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Effect of different extenders and cryoprotectants on fertilizing ability of cryopreserved scaly carp (*Cyprinus carpio*) sperm

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Abstract The present study aimed to explore effect of different ionic and sugar based extenders and cryoprotectants on motility, motility duration and fertilization ability of cryopreserved scaly carp (*Cyprinus carpio*) sperm. For cryopreservation experiments, three different extenders (E-1:75mmol/l NaCl, 70 mmol/l KCl, 2 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 20 mmol/l Tris, pH: 8; E-2: 350 mM glucose, 30 mM Tris, pH: 8;E-3: 300 mM glucose, 10% egg yolk, pH: 4.5) containing different CPAs such as DMSO, DMA and glycerol at 10% concentration were used. Following dilution of sperm at ratio of 1:3, the samples were transferred into 0.25-ml straws and frozen in liquid nitrogen vapour (for 10 min at -120°C) and finally stored in liquid nitrogen (-196°C) tank. The frozen spermatozoa were thawed in a water bath at 30°C for 20s. Cell motility, motility duration and also fertilizing ability were determined in post-thawed samples. In spite of highest mean post-thaw motility (40.00±5.77%) was determined with extender E-3 supplemented with DMSO, the best mean motility duration was determined with DMSO containing E-2 extender as 106.67±43.33s. On the other hand, DMA supplemented E-2 extender provided highest mean fertility as 99.66±0.33%. According to results of the experiment, scaly carp sperm can be successfully cryopreserved with sugar based extenders supplemented with DMSO and DMA at 10% concentration.

Keywords extender, cryoprotectant, semen, *Cyprinus carpio*

1. Introduction

Carp is one of the most cultured fish species and there are many its subpopulations worldwide. On the other hand, many of carp species need restocking and conservation because of their exploitation in natural fresh water. Thus, cryopreservation of genetic structure can be one of the solutions in order to maintain their genetic variability and also conservation of their valuable strains [1].

Cryopreservation biotechnology allows conservation of sperm for subsequent usages and also supplying of good quality sperm permanently. Additionally, it enables genetic improvement of cultured fish stocks and also it can be incorporated in conservation programs and hatchery applications in aquaculture [2].

It is known that, cryopreservation deals with storage of biological materials at very low temperature (-196°C). Cellular viability can be maintained in a genetically consistent form at this low temperature [3]. Cryoprotectant and extender combinations are employed as media where sperm cells are kept for long-term preservation. Because these diluents protects sperm cells from cryoinjuries which occur due to cold and heat shock during freezing and thawing process.

Extenders are necessary to supply a energy source for the sperm cells, protect the cells against heath shock damages and also provide survivality of the cells [4], while cryoprotectants prevent ice crystal formations during freezing and thawing [5,6,7]. However, optimal extender composition varies depending on many factors such as age, spawning season and spermatological properties among fish species. Thus, formulation of suitable cryosolutions are very important for the successful cryopreservation process.

From this point of view, the objective of the present study was to test effect of three different cryoprotectant agents (DMSO, DMA and Glycerol) combined with three different extenders (ionic and glucose based) on motility, viability and fertilization ability of frozen-thawed scaly carp sperm.

2. Material and Methods

2.1. Adult fish and gamete collection

The adult fish was obtained from aquaculture production station of State Hydraulic Works in Adana during spawning season of scaly carp (*Cyprinus carpio*). In the pre-spawning period, the adult fish were kept in earthen ponds and subjected to natural photoperiod conditions. Broodstock was subjected to fasting for 24 h prior to gamete collection and adult fish was transported into the shadowed tanks (V=1000 L) with oxygenation and supplied with continuously (2.5 l/min) well-aerated fresh water at 22°C.

The adult fish was taken out from the water and their abdomen was dried. The brood fish were anaesthetized in a 50 µl/l solution of eugenol (Merck Chemical, Germany) before injections and stripping. Special care was taken to avoid contamination of gametes with water, urine or faeces was carefully avoided. Carp pituitary extract (CPE) which suspended in 0.65% NaCl solution, was injected into intramuscularly to the brood fishes. Adult males were injected with 1 mg kg^{-1} of body weight of CPE 24 h before stripping. Females were injected at 2.5 mg kg^{-1} body weight of the same hormone in two doses, which 10% of the total dose was administered 24 h before stripping while the remaining 90% was injected 12 h later. The sperm stripped by abdominal pressure from ten males was collected into 20-mL glass tubes, which were covered with parafilm and stored in a styrofoam box holding crushed ice (4±2°C). Spermatological properties of fresh sperm samples were evaluated following stripping in ten minutes at the laboratory.

2.2. Evaluation of sperm quality and dilution

Evaluation of sperm was performed at +4 ºC using a cold table. Sperm motility was activated by mixing 1 µl of sperm with 20 µl activation solution (0.3 %NaCl) on a glass slide using a phase contrast microscope (Olympus, Japan) at 400× magnification. Sperm motility was determined with three replications of samples. Samples below 85% motility were discarded. Sperm motility period was assessed using a sensitive chronometer (sensitivity: 1/100 s) by recording the time following addition of the activation solution to the sperm samples.

In order to determine spermatozoa density, sperm was diluted at a ratio of 1:1.000 with Hayem solution (5 g Na_2SO_4, 1 g NaCl, 0.5 g $HgCl_2$ and 200 ml bicine) and density was evaluated using a 100 µm deep Thoma haemocytometer (TH-100; Hecht-Assistent, Sondheim, Germany) at 400x magnification with Olympus BX50 phase contrast microscope (Olympus, Japan) and expressed as spermatozoa $x10^9$ ml^{-1} (three replicates). Sperm pH was measured using indicator papers (Merck). Following evaluation, selected sperm samples were pooled and stored at +4ºC until dilution and cryopreservation.

2.3. Cryopreservation

Sperm samples from 5 males showing >85 motility was employed for the experiments. Sperm and extenders were hold at 4°C before dilution. Pooled semen was diluted at 1:3 ratio with three different extenders (E-I: 75 mmol/l NaCl, 70 mmol/l KCl, 2 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 20 mmol/l Tris, (pH: 8) [1]; E-II: 350 mM glucose, 30 mM Tris (pH: 8) [8]; E-III: 300 mM glucose, 10% egg yolk (pH: 4.5) [9]). Each of these extenders supplemented with three different types of cryoprotectants (DMSO, DMA, Glycerol) separately at 10% ratio.

The diluted samples were drawn into 0.25-ml plastic straws (IMV, France) and were sealed with polyvinyl alcohol (PVA). Before freezing, the semen samples were equilibrated at 4 °C for 10 min and following placed on a tray, which floating on the surface of liquid nitrogen in a styrofoam box, was adjusted according to 3 cm above of the liquid nitrogen (-120 °C) and the sperm samples were frozen for 10 min. Then, the straws

containing sperm samples were kept in liquid nitrogen (-196 °C) container until thawing. For the aim of thawing, the straws were removed from the liquid nitrogen tank and submerged into a water bath at 30 °C for 20 s. Afterward, post-thaw sperm motility and periods were evaluated immediately.

2.4. Fertilization

Fertilization process was performed at spermatozoa to egg ratio of $1.7x10^6$ in 500 ml plastic containers using 30 ml activation solution, which containing 3 g urea and 4 g NaCl in 1 l distilled H_2O, at 22°C for 30 min through slow mixing of the eggs and sperm. Then, the eggs were washed with the tannic acid solution(0.5-0.7 g/l) in order to eliminate adhesiveness for 10 min and following washing with the water gently transferred to the labelled Zuger glass incubators with running water (20-22°C) where kept until eyeing (14-16 h) and hatching (3-4 d). Fertilization of the eggs was determined at cell division stage.

2.5. Statistical Analysis

Statistical analyses were carried out using SPSS 14 for Windows Statistical Software Package. Results are presented as means±S.E. and differences between treatment groups were analyzed by two-way analysis of variance (ANOVA). Significant means were subjected to a multiple comparison test (Mann-Whitney U) for post-hoc comparisons at level of α=0.05. Normal distribution of the data was verified prior to statistical analyses.

3. Results

Fresh semen volumes ranged between 4.5ml-36.4 ml and mean volume was 14.23±22.16 ml. Motility values ranged from 70% to 90% and mean values were determined as 82±7.27%. Mean viability period (s), density ($x10^9$/ml), and pH values were 575.36±624.63 s, 11.5±3 $x10^9$/ml and 7.7±0.4 respectively (Table 1).

Mean fresh (control) sperm motility was 82±7.27% and sperm cryopreserved with 10% DMSO and 10% DMA containing ionic based extender (E-1) showed the lowest mean post-thaw motility (10.0±0.0). On the other hand, the highest mean post-thaw motility (40.0±5.77%) was obtainedusing10% DMSO containing glucose-egg yolk based extender (E-3)($p<0.05$) (Figure 1).

While the highest mean post-thaw viability period (106.67±43.33 s) was determined with glucose based extender (E-II) containing DMSO, the lowest one (31.66±9.27 s) was determined with ionic based extender (E-I) containing DMA ($p<0.05$) (Figure 2).

The overall mean fertilization rate of frozen sperm was 97.7±0.63% while the best fertilization rate was 99.6±0.33% with sperm that was frozen with glucose based extender (E-2).On the other hand, motility and fertilization rates of frozen-thawed scaly carp sperm was not statistically different between the experimental groups ($p>0.05$). (Figure 3).

The hatching rate of larvaes is very low and hatching was not observed in all experimental groups. The highest hatching rate (10%) was determined in extender (E-III) containing DMA.

Table 1: Spermatological properties in fresh scaly carp sperm (n=11).

Fish No	Volume (ml)	Motility (%)	Viability Period (s)	Concentration ($x=10^9$/ml)	pH
1	7.3	90	225	14.2	7.9
2	11.2	70	327	10.1	7.7
3	36.4	80	342	9.3	8.0
4	4.5	90	620	14.5	7.9
5	11	80	330	10.9	7.7
6	27	90	540	11.0	8.0
7	10.1	85	1200	13.7	7.7
8	8	80	792	10.1	7.4
9	20	80	617	9.8	7.3
10	11	90	590	13.4	7.6
11	10.1	75	746	9.7	7.7
Mean±SD	14.23±22.16	82 ±7.27	575.36 ±624.63	11.5 ±3	7.7 ±0.4

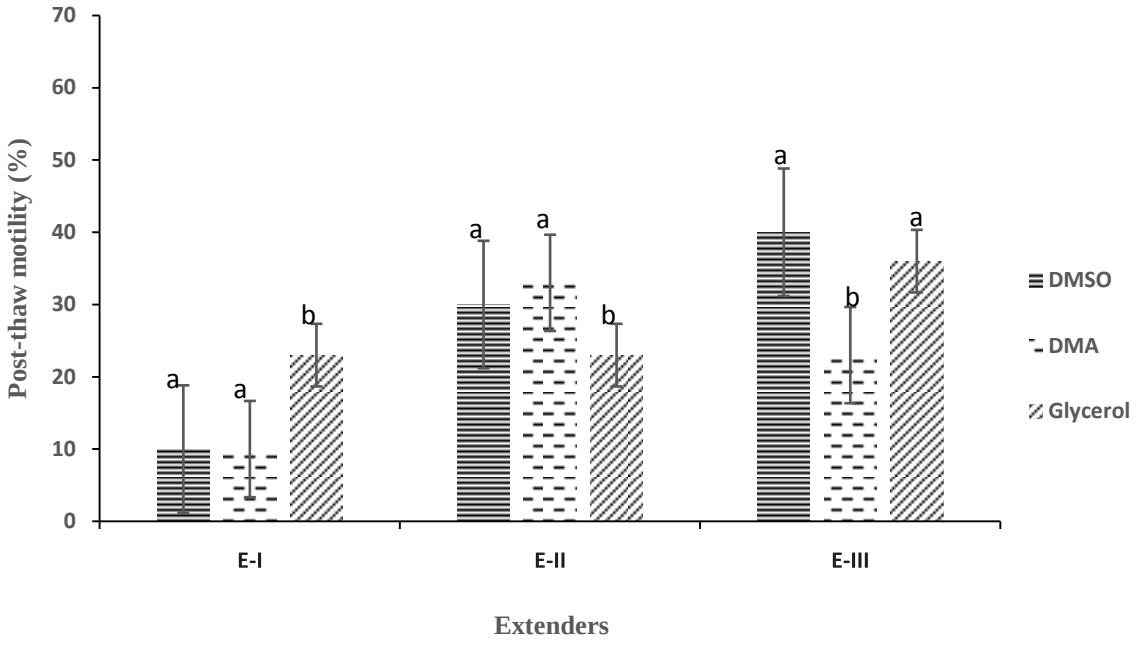

Figure 1: Effect of different extenders and cryoprotectants on post-thaw motility (%) of scaly carp sperm (n=3; mean±S.E.). Different letters indicate differences among datasets (ANOVA, p<0.05)

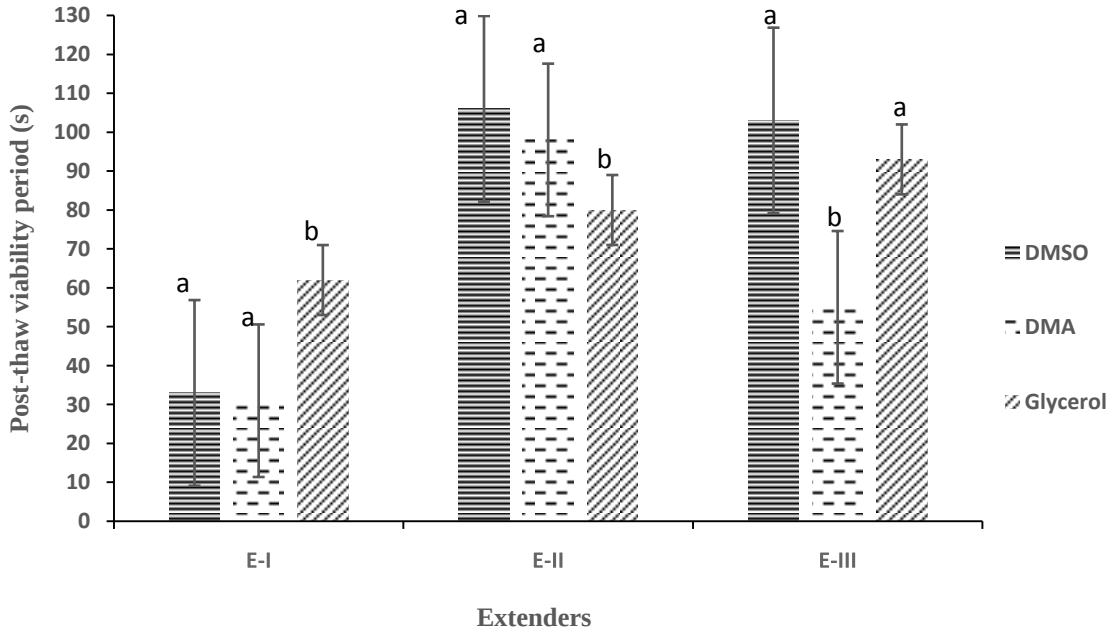

Figure 2: Effect of different extenders and cryoprotectants on post-thaw viability period (s) of scaly carp sperm (n=3; mean±S.E.). Different letters indicate differences among datasets (ANOVA, p<0.05).

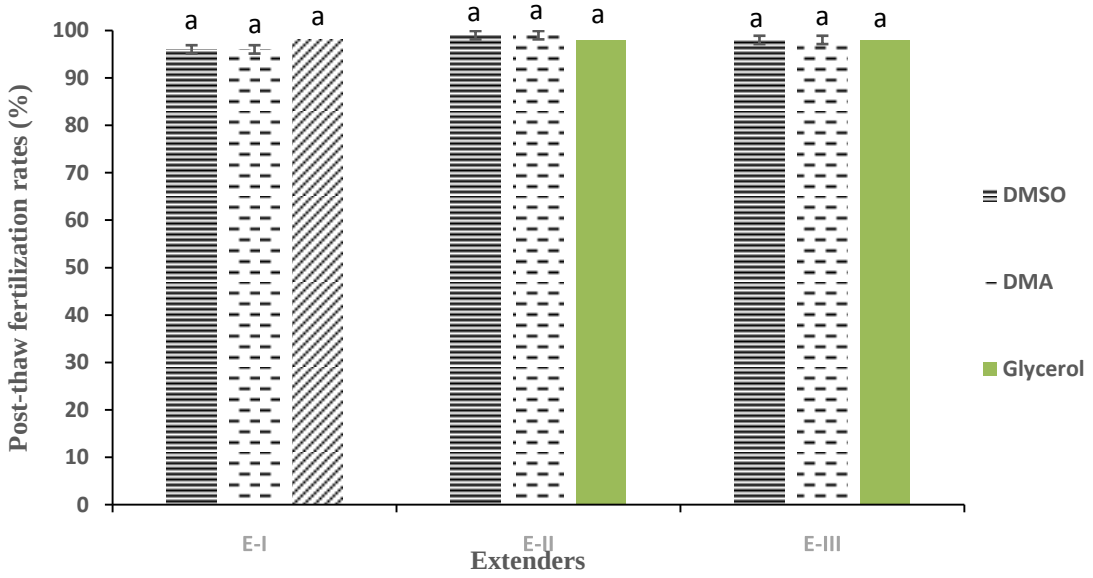

Figure 3: Effect of different extenders and cryoprotectants on post-thaw fertility of scaly carp Sperm (n=3; mean±S.E.). Different letters indicate differences among datasets (ANOVA, p<0.05).

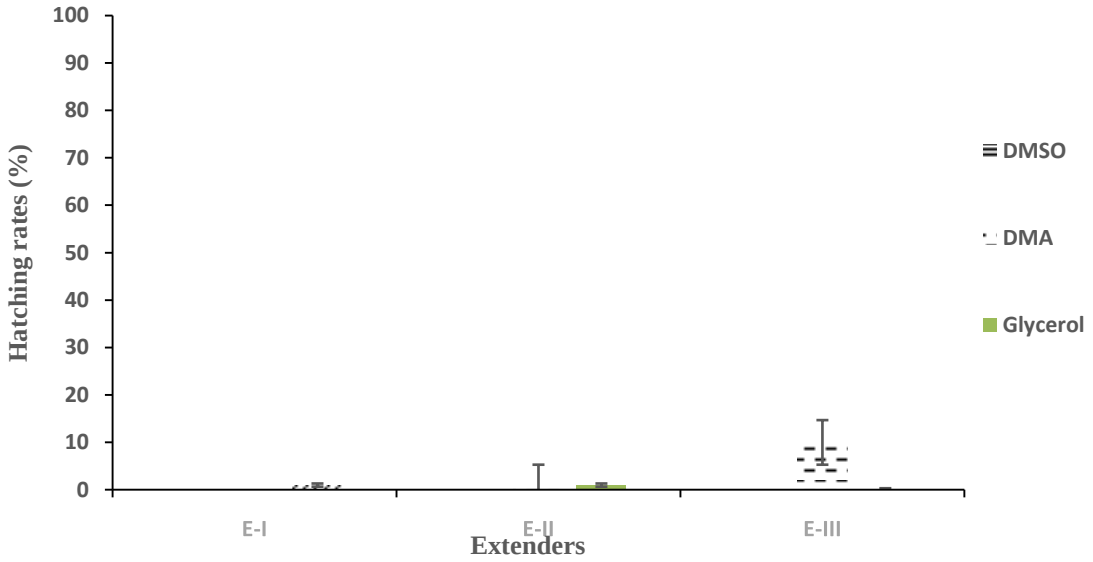

Figure 4: Effect of different extenders and cryoprotectants on post-thaw hatching (%) of scaly carp larvaes (n=3; mean±S.E.). Different letters indicate differences among datasets (ANOVA, p<0.05).

4. Discussion

Cryopreservation of fish sperm has been applied as an important tool in terms of carrying out of selective breeding programs and also conservation of important species in aquaculture. However, this process cause damages to the sperm cells [10]. It is well known that, extenders and cryoprotectants are key factors for the successful long-term conservation of sperm [11, 12].

Extenders have been employed to maintain higher motility following long-term preservation of spermatozoa, stabilise the immotile situation as in the seminal plasma during cryogenic storage [13] and also evolve fertility

[14]. Thus, extenders have been developed in accordance with ionic composition of the seminal plasma [15, 16] or sugar based extenders have been employed to improve sperm motility during cryopreservation. From this point of view, combinations of ionic, glucose-Tris and glucose-egg yolk based extenders and also DMSO, DMA and glycerol as cryoprotectants, were tested regarding post-thaw motility, viability period and fertilization capacity of frozen-thawed scaly carp sperm in the present study.

It should be noted that good knowledge of fresh sperm features of the brood fish is very important in commercial hatcheries in terms of artificial propagation and also in laboratories before the cryopreservation experiments. In this study, fresh scaly carpsperm was evaluated following hormonal application with carp pituitary extract (CPE). Mean sperm volume (14.23±22.16 mL), spermatozoa motility (82±7.27%), concentration (11.5±3 $x10^9$ spermatozoa mL^{-1}), viability period (575.36±624.63 s) and pH (7.7±0.4) for the males employed in this study were all within the range of *Cyprinidae* species [17,18, 19,20].

In this study, three extender and cryoprotectant combinations, which previously described, were tested. Composition of two glucose based extenders (E-II and E-III) were similar except presence or absence of egg yolk or Tris. Higher post-thaw motility results were attained with the extender combinations of glucose-egg yolk / DMSO and glucose-egg yolk / glycerol respectively. Similarly, higher post-thaw viability periods were achieved with the combinations of glucose-Tris / DMSO and glucose-Tris / DMA and also glucose-egg yolk / DMSO and glucose-egg yolk / glycerol respectively.

Glucose based extenders have been thoroughly investigated for the cryopreservation of fish semen for a number of fish species such as rainbow trout (*Oncorhynchus mykiss*) [21], Caspian brown trout (*Salmo trutta caspius*) [22], Atlantic salmon (*Salmo salar*)[23], brook trout (*Salvelinus fontinalis*) [24] and yellow croaker (*Pseudosciaena crocea*) [25]. Yıldız *et al*. [26] reported that molecular weight of sugar and buffer solution (such as tris) employed in the extenders could affect cryoprotective ability of the sugars. In this framework, glucose could playprotective role by interacting with the membrane lipids and proteins and also could decrease intracellular ice crystal formation risk causing cellular osmotic dehydration during cryopreservation [27]. Additionally, glucose supplies additional energy source for the sperm cells during recovery [28].

DMSO and DMA has proved higher post-thaw motility, viability period and fertility with glucose based extenders (E-II and E-III) instead of ionic based extender except fertility (E-I). The ionic structure of the extender was reported as the main factor for the controlling of sperm activation in many fish species and this could be the reason for the difference between sugar based extenders. DMSO and DMA, which are the permeable cryoprotectants regulating cellular dehydration by osmotically replacing water and reduces intracellular ice crystal formation. Rani and Munuswamy [29] reported that 10% DMSO was the optimum concentration for sperm viability and motility in the thawing semen of *Cyprinus carpio*. Similar results were obtained in common carp, when sperm motility and post-thaw motility duration were compared [1,17].

Cabrita *et al* (2001) demonstrated an increase in live spermatozoa in rainbow trout when different membrane stabilizers (BSA, egg yolk and soya) used in conjunction with permeating CPA when compared with freezing without these agents. Judycka e*t al* (2015) suggested the 0.1 M glucose as external CPA, that stabilizes the sperm cell membrane, in conjunction with 15% MeOH as an alternative extender for cryopreservation of Siberian sturgeon (*Acipenser baerii*) sperm. However, glycerol containing extender (E-III) demonstrated its positive effect on these parameters. Possible explanation of the higher results regarding E-III could be due to adding of egg yolk to the glucose based extenders. Furthermore, this might be caused by synergistic effect of glucose and cryoprotectants resulting in higher performance during cryopreservation. Additionally, sugar-based extenders can result in a jelly-like agglutination of spermatozoa in cryopreservation of cyprinid semen [2], which limits fertilization ability of sperm, the presence of this type formations were not observed in this study. Also it should be noted that when cryopreserved sperm is subjected to fertilization of fewer eggs with a higher ratio of extender, it will be easier for the sperm cells to reach the eggs. It is thought to have an important reason for the fertilization rates.

On the other hand, in spite of high fertilization results have been attained with the cryopreserved sperm in all tested groups, the results of the present study showed that a considerable decrease in the survival from the fertilized-egg stage to the hatching stage occurred in those treatments. Perez-Cerezales *et al*. [30] reported that sperm with damaged DNA can be viable and fertilize eggs. Cryopreservation of sperm could affect offspring

development by affecting the genetic and epigenetic information that is transmitted to the zygote [31]. Some authors reported that zygote may repair some degree of DNA fragmentation [30, 32, 33], but cryodamage can effect the embryo and offspring development [34, 35].

5. Conclusion

The findings of the present study indicated that combinations of glucose based extenders with DMSO and DMA has proved higher post-thaw motility, viability period and fertility. On the other hand, the results demonstrated revealed that scaly carp sperm is sensitive to cryodamage because of low embryo development and hatching. Thus, future studies should explore cryodamage and also the possible links between cryodamage, embryo development and offspring growth in scaly carp.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

References

[1]. Lahnsteiner, F., Berger, B., Horvath, A., Urbanyi, B., & Weismann, T. (2000). Cryopreservation of spermatozoa in cyprinid fishes. *Theriogenology*, 54: 1477-1498.

[2]. Irawan, H., Vuthiphandchai, V., & Nimrat, S. (2010). The effect of extenders, cryoprotectants and cryopreservation methods on common carp (*Cyprinus carpio*) sperm. *Animal Reproduction Science*, 122: 236–243.

[3]. Ashwood-smith, M.J. (1980). Low temperature preservation of cells, tissues and organs. Low temperature preservation of medicine and biology. Pitman medical. Kent, UK. 19-44.

[4]. Purdy, P.H. (2006). A review on goat sperm cryopreservation. *Small Ruminant Research*, 63: 215-225.

[5]. Gallant, R.K., Richardson, G.F., & McNiven, M.A. (1993). Comparison of different extenders for the cryopreservation of Atlantic salmon spermatozoa. *Theriogenology*, 40: 479-486.

[6]. Rana, K. (1995). Preservation of gametes. In: Bromage, N.R., Roberts, N.J. (Eds.), Broodstock Management and Egg and Larval Quality. Cambridge University Press, Cambridge, pp. 53–76.

[7]. Lahnsteiner, F., Patzner, R., & Wiesmann, T. (1996). Semen cryopreservation of salmonid fishes: influence of handling parameters on the post-thaw fertilization rate. *Aquaculture Research*, 27: 659–671.

[8]. Horvath, A., Miskolczi, E., & Urbanyi, B. (2003). Cryopreservation of common carp sperm. *Aquatic Living Resources*, 16: 457-460.

[9]. Tekin, N., Seçer, S., Akçay, E., & Bozkurt, Y. (2003). Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) semen. *The Israeli Journal of Aquaculture-Bamidgeh*, 55 (3): 208-212.

[10]. Bozkurt, Y.,Yavaş, İ., Bucak, M.N.,& Yeni, D. (2019). Effect of different cryoprotectants (Glycerol, Methanol and Dimethyl Sulfoxide) on post-thaw quality, viability, fertilization ability and DNA damage of cryopreserved Nile tilapia (O*reochromis niloticus*) spermatozoa. *CryoLetters*, 40 (1): 11-17.

[11]. Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., & Billard, R. (2000). Cryopreservation of sperm in marine fish. *Aquaculture Research*, 31: 231-243.

[12]. Chao, N.H., & Liao IC. (2001). Cryopreservation of finfish and shellfish gametes and embryos. *Aquaculture*, 197:161-189.

[13]. Tan Fermin, J.D., Miura, T., Adachi, S., & Yamauchi, K.(1999) Seminal plasma composition, sperm motility and milt dilution in the Asian catfish, *Clarias macrocephalus* (Gunther). *Aquaculture*, 171: 323-338.

[14]. Ohta, H., & Izawa, T. (1996). Diluent for cool storage of the Japanese eel (*Anguilla japonica*) spermatozoa. *Aquaculture*, 142: 107-118.
[15]. Villani, P., & Catena, C. (1991). Criopreservazione di gamete maschili di spigola (*Dicentrarchus labrax*). *Rivista Italiana di Acquacoltura*, 26: 217–222.
[16]. Asturiano, J.F., Pe´rez, L., Garzo´n, D.L., Marco-Jime´nez, F., Pen˜aranda, D.S., Vicente, J.S., &Jover, M. (2004). Physio-chemical characteristics of seminal and development of media and methods for the cryopreservation of European eel sperm. *Fish Physiology Biochemistry*, 30: 283–293.
[17]. Akçay, E., Bozkurt, Y., Seçer, S., & Tekin, N. (2004). Cryopreservation of mirror carp semen. *Turkish Journal of Veterinary and Animal Sciences*, 28 (5): 837-843.
[18]. Bozkurt, Y., & Seçer S. (2005). Effect of short-term preservation of mirror carp (*Cyprinus carpio*) semen on motility, fertilization and hatching rates. *The Israeli Journal of Aquaculture-Bamidgeh*, 57(3): 207-212.
[19]. Bozkurt, Y. (2006). Relationship between body condition and spermatological properties in scaly carp (*Cyprinus carpio*) semen. *Journal of Animal and Veterinary Advances*, 5 (5): 412-414.
[20]. Bozkurt, Y., Öğretmen, F., & Seçer, F.S. (2011). Effect of pH and ions on motility of scaly carp (*Cyprinus carpio*) spermatozoa. *Journal of Environmental Protection and Ecology*, 12(3): 990-998.
[21]. Tekin, N., Seçer, S., Akcay**,**E., Bozkurt, Y., & Kayam, S. (2007). Effects of glycerol additions on post-thaw fertility of frozen rainbow trout sperm, with an emphasis on interaction between extender and cryoprotectant. *Journal of Applied Ichthyology*, 23(1): 60-63.
[22]. Moghanloo, S.K., Niksirat, H., Mojazi Amiri, B., & Mirtorabi, S.M. (2007). *The effects of extender type, freezing and thawing rates on fertility of the cryopreserved semen of the Caspian brown trout (Salmo trutta caspius). Iranian Journal of Fisheries Sciences*, 7(1): 111-128.
[23]. Dziewulska, K., Rzemieniecki, A., Czerniawski, R., & Domagała, J. (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.
[24]. Lahnsteiner, F., Mansour, N., & Kunz, F.A. (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.
[25]. Cheng, S., Sheng, Z., Zhu, J-Q., & Wu, X.F. (2016). Effects of trehalose, bovine serum albumin, and sucrose, on the integrity of the plasma membrane of *Pseudosciaena crocea* semen after cryopreservation. *The Israeli Journal of Aquaculture-Bamidgeh*, 68: 1219.12.
[26]. Yıldız, C., Kaya, A., Aksoy, M., & Tekeli, T. (2000). Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. *Theriogenology*, 54: 579-585.
[27]. Agca, Y., Gilmore, J., Byers, M., Woods, E.J., Liu, J., & Critser, J.K. (2002). Osmotic characteristics of mouse spermatozoa in the presence of extender and sugars. *Biology of Reproduction*, 67: 1493-1501.
[28]. Rodina, M., Gela, D., Kocour, M., Alavi, H.S.M., Hulak, M. & Linhart, O. (2007). Cryopreservation of tench, *Tinca tinca*, sperm: sperm motility and hatching success ofembryos. *Theriogenology*, 67: 931-940.
[29]. Rani, K.U., & Munuswamy, N.(2014). Effect of DNA damage caused by cryopreservation of spermatozoa using a modified single cell gell electrophoresis in the freshwater catfish *Pangasianodon hypophthalmus* (Fowler, 1936). *Journal of Coastal Life Medicine*, 2: 515-519.
[30]. Pérez-Cerezales, S., Martínez-Páramo, S., Beirão, J., & Herráez, M.P. (2010). Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. *Reproduction*, 139: 989-997.
[31]. Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., & Robles, V. (2014). Factors enhancing fish sperm quality and emerging tools for sperm analysis. *Aquaculture*, 432: 389-401.

[32]. Fernández-Díez, C., González-Rojo, S., Lombó, M., & Herráez, M.P. (2018). Tolerance to paternal genotoxic damage promotes survival during embryo development in zebrafish (*Danio rerio*). *Biology Open*, 7: bio030130.
[33]. Ménézo, Y., Dale, B., &Cohen, M. (2010). DNA damage and repair in human oocytes and embryos: a review. *Zygote*, 18: 357-365.
[34]. Labbé, C., Martoriati, A., Devaux, A., & Maisse, G. (2001). Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. *Molecular Reproduction and Development*, 60: 397-404.
[35]. Aitken, RJ, & Krausz, C. (2001). Oxidative stress, DNA damage and the Y chromosome. *Reproduction*, 122: 497-506.