Iranian Journal of Fisheries Sciences 19(5) 2293-2307 2020
DOI: 10.22092/ijfs.2018.119818.

Cryopreservation of Mesopotamian catfish (*Silurus triostegus* H., 1843) spermatozoa: Effects of diluents and osmotic pressure on spermatozoa DNA damage, rate and duration of motility

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Received: October 2017 Accepted: March 2018

Abstract

This study was performed to evaluate the effectiveness of three different conditions of osmotic pressure (325, 365 and 385 mOsm kg^{-1}) in combination with dimethyl sulfoxide (DMSO) and NaCl or glucose on spermatozoa DNA damage, rate and duration of motility. Sperm was collected from eight healthy mature Mesopotamian catfish, evaluated microscopically and pooled at 25 °C. The pooled sperm samples were diluted to a final concentration of 1/3 (sperm/diluents) in NaCl and glucose based extender (10% cryoprotectant and 10% egg yolk (EY) into 80% diluents) and separated into groups of 3 different osmotic pressures **(**325-365-385 mOsm kg^{-1}**)**. Equilibrated sperm was frozen in 0.25 mL straws. Sperm samples were tested for post-thaw sperm motility, duration of motility, DNA damage, and apoptotic index. The highest spermatozoa motility rates were obtained with glucose and NaCl diluents at osmotic pressures of 365 and 385 mOsm kg^{-1} (p<0.01). The spermatozoa motility duration was found to be the highest in glucose and NaCl diluents at 365 mOsm kg^{-1} osmotic pressure (p<0.01). The post-thawing live spermatozoa rate was determined to be the highest in the sperm frozen with glucose at 385 mOsm kg^{-1}. The apoptotic cell rate was determined to be the highest in the sperm frozen with glucose at 385 mOsm kg^{-1} osmotic pressure. The necrotic cell rate was found to be the highest with 2.08±0.39% when frozen with the glucose diluent at 325 mOsm kg^{-1} pressures. It is concluded that the glucose solution with low osmolality had a harmful effect on the spermatozoa.

Keywords: *Silurus triostegus*, Cryopreservation, DNA damage, Osmotic pressure

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[DOR: 20.1001.1.15622916.2020.19.5.16.6]

Introduction

The catfish belonging to the Siluridae family of the order Siluriformes is represented by approximately 11 genera and 97 species. In Turkey, there are two species of Siluridae spread in Europe and Asia; the European catfish (*Silurus glanis* L., 1758) and the Mesopotamian catfish (*Silurus triostegus* H., 1843) (Froese and Pauly, 2008)

The Mesopotamian catfish, which is found abundantly in Karakaya and Atatürk Dam Lakes on the Euphrates River, is consumed by the local community with pleasure (Ünlü and Bozkurt, 1996). There are many studies on the biological characteristics of the species in question in Syria, Iraq, and Iran (Eealart, 1954; Beckman, 1962; Al-Hassan and Al-Sayab, 1994; Hashemi and Ansary, 2012). A limited number of studies was conducted on the biological characteristics in Atatürk Dam Lake by Oymak *et al.* (2001), on heavy metal accumulation by Karadede *et al.* (2004), on trace metal accumulation by Mol *et al.* (2010) and Olgunoğlu and Olgunoğlu (2011), on the European catfish (*S. glanis*) and the comparison of its anatomical and morphological characteristics by Ünlü *et al.* (2012).

The European catfish (*S. glanis*) has been farmed intensively for many years (Linhart and Proteau, 1993). On the other hand, there are no studies on the species *S. triostegus*, except for a small-scale study in which the artificial insemination of *S. triostegus* belonging to the same family was performed (Şahinöz *et al.*, 2007) and a study on the cryoprotectant effect on freezing of its sperm (Doğu *et al*., 2016).

One of the most important steps in investigating farming potential is to standardize the cryopreservation protocols to protect gene resources and meet the alternative protein needs of this species which is endemic to the Euphrates-Tigris basin. In sperm freezing protocols, diluents, cryoprotectants, the duration of equilibration, freezing and thawing rates are determined as the key factors playing an important role. In addition to being the environment in which spermatozoa can remain immobile before freezing, diluents play an important role in the regulation of ionic composition, osmotic pressure, and pH of the environment. Furthermore, it has been reported that the use of Mounib solution together with egg yolk as a diluent for freezing the catfish sperm gives positive results (Baulny *et al*., 1999).

Cryoprotectants used to prevent the damage during freezing are required to be water-soluble and with low toxicity. Although many different diluents have been used in the studies, it has been reported that DMA gives the best results in European catfish compared to DMSO, glycerol, methanol or propylene glycol (Baulny *et al.*, 1999).

Extending the duration of the equilibration, which varies from species to species, is not usually a situation preferred by sperm. As in the case with all other freshwater fish, it is stated in sperm freezing studies carried out with European catfish that the duration of the equilibration can vary between 15 min and 30 min depending on the variety and ratio of cryoprotectants (Wayman and Tiersch, 2000).

Freezing rates and duration play an important role in the formation of intracellular and intercellular crystallization. If the cooling rate is too

[DOR: 20.1001.1.15622916.2020.19.5.16.6]

fast during the freezing process, water in the cell cannot go out, and intracellular icing will occur, leading to the death of spermatozoa. On the contrary, if it is too slow, there will be an increase in osmolality and dehydration of the cell while the harmless extracellular icing can lead to cell damage or death. The low cooling rate is preferred to prevent the ice formation. However, the biggest deficiency of this method is that it causes cytolysis by leading to the denaturation of lipoproteins in the membrane of cells exposed to high osmolality for a long time (Lovelock, 1957). Therefore, the freezing rate must be adjusted optimally to prevent crystallization. While different cooling techniques have been tried, programmable cooling applications have been proposed for European catfish in recent years (Viveiros *et al.*, 2011).

Similarly, the thawing rate is another factor to be considered to minimize crystallization. The ratio of living cells after freezing and thawing is related to the ratio of the formation of ice crystals in the cell. The formation of ice crystals is prevented by applying the cooling and heating ratios, which minimize the amount of intracellular water causing ice formation and using suitable preservatives (Kadıoğlu, 2011). In general, the frozen catfish sperm is thawed in a water bath at 25-40 ºC in 5 seconds to 5 minutes (Bokor, 2009; Viveiros *et al.*, 2011).

Cell death in living creatures is caused by one of two different processes called apoptosis or necrosis. Necrosis is the death of the cell due to an acute dysfunction that develops as a result of the excessive trauma or damage in the cell. Since the control of the ion flow in the cell is rapidly lost, it is regarded as a passive and destructive process. The loss of the ion flow control causes water to enter the cell in excessive amounts and cells and organelles to swell without consuming energy and undergoing cytolysis. The flow of the dead cell content into the extracellular spaces and its infiltration to the pro-inflammatory cells leads to the death of neighbor cells and spread of the tissue damage (Taraphdar *et al.*, 2001).

Apoptosis is a phenomenon during which the cell destroys itself in a programmed way to prevent the formation of cancer-like diseases in cells of which DNA is damaged and cannot be repaired by the repair mechanism. Furthermore, apoptosis is the self-protection mechanism of the cell against various environmental factors. In this study, apoptosis occurring in fish sperms after cryopreservation was examined morphologically with the Dual AO/EB Stain fluorescence method (Liu *et al.*, 2015). In this method, Acridine Orange (AO) and Ethidium Bromide (EB) dyes, which can attach to the DNA well and have fluorescence properties, are used. This method can be used to identify the changes that occur during apoptosis in the cell membranes and nuclei peculiar to apoptosis (Gherghi *et al.*, 2003). This method can also differentiate the cells at different stages of apoptosis (Leite *et al.*, 1999; Baskić *et al.*, 2006). Moreover, in recent studies, it has been determined that this method is more open-ended and shows results in a short time compared to other expensive and prolonged methods, and it has the same precision (Liu *et al.*, 2015).

In this study, the effects of Mesopotamian catfish sperm, which was frozen using different osmotic pressure

[DOR: 20.1001.1.15622916.2020.19.5.16.6]

diluents, on the motility duration, spermatozoa motility rate, and spermatozoa DNA damage, were investigated. In our study, in addition to the previous methods used in the examination of sperm, spermatozoa DNA damage was determined more practically, and its relation with the spermatozoa quality was attempted to be revealed.

Materials and methods

Sperm collection and examination of spermatological characteristics

Mesopotamian catfish (n=8) were caught with fishing lines from Atatürk Dam Lake in April 2016 and those with an average weight of 2.50 kg and length of 90.00 cm, were used in our study. The fish were brought to the laboratory in fiberglass tanks in the lake water. The vertebra was used to estimate the age of the fish. In the age estimations made with a stereomicroscope, it was determined that the individuals were aged 6-7 years (Nikon SMZ 2T stereo). The physicochemical parameters of the lake water during the sampling period were determined by using a YSI 85 measuring device.

In the examination of fresh sperm, spermatozoa motility rate (%), spermatozoa motility duration (s), spermatozoa concentration ($\times 10^9$ ml^{-1}), sperm pH, and seminal plasma osmotic pressure (mOsm kg^{-1}) parameters were examined. Spermatozoa motility rate was visually measured according to the method determined by Aas *et al.* (1991), and the rate of spermatozoa with progressive motility was determined as %. Spermatozoa motility assessments were performed at x400 magnification under a light microscope and at 26°C on a heating table. Spermatozoa motility rate (%) and spermatozoa motility duration (s) were determined by keeping a chronometer until 5% motile spermatozoa remained from the spermatozoa activated by adding 50 µL of activation solution to 0.5 µL of sperm on a lame (26 mm x 76 mm). 50 mM of NaCl was used as the activation solution. The count of the spermatozoa concentration was performed with the Thoma lame (Thoma chamber, American Opticals, Buffalo, NY) using the hemacytometric method, and the number of spermatozoa was stated to be x 10^9 mL^{-1}. The sperm pH was measured using an indicator paper (pH: 0–14; Merck, Germany) and checked with Model GLP 21 pH meter (Crison, Barcelona). All examinations were repeated twice.

Since sperm could not be obtained from the catfish with the abdominal massage method, the gonads were placed into a petri dish, sliced, and kept waiting in 277 mOsm kg^{-1} glucose and 277 mOsm kg^{-1} NaCl solutions for 10 minutes. The testicular sperm solution was transferred to a tube with a pipette and kept waiting for 5 minutes. The fluid on the surface was removed. After centrifuging 800 g of the testicular sperm solution for 5 minutes, the supernatant was removed (Hettich Zentrifugen, Universal 320R, Germany), and the osmotic pressure of the seminal plasma was determined to be 350 mOsm kg^{-1} (Single-Sample Osmometer, Advanced, Model 3250). The glucose and NaCl solutions were at this osmotic pressure with a pH of 8.3 and the sperm in the tube were diluted equally. Thus, the sperm solution to be frozen was prepared. The sperm samples with the motility rates above 80% were used in our study. The

[DOR: 20.1001.1.15622916.2020.19.5.16.6]

results of examination of the fresh sperm obtained from the fish before and after placing the immobilization solution are presented in Tables 1 and 2.

Table 1: Diluent rates used in the study.

Glucose (%)	NaCl (%)	Osm. Pres. (mOsmkg^{-1})	Dis. Wat. (mL)
5.30	0.95	325	100
6.00	1.00	365	100
6.40	1.11	385	100

Osm. Pres.: Osmotic pressure, Dis. Wat.: Distilled Water

Table 2: Body weight, length and fresh spermatological characteristics of the captured *Silurus triostegus*

Parameters	Samples	Mean±SEM	Range
Body weight (g)	8	2500.00±100.40	2000.00-3000.00
Body length (cm)	8	90.00±10.30	80.00-100.00
Spermatozoa motility rate (%)	8	76.53±0.90	70.00-90.00
Duration of spermatozoa motility (sn)	8	990.70±67.80	180.00-2541.00
Spermatozoa concentration ($\times 10^9$ mL^{-1})	8	12.58±2.00	10.60-16.20
Sperm pH	8	8.33±0.19	8.00-8.60
Seminal plasma osmolality (mOsm kg^{-1})	8	349.00±1.98	47.00-352.00

Diluents

The combinations of Dimethyl Sulfoxide (DMSO- Merck K35155112 550) and two different diluents (Glucose-Merck 108342.1000 ve NaCl-Merck 106404.1000) at 3 different osmotic pressures were used **(**325-365-385 mOsm kg^{-1}**)**. The final level of freezing solutions was prepared by adding 10% cryoprotectant and 10% egg yolk (EY) into 80% diluent. The rates of glucose and NaCl diluents used in our study are given below.

According to this, six different groups were created: Group I; Glucose+DMSO+YS+325 mOsm kg^{-1}), Group II; NaCl+DMSO+YS+325 mOsm kg^{-1}), Group III (Glucose+DMSO+YS+365 mOsm kg^{-1}), Group IV (NaCl+DMSO+YS+365 mOsm kg^{-1}), Group V (Glucose+DMSO+YS+385 mOsm kg^{-1}), and Group VI (NaCl+DMSO+YS+385 mOsm kg^{-1}).

Freezing

The sperm taken from the fish were mixed, and a sperm pool was created. Subsequently, the sperm was divided into equal amounts for the number of the groups. The sperm and diluents were kept at the same temperature (24-26 °C). These were diluted at the rate of 1:3 (sperm:diluent) with the sperm diluent and drawn into 0.25 mL straws. They were cooled to 4°C in 1 hour in the refrigerator and were equilibrated for 30 minutes at this temperature. Then, they were frozen 4 cm above the surface of liquid nitrogen for 5 minutes in a styrofoam box and placed in a tank filled with liquid nitrogen at -180°C. The frozen sperm were stored in liquid nitrogen for 15 days.

Thawing

The sperm were thawed in a 40°C water bath for 15 seconds. After thawing, the motility rate and the motility duration were

[DOR: 20.1001.1.15622916.2020.19.5.16.6]

visually determined under the light microscope. 50 mM of NaCl (20 mM Tris–HCl, pH 8.0) was used as the activation solution.

Measurement of the DNA damage level in spermatozoa

In our study, Acridine Orange (AO-Sigma A8014 St. Louis, MO., USA), which dyes the cell DNA, and Ethidium Bromide (EB-Sigma E7637 St. Louis, MO., USA), which only dyes apoptotic or necrotic cells of which membrane integrity has deteriorated, were used to determine the Apoptotic index. For this purpose, 10 µg ml^{-1} of AO and EB stock solutions were prepared and diluted at the rate of 1/9 in PBS (Phosphate Buffered Saline, Cellgro 21-040-CM Manassas, VA, USA). 50 µl of AO and 50 µl of EB mixture solution was added to 10 µl of the sperm cell suspension ($120x10^6$ spermatozoa ml^{-1}) and incubated at room temperature for 5 minutes. Subsequently, it was placed on a lame, and the morphological changes of apoptosis in the cells were assessed by imaging under a fluorescent microscope (40×) (Olympus ckx 53-EM480 X350). With this method, three cell groups can be detected using double dyeing. Live cells appeared nucleus green because the integrity of the cell membrane was not deteriorated; apoptotic cells appeared orange because the integrity of the cell membrane was partially deteriorated, and necrotic cells appeared red because the integrity of the cell membrane was completely deteriorated. At least 200 cells were counted from each sample.

Data analysis

All values were expressed as means±standard error of mean (SEM). Statistical analyses were conducted using the SPSS 16 software program. Sperm motility and duration parameters were tested for normal distribution using the univariate procedure; all parameters showed a normal distribution. Data were tested for significant differences using analysis of variance (ANOVA), followed by the Tukey test, when applicable. The level of significance for all statistical tests was set at 0.05.

Results

In this study, the freezing ability of the sperm of Mesopotamian catfish was examined using two different diluents at three different osmotic pressures. The temperature, oxygen and pH of the lake water from which the fish were captured were approximately 15.50 °C, 7.98 ppm, and 8.50 (YSI 85), respectively. Table 2 shows the biometric characteristics of the samples studied and the spermatological characteristics obtained from the examination of the sperm. In Table 3, the results of the examination of the fresh sperm treated with two different diluents are given. According to these results, the motility rates of the sperm diluted with glucose were 75.26±1.37%, and the motility durations were 110 minutes. While the motility rates of the sperm diluted with NaCl were higher, the motility durations were determined to be 3.5-4 minutes.

[DOR: 20.1001.1.15622916.2020.19.5.16.6]

Table 3: Examination results of the fresh sperm treated with two different diluents.

Dil.	Spz. Mot. Rat. (%)	Spz. Mot. Dur. (min)	Sperm pH	Osm. Pres. (mOsm kg^{-1})
Glucose	75.26±1.37	110.00±4.29	8.30±0.12	349.00
NaCl	85.03±3.68	3.75±2.41	8.30±0.18	349.00

Dil.: Diluent, Spz.: Spermatozoa, Mot.: Motility, Rat.:Rate, Dur.: Duration, Osm. Pres.: Osmotic Pressure

There was no significant effect on the spermatological characteristics examined in the freezing performed at different osmotic pressures with glucose and NaCl diluents (Table 4).

Table 4: Post-thawing assessment of the *Silurus triostegus* sperm frozen with glucose and NaCl diluents

Diluents	n	Spermatological characteristics and SPZ. DNA damage levels				
		SPZ. MOT. RAT. (%)	SPZ. MOT. DUR. (S)	Live SPZ. (%)	Apoptotic SPZ. (%)	Nekrotic SPZ. (%)
Glucose	8	54.68±2.51	798.09±57.50	61.94±3.91	36.60±1.27	1.46±0.94
NaCl	8	49.24±2.33	719.63±55.98	61.44±4.71	37.47±1.69	1.08±0.79
P	8	>0.05	>0.05	>0.05	>0.05	>0.05

Spz.: Spermatozoa, Mot.: Motility, Rat.: Rate, Dur.: Duration

In our study, the highest spermatozoa motility rates were obtained with glucose and NaCl diluents at osmotic pressures of 365 and 385 mOsm kg^{-1} (365 mOsm kg^{-1} glucose: % 65.38±11.02; 365 mOsm kg^{-1} NaCl: % 64.62±10.95, 385 mOsm kg^{-1} glucose: % 61.54±9.15, 385 mOsm kg^{-1} NaCl: % 71.54±14.51) (p<0.01). Although a motility rate of 43.08±4.24% was observed in the NaCl diluent at 325 mOsm kg^{-1} osmotic pressure, this rate was observed to be the lowest in the glucose diluent at 325 mOsm kg^{-1} osmotic pressure (% 27.69±7.93) (Fig. 1).

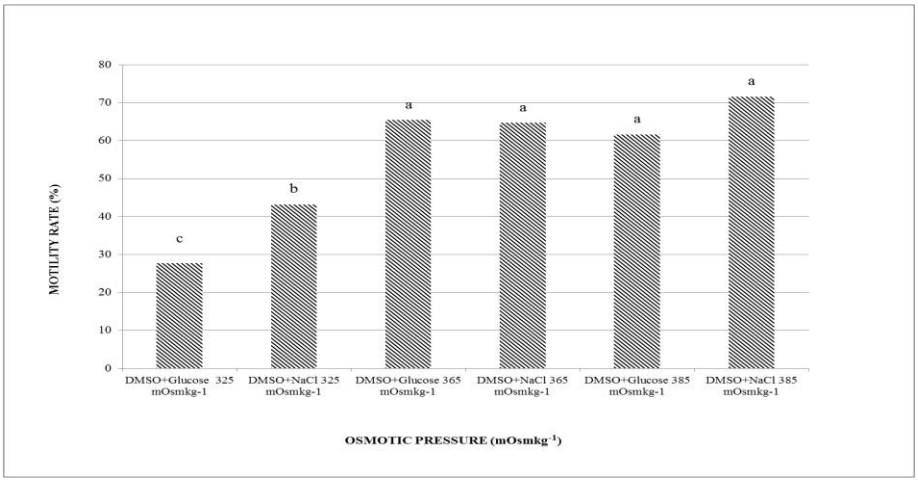

Figure 1: Results of the post-thawing motility rate of *Silurus triostegus* sperm according to different diluents.

The spermatozoa motility duration was found to be the highest in glucose (1420.38±166.69 s) and NaCl (1361.23±153.71 s) diluents at 365 mOsm

[DOR: 20.1001.1.15622916.2020.19.5.16.6]

kg^{-1} osmotic pressure (p<0.01). While spermatozoa motility durations of 582.08±47.73 sec and 604.38±64.05 sec were determined in glucose and NaCl diluents at 385 mOsm kg^{-1} osmotic pressure, respectively. This value was recorded to be 495.69±88.83 sec in the diluent at 325 mOsm kg^{-1} osmotic pressure. The spermatozoa motility durations of the glucose diluent at 325 mOsm kg^{-1} osmotic pressure decreased significantly (224.46±66.20 s) (Fig. 2).

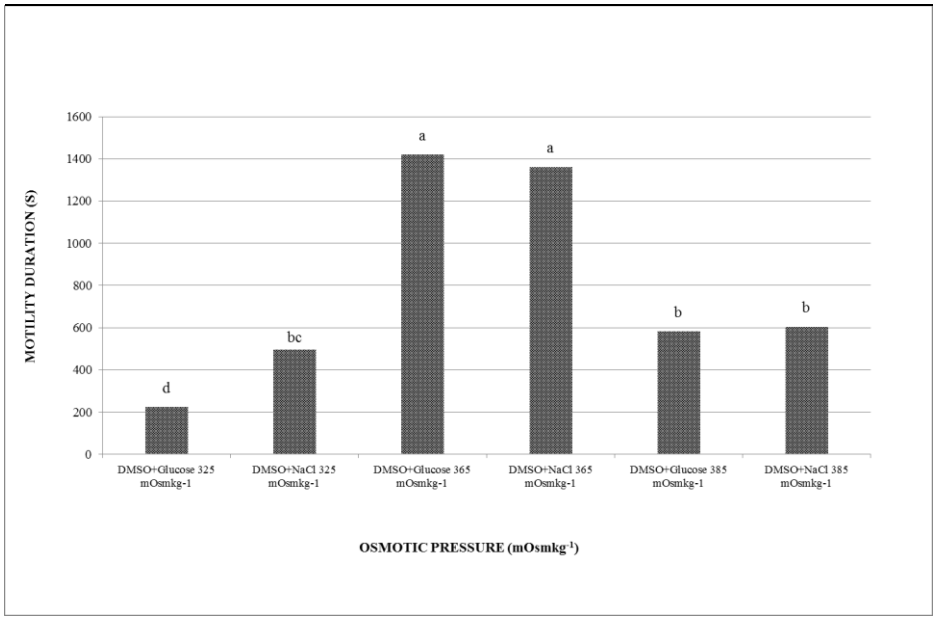

Figure 2: Results of the post-thawing motility duration of *Silurus triostegus* sperm according to different diluents.

The post-thawing live spermatozoa rate was determined to be the highest in the sperm frozen with glucose at 385 mOsm kg^{-1} (70.76±8.87%). However, the live spermatozoa rate of 65.33±4.73% was determined in the NaCl diluent at 325 mOsm kg^{-1} osmotic pressure, of 60.19±8.87% was determined in the NaCl diluent at 365 mOsm kg^{-1} osmotic pressure, and of 53.74±8.87% was determined in the NaCl diluent at 385 mOsm kg^{-1} osmotic pressure. The lowest rate was obtained in the spermatozoa frozen with the glucose diluents at 325 and 365 mOsm kg^{-1} (45.63±8.87% and 33.07±8.84%) (Figure 6). A live spermatozoa image obtained under the fluorescence microscope after dyeing with AO and EB is given below (Fig. 3).

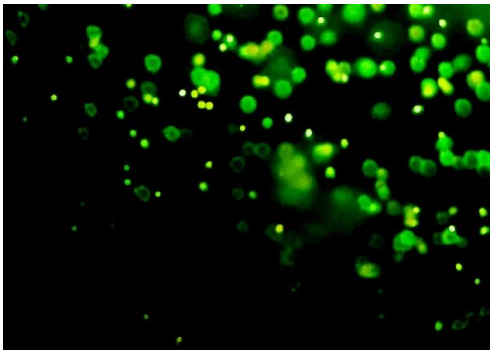

Figure 3: Post-thawing live cell image of *Silurus triostegus* sperm according to different diluents.

While the apoptotic cell rate was determined to be the highest in the sperm frozen with glucose at 385 mOsm kg^{-1} osmotic pressure (64.84±6.26%), it was

[DOR: 20.1001.1.15622916.2020.19.5.16.6]

determined to be the lowest in the sperm frozen with the glucose diluent at 385 mOsm kg^{-1} osmotic pressure (28.35±6.29%). This rate was calculated to be 54.05±6.29% in the glucose diluent at 365 mOsm kg^{-1} pressures, 45.96±6.29% in the NaCl diluent at 385 mOsm kg^{-1}, 39.09±6.29% in the NaCl diluent at 365 mOsm kg^{-1}, and 33.40±3.35% in the NaCl diluent at 325 mOsm kg^{-1} (Fig. 6). The apoptotic spermatozoa image obtained under the fluorescence microscope after dyeing with AO and EB is given below (Fig. 4).

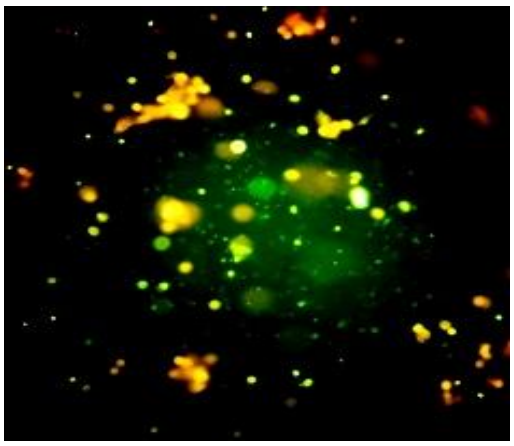

Figure 4: Post-thawing apoptotic cell image of *Silurus triostegus* sperm according to different diluents.

The necrotic cell rate was found to be the highest with 2.08±0.39% when frozen with the glucose diluent at 325 mOsm kg^{-1} pressures. Necrotic cells were observed at the rate of 1.26±0.21% with the NaCl diluent at 325 mOsm kg^{-1} pressure, at the rate of 0.89±0.39% with the glucose diluent at 385 mOsm kg^{-1} pressure, at the rate of 0.72±0.39% with the NaCl diluent at 365 mOsm kg^{-1} pressure, and at the rate of 0.31±0.39% with the glucose diluent at 365 mOsm kg^{-1} pressure. The lowest necrotic spermatozoa rate was obtained in the sperm frozen with NaCl at 385 mOsm kg^{-1} pressure (Fig. 6). The apoptotic spermatozoa image obtained under the fluorescent microscope after dyeing with AO and EB is given below (Fig. 5).

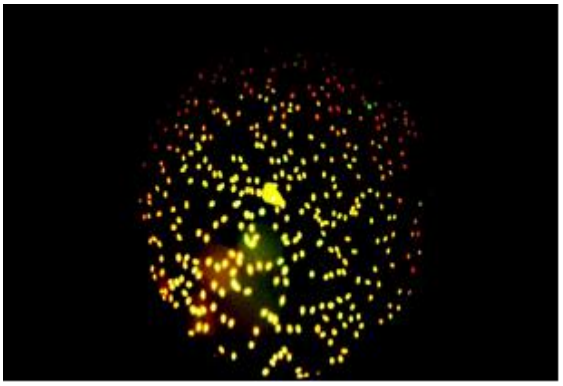

Figure 5: Post-thawing necrotic cell image of *Silurus triostegus* sperm according to different diluents.

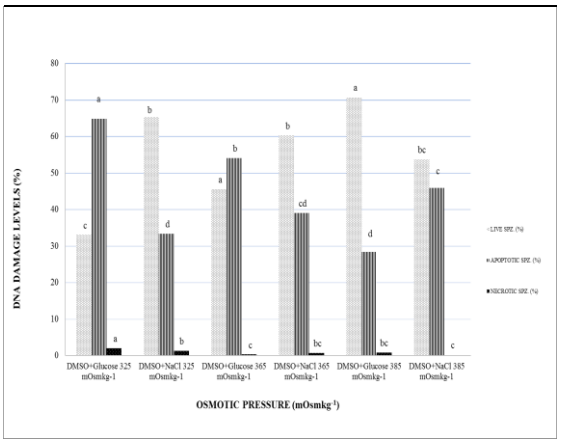

Figure 6: Results of the DNA damage levels of *Silurus triostegus* sperm according to different diluents.

Discussion

Sperm freezing can result in harmful effects on the structure functions of spermatozoa (Watson, 2000). During the freezing and thawing process, the fish spermatozoon is damaged due to the intracellular and extracellular ice crystals and osmotic shock. Intracellular ice crystals can affect the functions of intracellular organelles as well as the cell membrane. The outflow of water from the

[DOR: 20.1001.1.15622916.2020.19.5.16.6]

cell during freezing leads to an increase in the concentration of organic and inorganic substances in the cell and in the osmotic pressure (Martin *et al.,* 2004; Browne *et al.*, 2011). The increase in the intracellular melted material concentration and in the cryoprotectant amount can lead to a toxic damage (Said *et al.,* 2010).

In this study, the motility rates and motility durations decrease in freezing with NaCl and glucose solutions at low osmotic pressures of 325 mOsm kg^{-1} (Figs 1, 2). This shows that organic and inorganic diluents at the low osmolality reduce the motility rate and motility duration of spermatozoa.

The decrease in the spermatozoa motility is linked to the damage of the mitochondrial membrane. Mitochondria lead to a decrease in the ATP synthesis and motility with the oxidative phosphorylation. Motility determines the sperm quality as well as the fertilization ability of the spermatozoon (Rurangwaa *et al.,* 2004). In general, the spermatozoa motility and the motility duration of Cyprinidae frozen sperm decrease when compared to the fresh sperm (Lahnsteiner *et al.,* 2000; Linhart *et al.,* 2000; Martin *et al.,* 2004). In relation to freezing, the motility rate and motility duration of Mesopotamian catfish spermatozoa may be low due to cellular swelling at low osmotic pressures (Curry and Watson, 1994). This is especially prevalent in the glucose diluent.

As shown in Figure 6, in freezing performed with the glucose diluent with low osmolality (325 mOsm kg^{-1}), the live spermatozoa rate was low (33.07%). Martin *et al.* (2004) reported that cryopreservation significantly increased spermatozoa mortality. Researchers reported that while the PI permeability rate of the spermatozoa before cryopreservation was 28.0%, it increased to 50.8% after cryopreservation. Live cells have intact membranes and keep most of the dyes outside of the cell, and dyes in the damaged membranes of non-living cells are easily penetrated (Boroda *et al.,* 2016). Previous studies have revealed that cell integrity deteriorates after freezing and thawing (Segovia *et al.,* 2000; Martin *et al.,* 2004; Wang *et al.,* 2013). The morphologic damage and mitochondrial loss in the plasma membrane at the spermatozoon head result in a decrease in the viability of spermatozoa (Segovia *et al.,* 2000; Boonthai *et al.,* 2016). In this study, the rate of live spermatozoa was low in freezing performed only with the glucose diluent with low osmolality (325 mOsm kg^{-1}). The glucose diluent with low osmolality may not have preserved the integrity of the cell membrane, or the performance of some enzyme systems may have been affected (Boonthai *et al.,* 2016). Narayana (2008) have reported that the harmful effects of gentamicin, an aminoglycoside antibiotic, on the spermatozoa of rats occur as a result of the deterioration of the integrity of the cell membrane, a decrease in antioxidant enzymes and an increase in peroxide radicals. The Yo-Pro-1 analysis provides information about the cell membrane change as a result of the apoptotic process. It has been reported that the permeability of the live spermatozoa membrane after freezing increases for Yo-Pro-1 (Martin *et al.,* 2004).

In freezing performed with the glucose diluent at low osmolality (325 mOsm/kg^{-}

[DOR: 20.1001.1.15622916.2020.19.5.16.6]

[1]), apoptotic cell rates (64.84%) were significantly lower compared to other groups (Fig. 6). Necrosis and apoptosis are two forms of the cell death. Necrosis occurs as a result of damage and in a high number of cells. Necrosis leads to cell swelling and membrane ruptures. Apoptosis is a physiologically programmed cell death. It affects a single cell without being related to the surrounding tissue inflammation (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Martin *et al.*, 2004; Sabido, *et al.*, 2004). Normal spermatogenesis depends on the activity of apoptosis. In the testicles of adult mammals, 25-75% of the germ cells are degenerated and die. In the bull sperm, there was a significant increase in the rate of spermatozoa exposed to apoptosis after freezing. In a study conducted by Martin *et al.* (2004) on the bull sperm, it was found out that the apoptosis rate, which was 11.30% in pre-freezing spermatozoa, increased to 44.90% after freezing. Changes in the mitochondrial membrane are more responsible for the increase in apoptosis after freezing and thawing. Namely, entering of the apoptotic factors, associated with the mitochondrial deterioration due to cryopreservation, into the cytoplasm is held responsible for the increase in apoptosis (Baccetti *et al*., 1996; Martin *et al.*, 2004; Sabido *et al.*, 2004). It is reported that the low level of caspase, which is an indicator of apoptosis in mature sea urchin embryos, may be associated with the presence of some inhibitory proteins found in the total protein extracts after freezing, or the changes in the permeability in mitochondrial pores may have led to an increase in cell apoptosis due to the rise in the cytochrome-c release (Wang *et al.*, 2013). However, apoptotic cells are not permeable to *Propidiumiodide* (PI). Ejaculated spermatozoa have certain properties of apoptotic somatic cells. These include DNA fragmentation, chromatin condensation, acrosomal membrane lobulation, and mitochondrial swelling (Baccetti *et al*., 1996; Blanc-Layrac *et al.*, 2000; Martin *et al.*, 2004).

As shown in Figure 6, in freezing performed with the glucose diluent with low osmolality (325 mOsm kg^{-1}), the necrotic cell rate (33.07%) was significantly higher. It is known that sperm freezing and thawing have harmful effects on the live spermatozoa rate. During freezing, freezing of the intracellular water and cell water due to the thermal shock is regarded as the most common cause of the cellular dehydration and osmotic shock cell damage. It causes necrosis in the cell. In necrosis, elevated PI permeability is observed in dead cells (Dowell land Rinfret, 1960; Wyllie *et al.*, 1980; Curry and Watson, 1994; Lahnsteiner *et al.*, 2000; Linhart *et al.*, 2000; Watson, 2000). Necrosis is usually caused by extreme physiological conditions (e.g., hypothermia, hypoxia, etc.). In this case, mitochondria and the plasma membrane are damaged (Boroda *et al.*, 2016). The cellular damage may have increased due to recrystallization during thawing. The optimum freezing and thawing rate can be attributed to the dehydration process, prevention of ice formation during freezing, and the prevention of recrystalization during thawing (Chao and Liao, 2001; Froese and Pauly, 2008; Beirão *et al.*, 2011).

It was observed that after freezing of

[DOR: 20.1001.1.15622916.2020.19.5.16.6]

Mesopotamian catfish sperm with glucose and NaCl solutions at 325, 365, and 385 mOsm kg^{-1} and with DMSO, The necrotic cell rate was found to be the highest with 2.08±0.39% when frozen with the glucose diluent at 325 mOsm kg^{-1} pressures. These results of this experiment have shown that glucose and NaCl diluent at 365, and 385 mOsm kg^{-1} may be successfully used in Mesopotamian catfish sperm freezing.

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