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Research Article

Cryopreservation of Mirror Carp Semen

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Abstract: The effects of different extenders and cryoprotectants on the viability and fertilizing ability of frozen spermatozoa from the mirror carp, *Cyprinus carpio* L. (1758), were investigated. Semen was collected from anesthesized males by the abdominal massage method. Having determined the main spermatological properties (volume, motility, duration of motility, total spermatozoa number, concentration and pH), the pooled ejaculates were diluted with 3 extenders containing different cryoprotectants (15% DMSO, 15% DMA, 15% Glycerol) individually. One part semen was added to 3 parts extender. The diluted semen was packaged in 0.5 ml straws and left to equilibrate for 45 min at 4 °C. Following the equilibration, the straws were exposed to liquid nitrogen vapor for 10 mins and plunged into the liquid nitrogen. Afterwards frozen semen in the straws was thawed in a waterbath at 30 °C for 30 s to determine the motility and movement duration with regard to the post-thaw duration.

The success of freezing was assessed from post-thaw sperm motility, movement duration and fertilizing ability. According to the results, while the rates of motility after thawing were similar, the lowest movement duration (10 s) ($P < 0.001$) was found in extender 1, containing DMA and the highest fertilization rate (25.9%) ($P < 0.05$) was found in extender 3, again containing DMA. In conclusion, the extender-cryoprotectant interaction was important in the cryopreservation of mirror carp semen.

Key Words: Mirror Carp (*Cyprinus carpio*), semen, extender, cryopreservaton, fertilization

Aynalı Sazan Balığı Spermasının Dondurulması

Özet: Bu çalışmada dondurulmuş aynalı sazan balığı, *Cyprinus carpio* L. (1758), spermatozoonlarının canlılık ve fertilizasyon yeteneği üzerine farklı sulandırıcı ve cryoprotektanların etkileri araştırıldı. Sperma anestezi altına alınmış erkeklerden abdominal masaj yoluyla alındı. Alınan ejekülatların birincil spermatolojik özellikleri (miktar, motilite, motilite süresi, toplam spermatozoa sayısı, yoğunluk ve pH) saptandıktan sonra spermalar aynı kapta toplanarak (pooling), farklı cryoprotektan maddeler (% 15 DMSO, % 15 DMA, % 15 Glycerol) içeren üç farklı sulandırıcı ile sulandırıldı. Bir kısım spermaya üç kısım sulandırıcı eklendi. Sulandırılan spermalar 0,5 ml payetlere çekilerek 4 °C de 45 dk ekilibrasyonda tutuldu. Ekilibrasyon sonrası payetler sıvı azot buharında 10 dk dondurulduu ve sıvı azot içinde depolandı. Payetler içinde dondurulan sperma 30 °C su banyosunda 30 saniyede çözdürüldü ve çözüm sonrası motilite ve canlılık süreleri tesbit edildi.

Dondurma işleminin başarısı çözüm sonrası motilite, canlılık süresi ve fertilizasyon yeteneği ile değerlendirildi. Çalışmada elde edilen sonuçlara göre çözüm sonrası motilite oranları benzer bulunurken, en düşük canlılık süresi DMA içeren 1. sulandırıcıda (10 sn) ($P < 0.001$) en yüksek fertilizasyon yine DMA içeren 3. sulandırıcıda (% 25,9) ($P < 0.05$) bulundu. Sonuç olarak, aynalı sazan spermasının dondurulmasında sulandırıcı cryoprotektan etkileşiminin önemli olduğu gözlendi.

Anahtar Sözcükler: Aynalı sazan, sperma, sperma sulandırıcısı, spermanın dondurulması, fertilizasyon.

Introduction

It has been estimated that sperm from 200 fish species has been successfully cryopreserved (1). However, appropriate to each species, optimizations of technology are needed. The successful cryopreservation of fish spermatozoa might be used to increase the number of offspring from genetically superior males, aid in the transport of semen and provide a year-round

supply of male gametes. Furthermore, cryopreservation can increase the economic utilization of males and is a prerequisite for the establishment of gene banks (2).

There has been considerable research on semen preservation in teleosts. The cryopreservation of the sperm of carp has been attempted by many authors (3-7). Besides the influence of the variability in biological material on the fertilizing ability of frozen semen, the multiplicity of cryopreservation procedures affects the consistency of fertilization results. Differences in diluents, cryoprotectants and freezing techniques make it difficult to get variable estimations of the efficiency of various procedures (8,9).

Generally 2 types of extenders have been developed for the cryopreservation of fish spermatozoa: seminal plasma mimicking media (3) and simple carbohydrate-based solutions (5,10). Mostly dimethyl sulfoxide (DMSO) is used as the permeating cryoprotective agent for cryopreservation, but other cryoprotectants, like dimethyl acetamide (DMA), ethylene glycol, glycerol and DMSO-glycerol mixture, are also efficient (4,5,11). In particular, using an appropriate cryoprotectant solution protects cells from cellular disruption and membrane damage during freezing and thawing (12). Although the spermatozoa diluted with extenders are usually frozen as pellets on dry ice, they can also be frozen straws or ampoules in liquid nitrogen vapor (8,13). Freezing is applied immediately after dilution of the spermatozoa or after an equilibration time (10).

In numerous fish species with external fertilization, the duration of sperm motility is very short (14,15). The highest motility of the sperm is observed at the height of the breeding season (16). Studies on most fish species reported that the duration and motility of semen may show seasonal variation (17,18). Therefore sperm motility is an important component of a cryopreservation program in order to prevent poor quality semen samples prior to freezing and to estimate the fertility of the stored sperm after thawing. Most experiments in this field have focussed on finding appropriate extenders and cryoprotective agents for carp. In cyprinid fishes, sperm motility is often used to estimate semen quality and viability when spermatozoa are exposed to various experimental conditions (19). A relationship between motility and fertilizing capacity has been assumed by several authors (20,21). From this point of view, post-thaw motility, motility duration and fertilizing ability are very important for frozen cyprinid fish semen. The main purpose of this study was to investigate the effects of various extenders containing different cryoprotectants on post-thaw motility and fertilizing ability of frozen spermatozoa from mirror carp.

Materials and Methods

Adult Fish and Care

In this experiment, fifteen adult male mirror carp aged from 3 to 7 cultured at the State Hydraulic Works Fish Production Station in Bolu, Turkey, were used. In the pre-spawning period the parenteral brood fishes were kept separately in small pods and fasted 48 h prior to semen collection.

Gamete Collection

Semen was collected from anesthetized (0.1 g/l MS 222) males by manual abdominal stripping 12 h after a single injection of 2 mg/kg of carp pituitary extract (CPE) at 20-22 °C water temperature (22). Eggs were collected by hand stripping 10-12 h after a double injection of 3.5 mg/kg of CPE. The first injection, 10% (0.35 mg/kg) CPE was given 10 h before the second (3.15 mg/kg). Their abdomens and urogenital papilla were dried before stripping. Samples contaminated with fecal material or urine were discarded.

Evaluation of Motility, Movement Duration, Concentration and pH

Motility was evaluated using a light microscope at x200 magnification and was expressed as a percentage of motile spermatozoa. An activating solution of 0.3% NaCl was used for estimating motility. For the evaluation of motility, about 10 µl of semen was placed on a glass microscope slide and 100 µl of activation solution was added. Only samples showing high motility (>80%) were used for freezing. Movement duration was estimated using a sensitive chronometer (1/100 s). Motility and movement duration were evaluated according to the following criteria: 1) Mass progressive motility when most of the spermatozoa were still actively swimming with progressive movement, and 2) Total movement duration until most spermatozoa stopped swimming. The sperm concentration was estimated using the hemocytometric method and expressed as spermatozoa x 10^9 / ml. pH was measured using indicator papers.

Extenders and sperm cryopreservation

Semen collected from 13 males was pooled in equal amounts. The pooled semen was diluted at a ratio of 1:3 (1 part semen/3 parts extender) with 3 extenders containing 3 different cryoprotectants (15% DMSO, 15% DMA, 15% glycerol) resulting in 9 extenders. Extender 1, containing 0.75 g of NaCl, 0.02 g of KCl, 0.02 g of $CaCl_2$ and 0.02 g of $NaHCO_3$ supplemented to 100 ml with distilled water, was described by Kurokura et al. (3). Extender 2, containing 6 g of glucose, 0.3 g of NaCl and 0.05 g of $NaHCO_3$ supplemented to 100 ml with distilled water, was described by Zhang and Liu (23). Extender 3, containing 0.88 g of NaCl, 1.12 g of KCl and 0.36 g of Tris supplemented to 150 ml with distilled water, was described by Cognie et al. (5). The sperm and diluent were kept at 4 °C prior to dilution.

The diluted samples were drawn into 0.5 ml plastic straws (IMV, France) and were sealed with polyvinyl alcohol (PVA). Having been diluted, the samples were equilibrated for 45 min at 4 °C. After equilibration, the straws were placed on a Styrofoam rack that floated on the surface of liquid nitrogen in a Styrofoam box. The straws were frozen in liquid nitrogen vapor 3 cm above the surface of liquid nitrogen for 10 min. After 10 min the straws were plunged into the liquid nitrogen and stored for several days.

The frozen straws were thawed by plunging them into water at 30 °C for 30 s. Thawed sperm was activated using 0.3% NaCl and motility and movement duration were tested and recorded again.

Fertilization

All fertilization experiments were performed in triplicate at 22 °C. The fertilization solution (3 g of urea, 4 g of NaCl in 1 l of water) and the dry fertilization technique were used. Eggs were pooled from 7 females. Fertilization took place in dry plastic dishes and 100 g of eggs was placed into each dish. Batches of eggs (approximately 100,000 eggs) were inseminated with frozen or control semen (freshly collected semen). The sperm-egg ratio was approximately 250,000 sp/egg. Thawed spermatozoa were added over the eggs and gently mixed before activation with 20 ml of fertilization solution. After fertilization the eggs were stirred for 1 h and at the end of the stirring they were treated with tannic acid (0.5 g/l) for 30 s to remove the stickiness of eggs. Then the eggs were rinsed with hatchery water and placed into incubation trays. The experimental success was determined as the percent of swim-up larvae 3 days after fertilization.

Statistical analysis

Data are expressed as mean ± standard error. Differences in the means between treatments were tested using 2-way analysis of variance (ANOVA) by SPSS 10.0 software.

Results

The spermatological properties of the semen collected from 15 males are shown in Table. Semen volumes were rather variable and ranged from 1 to 40 ml and mean volume was 13.26 ml. Motility values were rather high and ranged from 70% to 95%. Samples 5 and 11 were not used for the cryopreservation because their motility values were below than 80%. The mean motility value from 15 samples was 88.33%.

Frozen sperm showed a significant decrease in quality compared to fresh sperm (Figure 1). The motility decrease was similar for the 3 types of extenders tested and varied from 30% to 55% except for extender 1 containing DMA (10%). Semen frozen with extender 1 containing DMA showed a percentage of motile cells (10%) lower than the others. Semen frozen with extender 1 containing DMSO had the highest post-thaw motility (55%), but this parameter was not significant ($P > 0.05$) with regard to the extender or cryoprotectant although the interaction between the extender and cryoprotectant was significant ($F = 4.105$).

Movement duration in fresh semen also showed significant differences with regard to the frozen spermatozoa (Figure 2). It was observed that a decrease in movement duration occurred after cryopreservation. The longest durations were achieved when using extender 2. Differences beetween the means of movement durations were significant ($P < 0.001$) when the extender was taken into consideration. The interaction beetween the extender and cryoprotectant was significant ($F = 3.969$).

Fertility trials with frozen sperm showed significant differences from the controls. The fertilization results are shown in Figure 3 as percentages of swim-up larva rates. The fertilization of eggs with frozen semen with equilibration in extenders 1-3 containing 15% DMSO, 15% DMA and 15% glycerol individually resulted in 20.5,

Table. Spermatological properties of collected samples.

Sample no	Volume (ml)	Motility (%)	Motility Duration (min:s)	Concentration ($x10^9$/ml)	Total spermatozoa number ($x10^9$)	PH
1	24	90	10:35	24.625	591	8.0
2	40	95	11:26	12.375	495	8.0
3	18	80	7:24	16.925	304.6	7.5
4	11	90	9:05	18.750	206.2	8.0
5	2	70	5:18	22.800	45.6	8.0
6	1	90	14:18	18.325	18.3	7.5
7	9	90	5:15	23.500	211.5	8.0
8	14	95	10:30	16.100	225.4	8.0
9	8	90	8:16	22.700	181.6	8.0
10	12	90	15:30	21.250	255.0	8.0
11	20	75	8:28	12.400	248.0	8.5
12	9	90	13:40	11.800	106.2	8.5
13	7	90	5:50	13.850	96.9	8.5
14	16	95	12:10	12.900	206.4	8.0
15	8	95	12:10	11.700	93.6	8.5
X ± sx	13.26 ± 2.51	88.33 ± 1.68	9.31 ± 0.90	17.33 ± 1.22	219.02 ± 40.4	8.06 ± 0.08

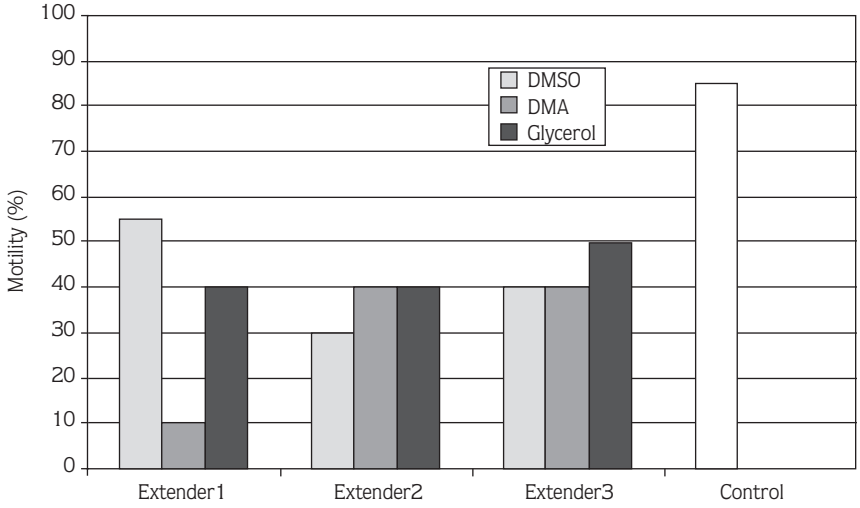

Figure 1. Post-thaw motility of frozen spermatozoa.

10.4, 15.4, 15.5, 18.7, 19.8, 22.1, 25.9 and 15.0 swim-up larva rates, respectively (Figure 3). Extender 3 containing DMA gave the best fertility (25.9%). The mean values were significantly different ($P < 0.05$, $F = 4.254$) with regard to the extender. However, the same values did not differ ($P > 0.05$, $F = 0.965$) with regard to the cryoprotectant. The interaction between the extender and cryoprotectant was significant as with motility and movement duration values ($P < 0.05$, $F = 5.098$).

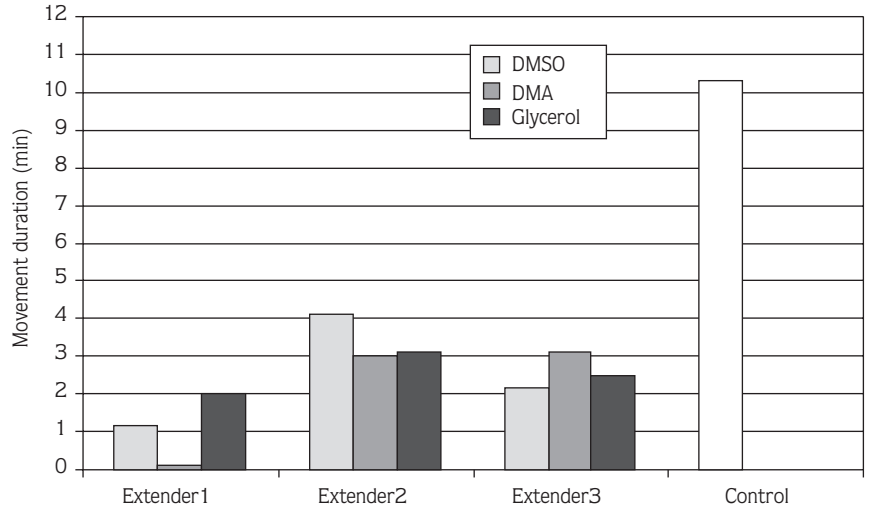

Figure 2. Post-thaw movement duration of frozen spermatozoa.

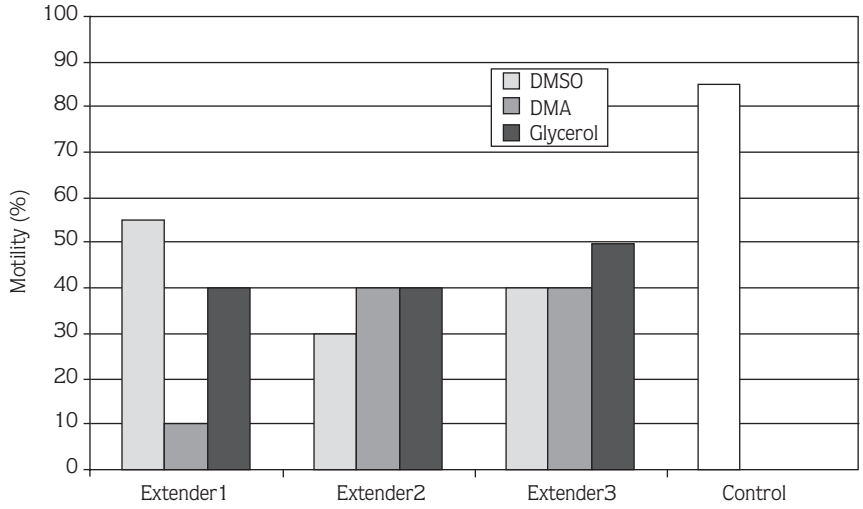

Figure 3. Fertilization rates from frozen or fresh spermatozoa.

Discussion

The cryopreservation of mirror carp semen has never been studied in Turkey. Limited amounts of data are available and the methods have only been adapted to cyprinid and salmonid species. The present paper describes the first attempt in Turkey to preserve the semen of mirror carp for longer periods of time.

The present work indicated that mirror carp spermatozoa can be successfully frozen using different extenders. These extenders have been used for fish spermatozoa cryopreservation since the 1980s elsewhere. Examination of the effect of extender constituents and cryoprotectants of diluted semen on the fertilizing ability of frozen semen yielded some interesting results. Strong cumulative effects of type of diluent and type of cryoprotectant demonstrate the multifactorial action of the extender on spermatozoal resistance against freezing injuries. These factors may not give general information about its effect on cryopreservation success but interactions might explain differences in the usefulness of extender constituents. For example, in this experiment, interactions between extenders and cryoprotectants were significant; 15%

addition of DMA to extender 1 was harmful to viability, but beneficial to fertility when using extender 3. Therefore the interaction between extender constituents is an important point to take into consideration.

Successful cryopreservation of mirror carp semen was achieved with the use of DMSO (19,23,24), DMA (25), glycerol (5) and ethylene glycol (4,11) as permeating cryoprotectants. Cabrita et al. (26) reported that dilution of the cryoprotectants causes a significant increase in fragility when the cells are exposed to hypo-osmotic shock. In this study, no significant differences were observed between the cryoprotectants. The extender and cryoprotectant have a higher osmolality than the semen samples, and so dilution of hypo-osmotic solutions for fertilization will cause greater osmotic stress. It was thought that this condition affected the fertilization results.

Post-thaw motility is one of the most important indicators of the success of a freezing protocol. Mirror carp spermatozoa motility was affected during cryopreservation. The best motility values were obtained with extender 1 containing DMSO but the fertility results in the same extender were no better than the others. The proportion of motile cells decreased faster with time in thawed sperm samples than in fresh ones. Furthermore, movement duration was also affected. Similar results for the motility parameters of frozen thawed spermatozoa were reported in fish in some experiments (3,4,9,21).

Seminal plasma mimicking media are popular in the literature for the cryopreservation of carp sperm (10,19,24,27,28). Kurokura et al. (3) used seminal plasma mimicking media as a buffer in their extenders during experiments on common carp sperm cryopreservation. Magyary et al. (29) modified Kurokura's extenders, but did not change the concentration of $NaHCO_3$. Tris is the most common buffer solution, not only for cyprinidae but also for other fish species (11). Carbohydrate-based solutions have also been found to be effective in some experiments (6,25,30,31). Cognie et al. (5) reported fertilization rates obtained from common carp semen frozen with Menezo-inra B2 containing glycerol or DMSO of 30% and 40%, respectively. Zhang and Liu (23) reported a 73.8% fertilization rate from grass carp semen frozen with glucose-DMSO.

The present comparison of different extenders indicated that better post-thaw motility does not necessarily indicate better fertilizing ability. The decrease in fertilization percentage can be explained by the lower sperm to egg ratio and the toxic effects of cryoprotectants. The toxic effect of cryoprotectants on eggs and embryos at room temperature has been reported in common carp (32). In addition, some authors recommend having an equilibration time after dilution, allowing cryoprotectants to penetrate the spermatozoa before cryopreservation (6,21). However, some authors reported this did not improve cryopreservation success in fish (4,9,19). In the present work, diluted samples were equilibrated for 45 min at 4 °C.

In this study, the inseminations were carried out using 250,000 thawed spermatozoa per egg. Munkittrick and Moccia (2) and Billard (13) reported that the successful fertilization of eggs with frozen semen requires up to 3×10^6 sperm per egg, and when using fresh semen requires 200,000 sperm per egg. Under these conditions, the fertilizing capacity of frozen-thawed spermatozoa was lower than that of fresh sperm. This may reflect the change in motility and movement duration observed after the cryopreservation process. However, this fertility decrease can be compensated for by providing a higher spermatozoa to egg ratio. However, Lubzens et al. (9) reported that attempts to increase the number of fertilized eggs by using larger amounts of spermatozoa were not successful.

The reason for these differences may be attributed to semen collection, semen quality, egg quality, extender compositions, cryopreservation procedures and insemination doses.

In conclusion, the highest post-thaw motility for semen frozen with extender 1 (seminal plasma mimicking media) containing DMSO was 55%. The longest movement duration was 4 min 10 s in extender 2 (NaCl, $NaHCO_3$, glucose) containing DMSO. However, the fertilization rate in extender 3 (NaCl, KCl, Tris) containing DMA was the highest (25.9%). In addition, significant interactions between extender constituents and cryoprotectants in fertilization success were observed. Thus the cumulative effects of type of diluent and type of cryoprotectant may improve the fertilizing ability of frozen mirror carp spermatozoa.

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