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Araştırma Makalesi
Research Article

The Effect of Grape Molasses as an Extender on Motility, Viability and Fertility in Rainbow Trout (*Oncorhynchus mykiss* W., 1792) Sperm

Zafer DOĞU[1*], Faruk ARAL[2], Erdinç ŞAHİNÖZ[1], Füsun TEMAMOĞULLARI[3]

[1]Harran University, Bozova Vocational High School Department of Fisheries, Bozova, 63300 Şanlıurfa, Turkey.
[2]Niğde University, Bor Vocational High School, Bor-Niğde, Turkey.
[3]Harran University, Faculty of Veterinary, Dept. of Pharmacology and Toxicology, 63300 Şanlıurfa, Turkey.

*Corresponding Author Tel.: +90 414 318 30 00/2742
E-mail: zaferdogu@harran.edu.tr

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Abstract

In this study, the effects of three different dosages of grape molasses (5, 7, 10 %) as a diluent were investigated on post-thaw motility, sperm viability and fertilization rate of frozen spermatozoa from the rainbow trout (*Oncorhynchus mykiss*). Semen from six male fish sperm was diluted with in extenders containing 5, 7, and 10% grape molasses with dimethyl sulfoxide 7%. The semen was frozen in 0.25 ml straws in liquid nitrogen vapor and stored in liquid nitrogen for 2 weeks. From the point of grape molasses effect over sperm motility, grape molasses level of 10 % was found more beneficial ($P<0.001$), and there was no significant difference between various level of grape molasses on fertilization rate ($P>0.05$). But, the 10% grape molasses solution was better (96.4%) than the glucose solution which has the highest fertilization rate ($P<0.05$). As a result of the study, the level of 10% grape molasses is given that might be used to freezing rainbow trout sperm.

***Keywords*:** Aquaculture, freezing, cryopreservation, milt.

Öz

Gökkuşağı Alabalık (*Oncorhynchus mykiss* W., 1792)Spermasında Motilite, Canlılık ve Fertilizasyon Üzerine Bir Sulandırıcı Olarak Üzüm Pekmezinin Etkisi

Bu çalışmada, gökkuşağı alabalıklarının (*Oncorhynchus mykiss*) dondurulan spermasına % 5, 7 ve 10 oranında katılan pekmezin spermatozoa motilitesi, canlı spermatozoa oranı ve döllenme oranı üzerine etkisi incelendi. Altı adet erkek balık sperması % 7 dimetil sülfoksit ile birlikte % 5, 7 ve 10 oranında pekmez içeren sulandırıcı ile sulandırıldı. Spermalar 0.25 ml payetlerde, sıvı azot buharında dondurularak 2 hafta boyunca sıvı azot içerisinde muhafaza edildi. Spermatozoa motilitesi üzerine pekmez etkisi açısından, %10 oranında üzüm pekmezinin ($P<0.001$) daha faydalı olduğu ve ($P>0.05$) döllenme oranı üzerine pekmezin tüm oranlarında önemli bir fark bulunamadığı saptandı. Ancak, % 10 oranında kullanılan pekmez ile dondurulan spermalardan elde edilen döllenme oranı glikoz sulandırıcısı için elde edilen en yüksek değerden daha iyi oldu (96.4%). Çalışma sonuçlarına göre, % 10 oranında pekmezin gökkuşağı alabalıklarının spermalarının dondurulmasında kullanılabileceği kanaatine varıldı.

***Anahtar Kelimeler*:** Yetiştiricilik, dondurma, kriyoprezervasyon, sperma.

Introduction

The cryopreservation of fish sperm has several benefits, including storage, transportation, stock protection, conserving biodiversity and aquaculture, the protection of genetic resources; these are crucial in terms of the Execution of scientific studies (Cabrita et al.,

2005). Thus, studies of improvements in optimal semen preservation techniques and media are gaining importance day-by-day (Stein and Bayrle, 1978; Holtz, 1993; Sarvi et al., 2006; Ekici et al., 2012; Ciereszko et al., 2014).

One of the most important solutions used in semen freezing is extenders. An extender acts as a cryopreservation diluent, the purpose of which is to supply the sperm cells with energy, protect them from temperature-related damage, and maintain a suitable environment in which the sperm can survive the cryopreservation process (Holtz, 1993; Vishwanath and Shannon, 2000). In this context, an extender is a medium used to dilute sperm, while a cryoprotectant is a material that is added to the extended sperm dilutions to protect the sperm from cold temperatures and cryoprotectant toxicity during cryopre-servation (Muchlisin et al., 2004).

Stein and Bayrle (1978) achieved over 70% success rate for different freshwater species in the breeding season, including rainbow trout (*Salmo gairdneri* Richardson), the brown trout (*Salmo trutta forma fario* L.), the brook trout (*Salvelinus fontinalis* Mitchill), the Danube salmon (*Hucho hucho* L.), the grayling (*Thymallus thymallus* L.), the pike (*Esox lucius* L.), and the carp (*Cyprinus carpio* L.) using an extender containing 100 mg of glucose. There was always high motility in the dilution medium before and after freezing. Stoss and Refstei (1983) found that a very simple extender containing 0.3M glucose and 10% DMSO produced successful results in the cryopreservation of sperm from the Atlantic salmon and sea trout in fertilization tests with frozen sperm, which could support the concept of glucose supporting the spermatozoa.

Similarly, a simple and effective cryopreservation procedure using an extender containing 0.1, 0.2, and 0.3 M glucose and 10% methanol was established for rainbow trout semen, securing 60% post thaw motility compared to 90% in fresh semen (Ciereszko et al., 2014). Judycka et al. (2015) researched the 0, 0.10, 0.15, 0.20 and 0.30 M glucose in methanol extender and found that the beneficial effect of glucose for semen cryopreservation was related to its concentration with a quite narrow optimal range of 0.1 to 0.15 M in Siberian sturgeon (*Acipenser baerii*).

Their study also indicates that the use of a simple extender containing 0.1 M glucose in 15% methanol can be an alternative cryopreservation method for sturgeons. They therefore concluded that the concentration of glucose is important for cryopreservation efficiency.

A glucose extender that supplements energy is considered as a possibility for encouraging the motility of spermatozoa even after cryopreservation. It has been shown that fish spermatozoa are capable, to a limited extent, of using exogenous energy sources (Mounib, 1978; Harvey and Kelley, 1984). One of the energy sources studied is glucose; a non-permeating co-cryoprotectant (Betsy et al., 2015). Glucose is the chief substance utilized for energy in fish semen (Hamner and Charles, 1969).

Longer motility in the spermatozoa of guppy was noticed when extracellular glucose was given (Gardiner, 1978). Similarly, the addition of glucose to the extender enhanced the quality of the milt and its storage duration (Ponniah et al., 1999). For green swordtail (*Xiphophorus helleri)*, the addition of glucose yielded higher and longer motility for fresh and cryopreserved sperm when the concentration was kept below 11.0 mM. The effect was not seen when the concentration of glucose was higher than 11.0 mM (Dong et al., 2006).

Grape molasses is a traditional Turkish product made out of condensed grape juice. It is produced by simply boiling the juice without the addition of sugar or other food additives (Kaya and Belibağlı, 2002). It contains high amounts of sugar, minerals and organic acid, and is very important for human nutrition (Bozkurt et al., 1999; Yoğurtçu and Kamışlı, 2006; İnan et al., 2011).

In recent years, the consumption of grape molasses has rapidly increased due to its beneficial composition of minerals, carbohydrates, organic acids, phenolic compounds, flavonoids, and proteins. The major constituent of grape molasses is carbohydrates, which is the main source of energy in the body. Moreover, the carbohydrates in grape molasses generally exist in the form of glucose and fructose. Thus, the phenolic compounds and flavonoids in grape molasses have a positive effect on human health due to their antioxidant and antimutagenic properties (Turhan and Tetik, 2010).

Besides extender which is one of the important parameters used in cryopreservation, cryoprotectant is another important parameter. Dimethyl sulfoxide (DMSO), glycerol, methanol, dimethylacetamide (DMA), and propylene glycol are commonly used cryoprotectants for fish sperm cryopreservation (He and Woods, 2003). In this study, DMSO has been preferred for the cryopreservation of salmonid sperm since the cryoprotectants such as glycerol, ethylene glycol, propanediol, dimethy acetamide and methanol are less popular or have been used with limited success (Stoss and Holtz, 1981).

The objective of this work was to evaluate the freeze-thawing of rainbow trout milt, using a different rate of grape molasses as an extender.

Materials and Methods

Animals and Sperm Collection; The sperm were obtained in February 2014 from 2 years old male rainbow trout spawners that were cultured in the Department of Fisheries at Harran University in Turkey. The size of the fish (means ± S.E.) used for the study was 386.8 ±11.7 g in body weight and 14.08± 0.4 cm in total length. The sperm were collected near the fish cages through abdominal massage and kept on ice for no longer than three hours. The semen samples that had at least 80 % progressive motility were pooled. The sperm was kept at 4 ºC until they were frozen. Spermatozoa concentration, spermatozoa motility, and duration of motility were assessed after collection. The sperm from all the fish were pooled together to make heterogeneous sperm in order to avoid the influence of individual fish.

Experimental Design; Sperm from six young male fish were diluted in extenders containing 5, 7, and 10% grape molasses, 20% egg yolk and 7% DMSO, and a control extender containing 0.3 M glucose, 20% egg yolk and 7% DMSO, at 4 °C. The diluted samples containing grape molasses were drawn into 0.25 ml plastic straws (IMV, France). The samples were equilibrated in the plastic straws for 15 minutes at 4 ºC. Following the equilibration, the straws were placed on a styrofoam rack, floating on the surface of liquid nitrogen in a styrofoam box. The straws were frozen in liquid nitrogen vapor 3 cm above the surface of the liquid nitrogen (-120 ºC) for 10 minutes (Tekin et al., 2003). Then the straws were plunged into the liquid nitrogen (-196 ºC) and stored for two weeks. The straws were thawed in a water bath at 35°C for 30 seconds. The fertility and post-thaw motility of the spermatozoa in each treatment was independently assessed

by two observers using 12 straws (Tekin et al., 2003).

Assessment of spermatozoa concentration, spermatozoa motility and duration; The spermatozoa concentration was determined using a haemocytometer and expressed in terms of the number of cells x10^9 cell/ml. Fresh and post-thaw sperm motility was evaluated under a light microscope at 40x magnification immediately after mixing 1 µl of the semen with 250 µl of the activation solution on a microscope slide. An activation solution, 50 mM NaCl (20 mM Tris-HCl, pH 8.0), was used to estimate the motility rate. Sperm motility was evaluated from the semen with a progressive forward movement. Immotile sperm were defined as sperm without forward movement.

Sperm motility (progressive movement) in the samples was estimated subjectively on the basis of a percentage ratio of progressively (straight-line forward movement) moving spermatozoa, assessed in several view fields of a light microscope at 40 x magnification.

Three semen samples were frozen for each treatment and motility was measured three times for each freezing process. The duration of sperm motility was timed from the initial contact of the semen with the activation solution until the cessation of motility (expressed in seconds). The duration of sperm motility was subjectively evaluated as the time that elapsed from activation until 5% of the spermatozoa maintained forward swimming activity. The sperm motility observations were done at 5-7°C. To avoid variations from individual differences in subjective judgement, the same person performed all the sperm motility observations. The duration of progressive movement was also recorded and expressed in seconds (Aral et al., 2005).

Viability assessment; Viability assessments using eosine-nigrosine stained semen smears were conducted following Jeyalectumie and Subramoniam's example (1989). In the first case, 25 µL of eosin (0.5%) was added to 25 µL of nigrosin (10%) in 50 µL of sperm. The 100 µL solution was transformed into a smear and used to stain a microscope blade, then air-dried for observation under a 400 x magnifying optic microscope. Eosin-stained cells (rosaceous) were considered dead when there was no coloration in the live cells, but rather they were translucent. An average survival average percentage rate was obtained by counting a minimum of 100 cells per blade (two repetitions per sample) (Uberti et al., 2014).

Fertility test; For the fertilization process, a dry fertilization technique was used. Eggs were pooled from 10 females. Fertilization took place in dry plastic dishes and 200 eggs (about 20 g) were placed into each dish. Batches of eggs were inseminated with frozen stored semen for 2 weeks or fresh semen for the control sample. Eggs and sperm cells were gently mixed for 10 seconds. The sperm-egg ratio was approximately 0.25x10^6 spz/egg. Following insemination, 25 ml of fertilization solution (0.3% NaCl) was added to the sperm-egg mixture and left for 45 minutes to allow the eggs to swell. After the swelling, the eggs were rinsed in hatchery water (10°C) and batches were placed into vertical incubation trays. The success of the experiment was determined by the percentage of eyed-eggs 20-25 days after fertilization.

Statistical Analyses; Spermatozoa motility and duration obtained from each diluent were expressed as percentage (means ± SE) and s, respectively. Motility data were normalized through arcsine transformation and the results were analyzed using a one way analysis of varience (ANOVA) at tihe significance level $p<0.05$ after data was verified for normal distribution of variance.

Significant differences between treatments were detected using the Duncan's multiple range test ($P < 0.05$) generated by SPSS software (SPSS 10.0 windows).

Results

The effect of grape molasses on motility in rainbow trout (*Oncorhynchus mykiss*) (n = 10), the overall mean ± S.E. of motility duration (s), motility rate (%), sperm viability (%) and fertilization rate were found 69.92±3.00, 48.87±2.01, 78.85±1.58 and 95.27±0.27, respectively (Fig 1-4).

Motility Duration; Although, motility duration showed no significant differences throughout the groups of 0.3 M glucose and 10 % grape molasses, 5 % and 7 % grape molasses groups exhibited lower values than the 0.3 M glucose and 10 % grape molasses groups ($P < 0.01$) (Fig1).

Motility Rate**;** Post-thaw motility rate had no significant differences throughout the groups of 0.3 M glucose and 10 % grape molasses but the 5 %and 7 % grape molasses groups were lower values than the 0.3 M glucose and 10 % grape molasses groups ($P < 0.01$) (Fig2).

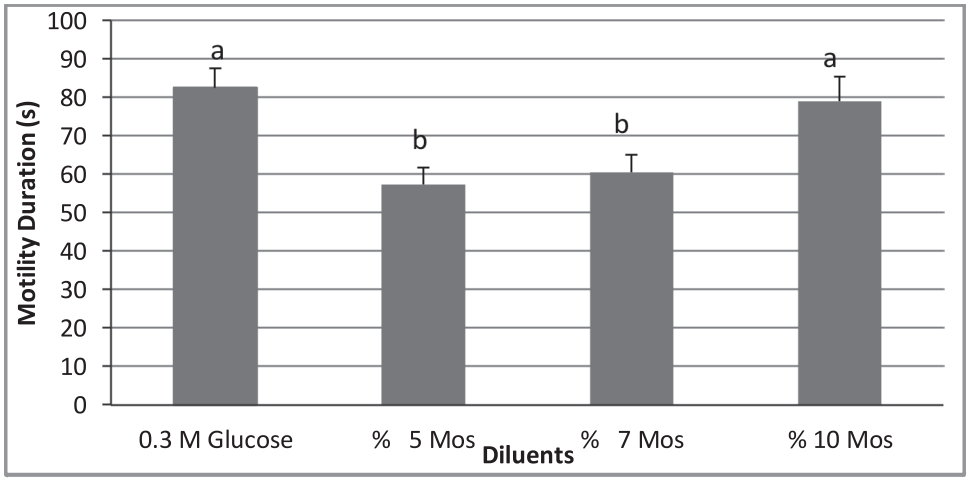

Fig 1. Motility durations of *O. mykiss* at different dose of grape molasses. Different letter superscripts indicate means that were significantly different ($P < 0.05$).

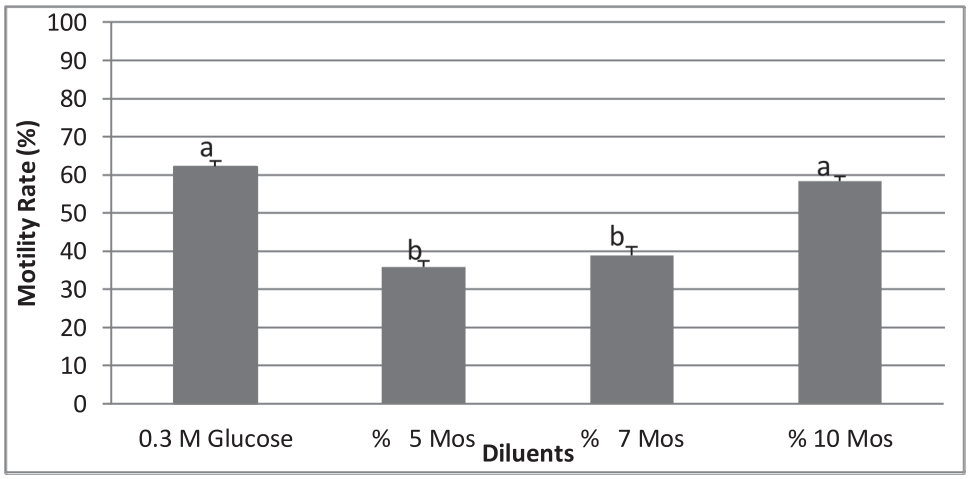

Fig 2. Motility rates of *O. mykiss* at different dose of grape molasses. Different letter superscripts indicate means that were significantly different ($P < 0.05$).

Sperm Viability; The lowest sperm viability was found in 5 % grape molasses group, however the highest values were observed in 0.3 M glucose. And also, there was no significant differences between 7 % and 10 % grape molasses groups (P<0.05) (Fig. 3).

Fertilization Rate; There were significant differences between three groups (P<0.05). A significant increase in fertilization rates was observed in post-thaw sperm. And also, the highest post-thaw fertilization rates were found in 10 % grape molasses group (Fig. 4).

Discussion

The major compound in grape molasses is carbohydrate (Republic of Turkey Ministry of Food, Agriculture and Livestock, 2015). The mechanism responsible for the effect of molasses is not fully understood. Grape molasses improves sperm motility, viability and fertilization.

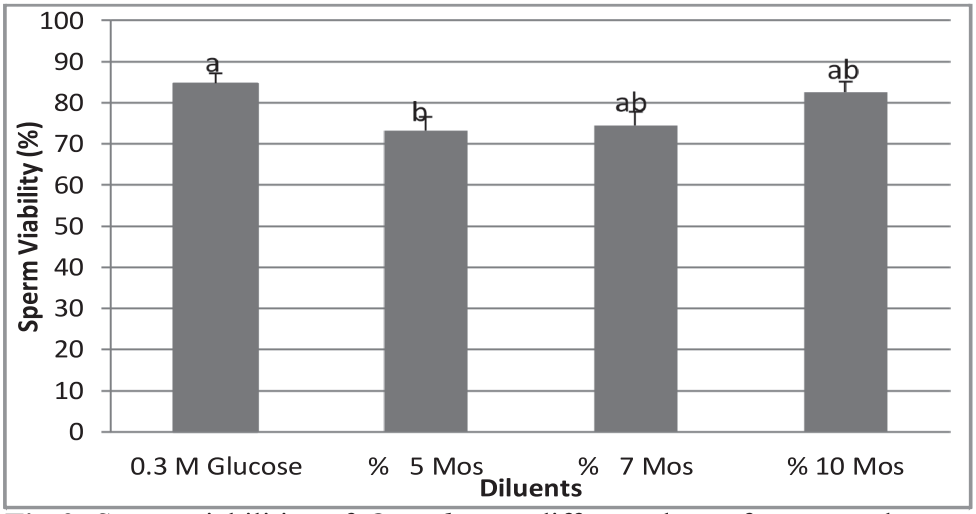

Fig 3. Sperm viabilities of *O. mykiss* at different dose of grape molasses. Different letter superscripts indicate means that were significantly different (P < 0.05).

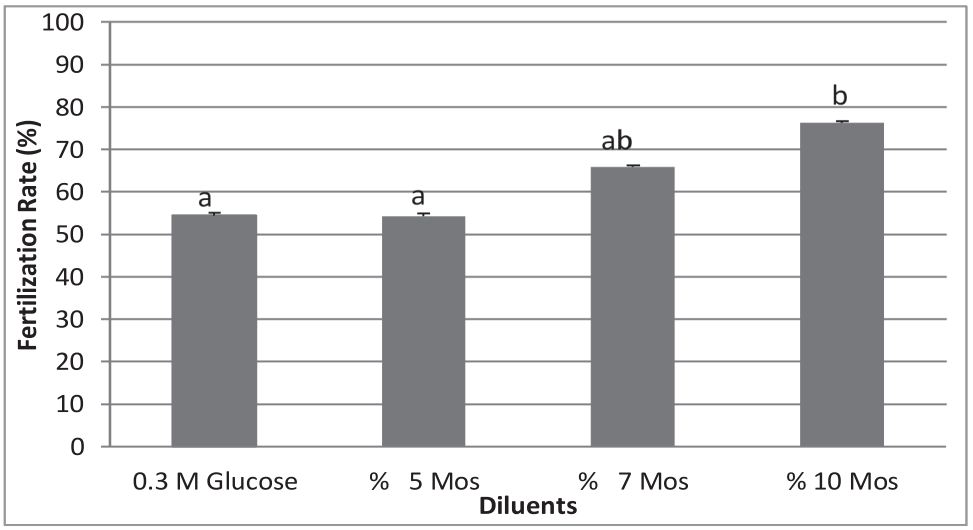

Fig 4. Fertilization rates of *O. mykiss* at different dose of grape molasses. Different letter superscripts indicate means that were significantly different (P < 0.05).

Low molecular weight non-permeable cryoprotectants, such as galactose, glucose, sucrose, trehalose or other sugars, have been widely used in freezing livestock sperm, as sugars cause dehydration before cooling, and so cause less intracellular ice crystal formation. The cryoprotective effects of sugars on sperm cells may differ according to the molecular weight of the sugars (Anchordoguy et al., 1987, Molinia et al., 1994); the saccharose, glucose and maltose in grape molasses do not enter the cell and so they increase the osmolarity of the extender and serve to protect the integrity of the plasma membrane (Gilmore et al., 1996, Woelders et al., 1997).

The results also indicate the possible influence of the sugar rate in post-thaw sperm motility; however, in this study, the different compounds of the grape molasses in the freezing extenders makes interpretation complicated. Grape molasses was selected because of previous determinations by other researchers that grape molasses shares characteristics with other sugar compounds (Bozkurt et al., 1999; Yoğurtçu and Kamışlı, 2006; İnan et al., 2011). In addition, glucose-based extenders have the same components, the only difference being the sugar rate. The molasses-based extender that was used was the most appropriate for the freezing of rainbow trout semen and had other additional components. These compounds include minerals, carbohydrates, organic acids, phenolic compounds, flavanoids, and proteins (Bozkurt et al., 1999; Yoğurtçu and Kamışlı, 2006; İnan et al., 2011; Turhan and Tetik, 2010; Republic of Turkey Ministry of Food, Agriculture and Livestock, 2015).

Glucose may help protect pets and fish sperm during the rapid cooling stage. The cryoprotective effect of glucose may change according to the molecular weight of the glucose; (Anchordoguy et al., 1987; Molinia et al., 1994). These substances cannot enter the cells, thus the increased osmolarity of the diluent can serve to protect the integrity of the cell membrane (Gilmore et al., 1996; Woelders et al., 1997). Molasses contains on average 60% sugar and 42% invert sugar (Üstün and Tosun, 1997). 100 grams of molasses contains 0.74 g saccharose, 29.37 g glucose, 28.81 g fructose, and 0.26 g maltose (Republic of Turkey Ministry of Food, Agriculture and Livestock, 2015). The glucose composition of semen extenders has been shown to be an important factor in the freezing of ram spermatozoa (Salamon, 1968).

Sperm motility, the duration of motility, and the number of live sperm were similar to that obtained with the glucose extender, but in this study, the fertilization rate seems to be higher in the frozen-thawed semen that has 10% rate of grape molasses.

The motility, viability and fertilization values observed with the 10% molasses extender were similar to those described by Stoss and Refstie (1983) and Stoss (1983), who used the same 0.3 M glucose extender and 7,5 % DMSO. This study confirms that low glucose and high saccharose and maltose molecular weight sugars added to 10% DMSO in a 10 % molasses extender improved the post-thaw parameters of the fish spermatozoa.

DMSO is the permeable cryoprotectant most commonly used for fish milt. It seems to regulate cellular dehydration by osmotically replacing intracellular water. This mechanism reduces the formation of intracellular ice crystals. Rani and Munuswamy (2014) reported that 10 % DMSO was the optimum concentration for sperm viability and motility in the thawing semen of common carp (*Cyprinus carpio* L.).

In our study, the concentration in every molasses extender was 10 %, indicating that there are no harmful effects of using molasses extenders with 10% DMSO as freezing extenders.

Plasma membrane integrity is another indicator of the success of freeze-thawing methods, as it is important for the fertilizing ability of spermatozoa (Reyes et al., 2002). The high percentage of viability in both the control and 10% grape molasses groups suggests that using a high percentage of grape molasses did not damage the spermatozoa. These results confirm those obtained with Atlantic salmon milt by Mounib (1978), who reported 80% sperm viability when stored in liquid nitrogen (-196°C). Freezing and thawing speeds are critical factors that affect sperm viability (Stoss, 1983).

The changes in viability were similar to the changes in motility. The proportion of spermatozoa with an intact plasma membrane was significantly higher in the semen cryopreserved in the control and in the 10% grape molasses groups, giving high protection to the spermatozoa. However, the sperm's motility percentage was lower than the percentage of sperm with an intact plasma membrane, suggesting that when studying the freezing-thawing method, motility may not be a reliable indicator of sperm viability; low motility after thawing does not always indicate cellular damage (Billard and Jensen, 1996; Reyes et al., 2002). Yıldız et al. (2000) researched the effects of glucose on dog sperm. They reported that the addition of monosaccharides could increase viability and the percentage of intact acrosomes.

The chemical composition of extender media for cryopreserving spermatozoa varies greatly, with simpler extenders containing only two or three substances, such as the salts most commonly used in fish spermatozoa cryopreservation studies, NaCl, KCl, and $CaCl_2$ (McAndrew et al., 1993). Hanks' Balanced Salt Solution, which includes mineral salts such as Sodium Chloride, Potassium Chloride, Magnesium Sulfate, Calcium Chloride, and Sodium Bicarbonate, could be used to freeze the sperm of Atlantic cod (*Gadus morhua*).

In addition, Erdahl and Graham's extender, which includes salts such as $CaCl_2$, $MgC1_2$, KCI, NaC1, could be used to freeze the sperm of rainbow trout, *Oncorhynchus mykiss*, while BTS (Beltsville Thawing Solution), a medium characterized by its low potassium level, could be used to freeze pig sperm. It provides the continuous pumping of sodium-potassium and prevents the decrease of intracellular potassium that maintains spermatozoa motility. Furthermore, it decreases the spermatozoa motility increases decreasing by the lipid peroxidation of potassium (Alvarez and Storey, 1982).

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