubation, maintain osmotic pressure of the diluent, and act as cryoprotectant. It is known that storage temperature, molecular weight of the sugar, and the type of buffer used in the extender affect cryoprotective ability of the sugars (Yıldız et al., 2000). In addition, the sugars play a cryoprotective role by interacting with membrane lipids and proteins and also decreasing the risk of intracellular ice crystal formation, which causes cellular osmotic dehydration during cryopreservation (Agca et al., 2002). Monosaccharides and disaccharides cannot diffuse through the membrane, and it is believed that their cryoprotective effect is exerted by creating an osmotic pressure that induces cell dehydration and a reduced incidence of intracellular ice formation (Fuller, 2004).

The cryoprotective properties of various sugars against freezing-thawing induced damage have been documented in various cell types (Yıldız et al., 2000; Storey et al., 1998). The effect of sugar supplementation into the freezing extenders on post-thaw semen quality has been reported in large yellow croaker *Pseudosciaena crocea,* (Cheng, 2016), rainbow trout *Oncorhynchus mykiss*, (Tekin et al., 2007), Caspian brown trout *Salmo trutta caspius,* (Moghanloo et al., 2007), Atlantic salmon *Salmo salar,* (Dziewulska et al., 2011), and brook trout *Salvelinus fontinalis,* (Lahnsteiner et al., 2011). Standardization and simplification of the cryopreservation procedure using different sugar types for common carp sperm are needed for commercial applications.

For this purpose, this study was designed to compare the effect of several monosaccharide (glucose, fructose, xylose) and disaccharide (lactose, maltose, sucrose and trehalose) sugar types used in the ionic based extenders on quality (motility, duration of motility, and viability) and fertilization ability of frozen/thawed common carp spermatozoa.

Materials and Methods

Reagents. Additives and other chemicals used in this study were obtained from a local representative of Sigma-Aldrich Chemicals Company (St. Louis, MO, USA).

Broodstock handling and gamete collection. Common carp broodstock were collected from wintering ponds by seining, and transported into the hatchery 48 h prior to gamete collection. In the hatchery, healthy male (2845.2±24.2g; n=9) and female (3275.4±12.4g; n=5) broodfish were held separately in shadowed tanks (V=1000 L) and supplied with well-aerated water (2.0 L/min) at 23±0.5°C.

The brooders were anesthetized in 5 ppm quinaldine (Reanal Ltd., Budapest, Hungary) solution for a few minutes. Sperm was collected from nine males by manual abdominal stripping 12 h after a single injection of 2 mg/kg of (CPE) carp pituitary extract (Argent Labs., Redmond, WA, USA) at 23±0.5°C water temperature. For sperm collection, the urogenital papillae of mature male fishes were carefully dried and sperm was hand-

stripped directly into test tubes which were placed in a styrofoam box containing crushed ice (4°C). Contamination of sperm with water, urine, or feces, was carefully avoided. Eggs were collected by hand stripping following injection of CPE at 3.5 mg/kg ratio. To achieve this 10% of the total amount (0.35 mg/kg) was applied 10 h before the second (3.15 mg/kg) injection of CPE (Bozkurt et al., 2012). Abdomenal and urogenital papillae of female broodstock were dried before stripping. Egg samples contaminated with fecal material or urine were discarded. Only transparent, well rounded, and unwrinkled eggs were used for fertilization.

Semen examination. A preliminary semen standard analysis of osmolarity (Wescor, USA), pH (micro pH 2000 Crison), and cell motility, was performed within 15 min of sampling to discard bad quality and contaminated samples. Sperm motility was determined subjectively using light microscope with phase-contrast attachment (Olympus BX50, Tokyo, Japan) at 400x magnification. Samples were activated by mixing 1 µl of sperm with 20 µl activation solution (AS) (45 mM NaCl, 5 mM KCl, and 30 mM Tris-HCl, pH 8.2) (Horvath et al., 2003) on a microscope glass slide.

Motility percentages were defined as the percentage of spermatozoa moving in a forward motion (Vuthiphandchai and Zohar, 1999). Sperm cells that vibrated in place were not considered to be motile. For cryopreservation experiments, sperm samples showing below 80% motility were discarded. Following addition of the activation solution to the sperm samples, duration of sperm motility was determined using a sensitive chronometer (sensitivity: 1/100 s) recording the time until forward motion of spermatozoa ceased. Approximately twenty spermatozoa were counted in each field of view on a microscope slide. This was repeated three times (three replicates) for a total of sixty spermatozoa.

Spermatozoa density was determined according to the hemacytometric method. Sperm was diluted at ratio of 1:1000 with Hayem solution (5 g Na_2SO_4, 1 g NaCl, 0.5 g $HgCl_2$, 200 ml bicine) and density was determined using a 100 µm deep Thoma hemocytometer (TH-100, Hecht-Assistent, Sondheim, Germany) at 400x magnification with Olympus BX50 phase contrast microscope (Olympus, Japan) and expressed as spermatozoa $x10^9$/mL (three replicates). The counting chambers were kept moist for at least 10 min before cell counting (Tekin et al., 2003). Sperm pH was measured using indicator papers (Merck, 5.5-9) within 30 min of sampling.

Semen freezing and thawing. Samples were diluted 1:5 in an extender composed of 75 mmol/L NaCl, 70 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$ and 20 mmol/L Tris, (pH 8.0) (Lahnsteiner et al., 2000). The extender contained 10% DMSO and supplemented with monosaccharides (glucose, fructose, xylose) and disaccharides (lactose, maltose, sucrose, and trehalose) sugar types at concentration of 0.3 M separately to provide equal osmotic pressure for each experimental group while the control group did not contain any sugar type. The diluted sperm concentration was around 1 x 10^9 cells/ml extender and this dilution was high enough to avoid damage due to sperm compression during freezing and thawing (Lahnsteiner et al., 2000). Extended semen was equilibrated at 4°C for 10 min and then drawn into 0.25-ml plastic straws (IMV France) that had been pre-cooled at 4°C and sealed with polyvinil alcohol (PVA).

The labeled straws were then laid horizontally on a tray above liquid nitrogen in a styrofoam box (inner dimensions 54 x 22 cm, height 33 cm). The straws were frozen in the vapour of the liquid nitrogen, 4 cm above the surface of the liquid, for 10 min and then plunged into the liquid nitrogen and stored for 3 days. (Yavas et al., 2014). The frozen straws were thawed in a water bath of 40°C for 5 s and then gently agitated during thawing. All cryopreservation and thawing trials were triplicated. Thawed sperm was activated by mixing 1 µl of sperm with 20 µl (AS) on a microscope glass slide and observed microscopically for determination of post-thaw motility, motility duration, and viability (three replicates). To assess live/dead sperm percentage, eosin-nigrosin preparations were made according to the method of Bjorndahl et al. (2003). A total of 300 sperm cells was counted on each slide at x1000 magnification.

Fertilization. Pooled eggs from 5 mature females were used to determine fertilization rates. Following microscopic control of egg quality, cryogenic straws randomly selected from the liquid nitrogen tank were used to fertilize eggs. Egg samples (about 1.000 eggs) were inseminated in dry Petri dishes with fresh or frozen sperm immediately after thawing at a spermatozoa-egg ratio of $1x10^6$:1 (Lahnsteiner et al., 2003). Thawed sperm was added to the eggs and gently mixed before activation with 20 ml fertilization solution (3 g urea and 4 g NaCl in 1 L distilled water). The eggs were then lightly stirred for 30 min after which they were washed with hatchery water (23±0.5°C, 8 mg/l O_2). They were then treated with tannic acid (0.5 g/l) for 30 s to remove stickiness of the eggs. The eggs were rinsed with hatchery water again and transferred into labeled Zuger glass incubators supplied with flow-through hatchery water (23±0.5°C). Living and dead eggs were counted in each incubator during incubation and the dead eggs were removed. The fertilization (number of 4-cell stage embryos/number of total eggs) and eyed egg (number of eyed egg embryos/number of total eggs) rates were determined under a stereomicroscope at 20-fold magnification. Fertilization trials were triplicated.

Statistical Analysis. Results are presented as mean±SE. Data for percentage of sperm motility and fertilization were transformed by angular transformation prior to statistical analysis by SPSS 10.0 software. Differences between parameters were analyzed by repeated analysis of variance (ANOVA). Significant means were subjected to a multiple comparison test (Duncan) for post-hoc comparison at level of α = 0.05. All analyses were carried out using SPSS 10 for Windows statistical software package.

Results

The mean progressive motility value of the fresh sperm samples was 87.2±0.45%. Mean progressive motility duration (s), spermatozoa density ($x10^9$/ml), and pH values were determined as 57.2±0.4s, $21.2x10^9$/ml and 7.5±0.50 respectively. The post-thaw progressive motility (%), duration of progressive motility (s), and viability (%) rates of the common carp sperm are presented in Figures 1, 2 & 3.

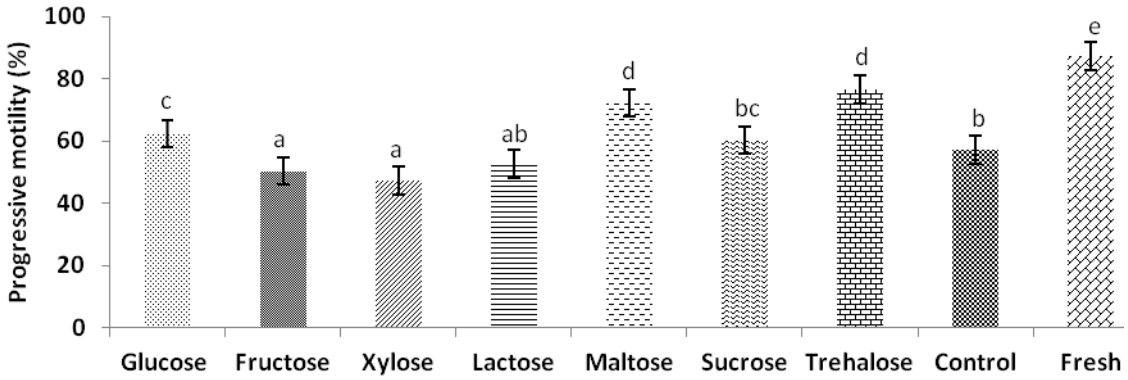

Fig. 1. Effect of different sugar types on post-thaw progressive motility of common carp (*C. carpio)* semen (n=3; mean±). Different letters indicate differences among the treatment groups ANOVA ($p<0.05$).

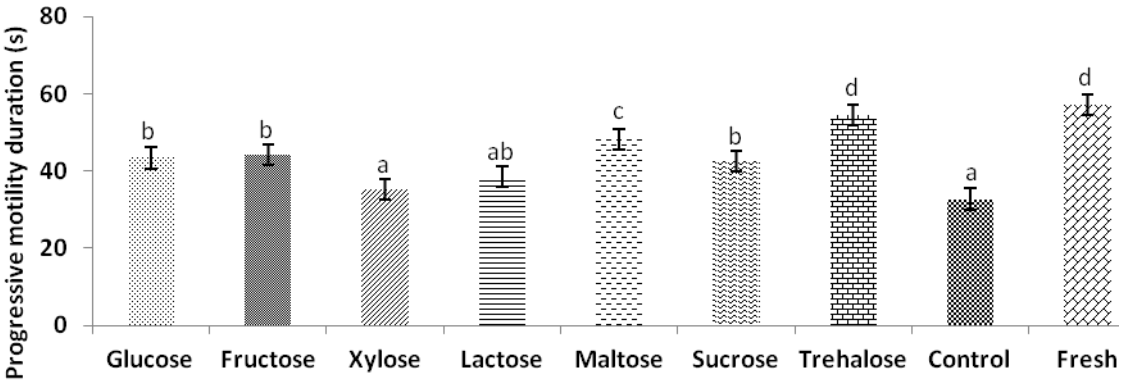

Fig. 2. Effect of different sugar types on post-thaw progressive motility duration of common carp (*C. carpio)* semen (n=3; mean±). Different letters indicate differences among the treatment groups ANOVA ($p<0.05$).

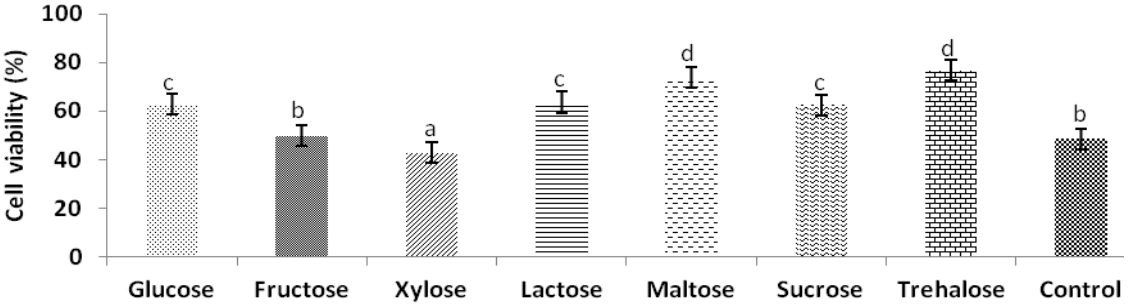

Fig. 3. Effect of different sugar types on post-thaw cell viability of common carp (*C. carpio)* semen (n=3; mean±). Different letters indicate differences among the treatment groups ANOVA ($p<0.05$).

The type of sugar significantly influenced motility, motility duration, and viability rates (*P<0.05*). Glucose, maltose, sucrose, and trehalose, provided higher motility compared to the sugar-free control in post-thaw samples. Trehalose provided highest progressive motility duration, and higher viable sperm rates were obtained with all sugar types except xylose. Xylose exhibited the lowest post-thaw progressive motility duration (35.2±1.4 s) among the sugar types.

Fertilization (%) and eyed-egg rates (%) of common carp spermatozoa which were cryopreserved with different sugar types in the extender, the control, and those of fresh samples are shown in Figures 4 & 5. The mean highest fertilization (78.2±1.4 %) and eyed egg rates (94.3±1.5 %) were determined using trehalose in the extender (*P<0.05*).

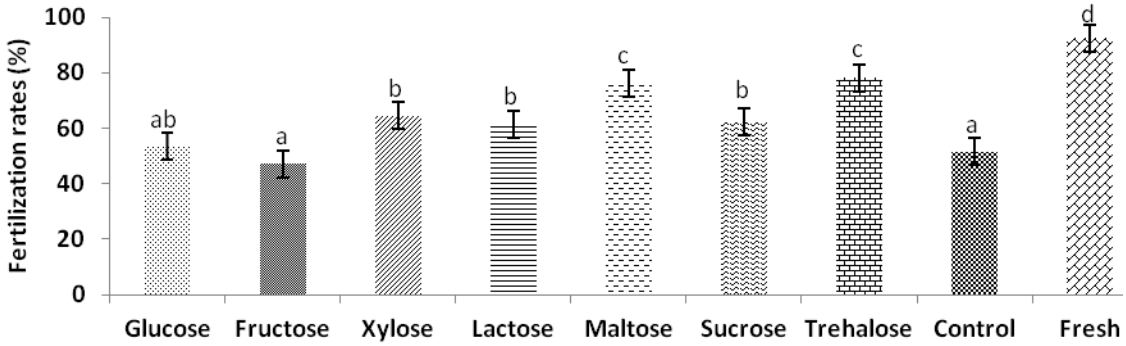

Fig. 4. Effect of different sugar types on post-thaw fertility of common carp (*C. carpio)* semen (n=3; mean±). Different letters indicate differences among the treatment groups ANOVA ($p<0.05$).

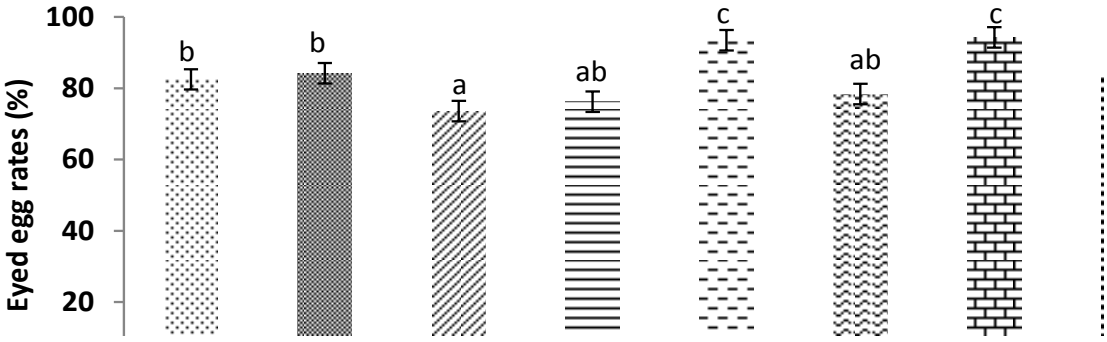

Fig. 5. Effect of different sugar types on eyed egg rates of common carp (*C. carpio)*(n=3; mean±). Different letters indicate differences among the treatment groups ANOVA (p<0.05).

Fig. 5 Effect of different sugar types on eyed egg rates of comn

Discussion

Cryopreservation of fish sperm is an important tool for conservation of biodiversity, efficient selective breeding, and also for synchronization of artificial reproduction. On the other hand, cryopreservation process causes irreversible cell damage resulting in significant reduction in viability and motility of spermatozoa (Li et al., 2010). Damage in sperm cells resulting in low post-thaw motility is induced by distinct mechanisms at each cryopreservation phase (Medeiros et al., 2002). The primary cause of such damage, also termed cryoinjury, are heat removal, pH fluctuations, cold shock, ice crystal formation, osmolarity effects, and cryoprotectant toxicity (Chao and Liao, 2001). Most studies to prevent such damage using sugar types as supplementary elements in the freezing extenders have been carried out with domestic animals (Malo et al., 2010; Naing et al. 2010). To our knowledge the number of studies dealing with detailed comparison of different sugar types (monosaccharids and disaccharids) on cryopreservation success of common carp spermatozoa is limited (Warnecke and Pluta, 2003; Irawan et al., 2010).

According to results of the present study, there were significant differences in post-thaw quality parameters of common carp spermatozoa in response to the sugar types added to the extender. It was determined that post-thaw motility of common carp sperm is low when compared to fresh sperm. This indicates that cryogenic injuries occur during the freezing and thawing process (Babiak et al., 2002). In post-thaw samples glucose as monosaccharide, and the disaccharides apart from lactose, provided higher motility compared to the sugar-free control. Some studies have shown that the cryoprotective effect of the sugars on sperm cells may differ according to chemical functionality and molecular weight (Devireddy et al., 2002). One explanation given is that since the molecular weight of disaccharides is higher they have a greater cryoprotective effect on common carp spermatozoa. Disaccharides can form hydrogen bonds with polar head groups of phospholipids, binding to the membrane interface and thereby replacing water molecules more efficiently than monosaccharides (Malo et al., 2010). In our study, trehalose (disaccharide) provided highest progressive motility duration (54.5±1.6 s) and better viable spermatozoa rates were obtained with all sugar types except xylose. Xylose (monosaccharide) also exhibited the lowest post-thaw progressive motility duration (35.2±1.4 s).

According to results of the some studies, the presence of disaccharides in the extender after freezing of fish spermatozoa was effective in prolonging post-thaw survival of semen (Warnecke and Pluta, 2003; Dziewulska et al., 2011). It was noted that the substitution of sucrose with trehalose results in slightly better post-thaw motility figures in common carp spermatozoa (Warnecke and Pluta, 2003). Using sucrose as a component of the extender improved post-thaw motility and motility durations in carp sperm (Irawan et al. 2010).

In this study, influence of various sugar types supplemented to the extender on post-thaw motility, viability, and fertilizing capacity of cryopreserved common carp (*Cyprinus carpio*) semen was investigated. In conclusion, it was determined that different sugar types supplemented with freezing extenders have different capabilities to protect spermatozoa during cryopreservation. The results of the present study clearly showed that better post-thaw spermatozoa viability and fertilization results might obtained by adding trehalose and maltose as disaccharide sugar component to the freezing extender for common carp semen cryopreservation for gene bank purposes or future use in selective breeding programs in aquaculture.

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References

Agca Y., Gilmore J., Byers M., Woods E.J., Liu J. and J.K. Critser, 2002. Osmotic characteristics of mouse spermatozoa in the presence of extender and sugars. *Biol. Reprod*., 67: 1493-1501.

Babiak I., Glogowski J., Dobosz S., Kuzminski H. and K. Goryczko, 2002. Semen from rainbow trout produced using cryopreserved spermatozoa is more suitable for cryopreservation. *J. Fish Biol*., 60: 561-570.

Bozkurt Y., Yavas I. and F. Karaca, 2012. Cryopreservation of brown trout (*Salmo trutta macrostigma*) and ornamental koi carp (*Cyprinus carpio*) sperm. In: *Current frontiers in cryopreservation*. Edited by: Katkov, I. pp. 293–304. Intech, Croatia. ISBN:978-953-51-0302-8, 462p.

Bozkurt Y., Yavaş I. and C. Yıldız, 2014. Effect of different avian egg yolk types on fertilization ability of cryopreserved common carp (*Cyprinus carpio*) spermatozoa. *Aquacult. Int*., 22: 131-139.

Bjorndahl L., Soderlund I. and U. Kvist, 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Hum. Reproduct*., 18: 813-816.

Chao N.H. and I.C. Liao, 2001. Cryopreservation on finfish and shellfish gametes and embryos. *Aquaculture*, 197: 161-189.

Cheng S., Sheng Z, Zhu J-Q. and X.F. Wu, 2016. Effects of trehalose, bovine serum albumin, and sucrose, on the integrity of the plasma membrane of *Pseudosciaena crocea* semen after cryopreservation. *Isr. J. Aquacult.-Bamidgeh,*68. 1219.12 pages

Devireddy R.V., Swanlund D.J., Olin T., Vincente W., Troedsson M.H.T., Bischof J.C. and K.P. Roberts K.P., 2002. Cryopreservation of equine sperm: optimal cooling rates in the presence and absence of cryoprotective agents determined using differential scanning calorimetry. *Biol. Reprod*., 66: 222-231.

Dziewulska K., Rzemieniecki A., Czerniawski R. and J. Domagała, 2011. Post-thawed motility and fertility from Atlantic salmon (*Salmo salar* L.) sperm frozen with four cryodiluents in straws or pellets. *Theriogenology*, 76: 300-311.

Fuller B.J., 2004. Cryoprotectants: the essential antifreezes to protect life in the frozen state. *Cryoletters*, 25: 375-388.

Horvath A., Miskolczi E. and B. Urbanyi, 2003. Cryopreservation of common carp sperm. *Aquat. Liv. Resour*., 16: 457-460.

Irawan H., Vuthiphandchai V. and S. Nimrat, 2010. The effect of extenders, cryoprotectants and cryopreservation methods on common carp (*Cyprinus carpio*) sperm. *Anim. Reprod. Sci*., 122: 236-243.

Kopeika E., Kopeika J. and T. Zhang, 2007. Cryopreservation of fish sperm. *Methods Mol. Biol*., 368: 203-217.

Lahnsteiner F., Berger B., Horvarth A., Urbanyi B. and T. Weismann, 2000. Cryopreservation of spermatozoa in cyprinid fishes. *Theriogenology*, 54: 1477-1498.

Lahnsteiner F., Berger B. and T. Weismann, 2003. Effects of media, fertilization technique, extender, straw volume and sperm to egg ratio on hatchability of cyprinid embryos, using cryopreserved semen. *Theriogenology*, 60 (5): 829-841.

Lahnsteiner F., Mansour N. and F.A. Kunz, 2011. The effect of antioxidants on the quality of cryopreserved semen in two salmonid fish, the brook trout (*Salvelinus fontinalis*) and the rainbow trout (*Oncorhynchus mykiss*). *Theriogenology*, 76 (5): 882-890.

Li P., Hulak M., Koubek P., Sulc M., Dzyuba B., Boryshpolets S., Rodina M., Gela D., Manaskova-Postlerova P., Peknicova J. and O. Linhart, 2010. Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, C*yprinus carpio* L. *Theriogenology*, 74: 413-423.

Malo C., Gil L., Gonzalez N., Cano R., Blas I. and E. Espinosa, 2010. Comparing sugar type supplementation for cryopreservation of boar semen in egg yolk based extender. *Cryobiology*, 61: 17–21.

Medeiros C.M.O., Forell F., Oliveira A.T.D. and J.L. Rodrigues, 2002. Current status of sperm cryopreservation: why isn't it better?. *Theriogenology*, 57: 327-344.

Moghanloo K.S., Niksirat H., Mojazi Amiri B. and S.M. Mirtorabi, 2007. The effects of extender type, freezing and thawing rates on fertility of the cryopreserved semen of the Caspian brown trout (*Salmo trutta caspius*). *Iran. J. Fish. Sci*., 7 (1): 111-128.

Naing S.W., Wahid H., Mohd Azam K., Rosnina Y., Zuki A.B., Kazhal S., Bukar M.M., Thein M., Kyaw T. and M.M. San, 2010. Effect of sugars on characteristics of boer goat semen after cryopreservation. *Anim. Reprod. Sci*., 122: 23-28.

Routray P., Dash S.N., Dash C., Swain P., Sarkar S.K. and N. Sarangi, 2008. Cryopreservation of silver barb *Puntius gonionotus* (Bleeker) spermatozoa: effect of extender composition, cryoprotective agents and freezing rate on their postthawing fertilization ability. *Aquacult. Res*., 39: 1597-1605.

Storey B.T., Noiles E.E. and K.A. Thompson, 1998. Comparison of glycerol, other polyols, trehalose and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation. *Cryobiology*, 37: 46-58.

Tekin N., Secer S., Akçay E. and Y. Bozkurt, 2003. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) semen. *Isr. J. Aquacult.-Bamidgeh,* 55(3):208-212.

Tekin N., Secer S., Akcay E., Bozkurt Y. and S. Kayam, 2007. Effects of glycerol additions on post-thaw fertility of frozen rainbow trout sperm, with an emphasis on interaction between extender and cryoprotectant. *J. Appl. Ichthyol.,* 23 (1): 60-63.

Urbanyi B., Horvath A., Varga Z., Horvath L., Magyary I. and F. Radics, 1999. Effect of extenders on sperm cryopreservation of African catfish. *Clarias gariepinus* (Burchell, 1822). *Aquacult. Res*., 30: 145-151.

Yavas I., Bozkurt Y. and C. Yıldız, 2014. Cryopreservation of scaly carp (*Cyprinus carpio*) sperm: effect of different cryoprotectant concentrations on post-thaw motility, fertilization and hatching success of embryos. *Aquacult. Int*., 22 (1): 141-148.

Yıldız C., Kaya A., Aksoy M. and T. Tekeli, 2000. Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. *Theriogenology*, 54: 579-585.

Vuthiphandchai V. and Y. Zohar, 1999. Age-related sperm quality of captive striped bass *Morone saxatilis*. *J. World Aquacult. Soc.,* 30 (1): 65-72.

Warnecke D. and H.J. Pluta, 2003. Motility and fertilizing capacity of frozen/thawed common carp (*Cyprinus carpio* L.) sperm using dimethyl-acetamide as the main cryoprotectant. *Aquaculture*, 215: 167-185.