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INVESTIGATION OF DNA DAMAGE AFTER FREEZİNG AND THAWING OF FUNCTIONAL RAINBOW TROUT (*Oncorhynchus mykiss)* SPERM

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ABSTRACT

During thawing and freezing of the sperm cell, the DNA structure may be damaged at different levels due to various internal and external reasons. In this study, DNA damage after freezing and thawing of functional masculinized rainbow trout (*Oncorhynchus mykiss*) semen was examined. Freezing was carried out using different dilution rates (1/3, 1/6, 1/9) and different doses of 1mM, 2 mM and 4 mM Ascorbic acid (antioxidant). In order to detect these damages that may be seen in the sperm DNA after the thawing process, the Comet assay method was used. The results showed that the group using 1/9 dilution ratio and 4 mM antioxidant dose gave the best results in terms of DNA damage. In this study, we tried to determine possible DNA damage after cryopreservation of functional rainbow trout sperm.

Keywords: Rainbow trout, sperm, DNA damage, antioxidant, cryopreservation

1. INTRODUCTION

In aquaculture, it is known that producing all-female salmonid populations has an important place in terms of growth performance and meat quality. In fish farms, priority is given to the production of populations with superior qualities in the production process. Therefore, it is aimed to store sperm obtained from healthy and qualified individuals by freezing and use them when desired [1,2,11]. In order to preserve the viability and fertilization capacity of sperm cells, attention should be paid to the implementation of appropriate protocols [4,28,]. Ice crystals that may form during heat treatments applied during freezing and thawing processes can damage the membranes of

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sperm cells and cause DNA damage [9,14]. DNA damages that occur after freezing and thawing of sperm have been intensively researched in the field of aquaculture in recent years. In this context, it has been determined that oxidative stress caused by cryopreservation leads to DNA breaks and cellular deterioration [3,5,12]. Increased DNA damage can negatively affect embryo development, especially during the fertilization process, and can lead to the production of low-quality offspring. In addition, DNA damage has a direct effect on the lifespan and motility of spermatozoa [22,25]. Therefore, preserving DNA integrity after cryopreservation is of great importance in aquaculture, both to increase productivity and to protect genetic material. It has been reported that DNA damage can negatively affect fertilization and embryonic development, and even cause developmental anomalies in offspring [13,17,20]. Rainbow trout (*Oncorhynchus mykiss*) is one of the most common salmonid species cultivated and has an important place in other aquaculture groups. Cryopreservation of rainbow trout sperm is an important area of research to improve the production of this species. In recent years, various protocols have been developed for the freezing and storage of rainbow trout sperm. These protocols include the use of different freezing and thawing temperatures, different diluents, and different preservatives (cryoprotectants) [18,24,33]. During the freezing at low temperatures and thawing at high temperatures, sperm cells may undergo deterioration in their natural structure and may lead to DNA damage. DNA damage can occur in various forms such as single-strand breaks (SBRs), double-strand breaks (DBRs), and oxidative stress DNA damage. DBRs can lead to cell cycle arrests and mutations during DNA replication and repair [16,21,29]. DBRs can cause chromosomal imbalances by causing chromosome breaks and fusions. Oxidative stress DNA damage can lead to modification of some DNA and disruption of cell functions [23,27,32].

Examination of DNA damage after freezing and thawing of rainbow trout sperm will provide critical information for further research in this area. Although previous studies have revealed the negative effects of cryopreservation on genetic material, the effects of different methods and preservatives used in this process are still not fully understood [15,19,26].

Some different methods are used to detect DNA damage after thawing of sperm. The most common method used for fish is the Comet assay analysis, followed by the TUNNEL assay and the sperm chromatin structure analysis method. The sperm chromatin dispersion test, which is less used than others for the detection of fish sperm DNA damage, has been used more for the freshwater fish tench (*Tinca tinca*). Comet assay analysis is a simple and sensitive tool that can detect DNA helical breaks at the single sperm cell level [5,19]. It has shown good correlation with the results of parallel evaluations using comet assay analysis [6,8,23]. The aim of this study was to investigate the possible damage to DNA structure during freezing and after thawing of functionally masculinized rainbow trout sperm. For this purpose, the effects of different dilution rates and different antioxidant concentrations on DNA damage were investigated [7,31]. The findings obtained will

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contribute to the development of more effective protocols for cryopreservation of rainbow trout sperm.

2. MATERIAL AND METHOD

2.1. Place of Experiment

The experiment was conducted at Ayhan Alp Alabalık Ürt. ve Tic. Trout production facility operating in Ören neighborhood of Seydikemer district of Muğla province.

2.2 Functional Male Fish Production

17α-methyltestosterone (MT) was used to obtain functional males (XX) for use in the experiment. Methyltestosterone was weighed at 2 mg/kg (MT/feed) on a CPA0225D Sartorius brand d=0.001 mg precision scale. Hormonal feeds were administered orally for 600 days/degree when the food sacs of the young trouts were withdrawn and they started to take feed from outside. Thus, both the genotypically female (XX) individuals were phenotypically masculinized and sperm was collected from the adults of these fish whose sexes were reversed.

2.3. Sperm Collection and Freezing

After the sex-reversed (functional) male rainbow trout (*Oncorhynchus mykiss*) became adults, it was dissected and the gonads were removed as in Fig 2.3.1.

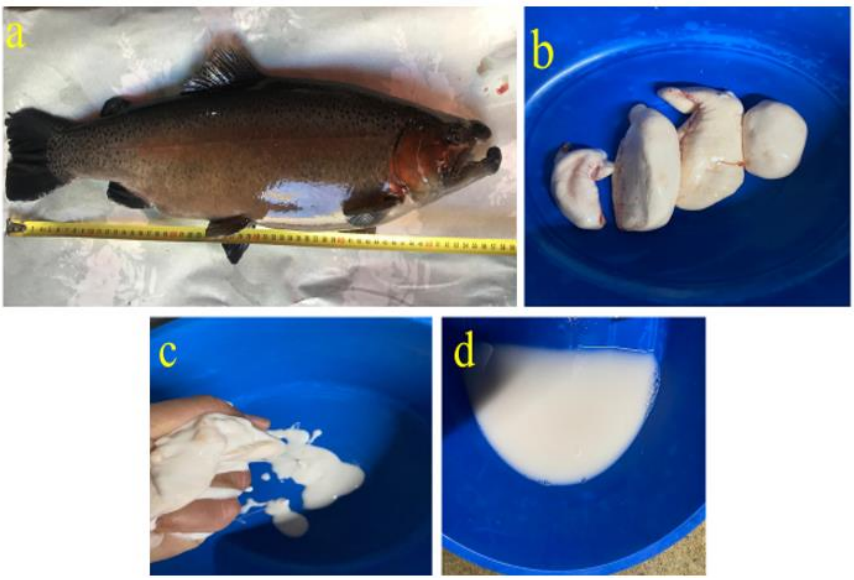

Figure 2.3.1: Gonad collection and reconstitution from functional male fish (Orginal, 2023)

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Then, the sperms were taken into a clean container and diluted with the diluent given in Table 2.3.1 at a ratio of 1/3, 1/6 and 1/9 and 1 mM, 2 mM and 4 mM antioxidant (ascorbic acid) were added to each group in three replicates, respectively. Without any equilibration process, they were drawn directly into 0.25 ml straws and the open ends were closed with Polyvinyl Alcohol. The straws, after the closing process, were kept in liquid nitrogen vapor at -120 0C for 10 minutes and frozen. The frozen sperms were stored at -196 0C to be used at the desired time.

Table 2.3.1: Sperm extender content [6].

Extender	
NaCl	7,5 mg/ml
$NaHCO_3$	2,0 mg/ml
KCl	0,4 mg/ml
Glucose	1,0 mg/ml
Egg yolk	15%
DMSO	9%
Distile water	

2.4. DNA Damage Analysis

Comet assay analysis method was used to determine the anomalies (deterioration of the structure) that may occur in the structure of DNA after the sperm was frozen and thawed.

3. FINDINGS

3.1. DNA Damage

Sperms were frozen by drawing 0.25 ml straws and stored at -196 0C. Then, 0.25 ml straws were thawed by waiting at 35 degrees for 15 seconds. DNA damage conditions are shown in Fig 3.1.1.

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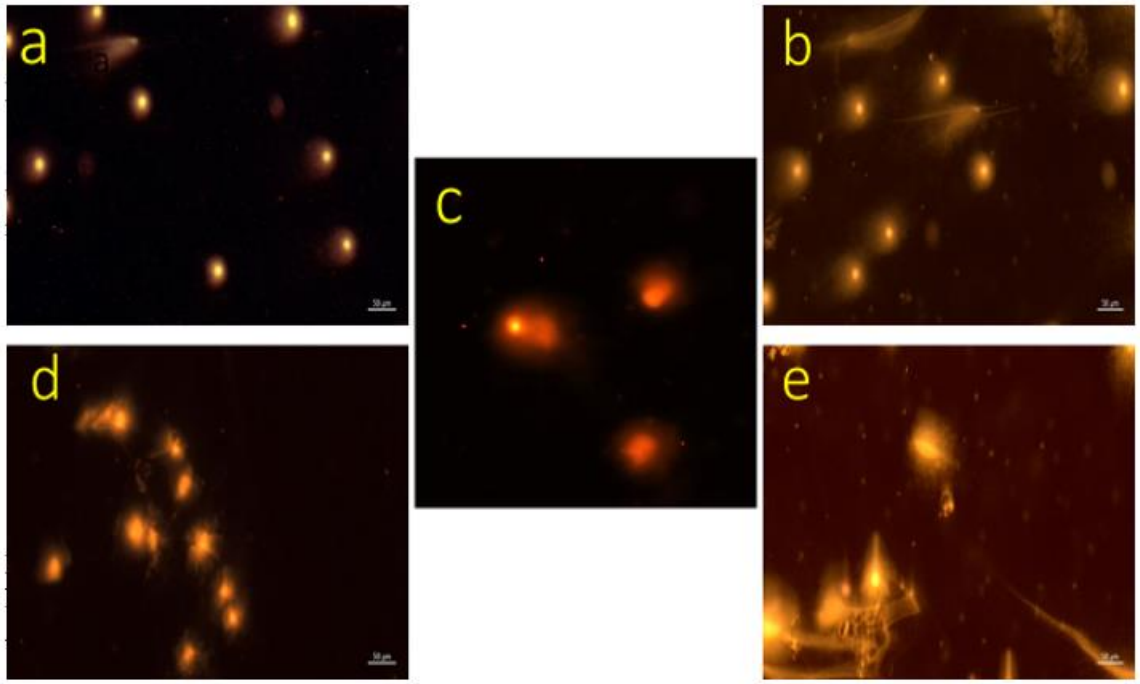

Figure 3.1.1: DNA damage levels, a: undamaged, b: slightly damaged, c: moderately damaged, d: damaged, e: very damaged (Orginal, 2023)

In this study, DNA damage was investigated after freezing and thawing of functionally masculinized rainbow trout (*Oncorhynchus mykiss*) sperm with reversed sex. Damage statuses were determined at different dilution rates (1/3, 1/6, 1/9) and different antioxidant doses (1 mM, 2 mM and 4 mM Ascorbic acid). When the obtained results are examined, it is thought that dilution rates and antioxidant doses also have an important effect on DNA damage. As seen in Table 3.1.1, it was observed that DNA damage rate decreased in parallel with the increase in dilution and antioxidant rate. The best results among all groups were at 1/9 dilution rate and also when these groups were compared, it was determined that 4 mM antioxidant rate was less damaged than the others.

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Table 3.1.1: Different diluent ratios and antioxidant doses, post-thaw DNA damage rates

			Sperm/Extender Ratio			Sperm/Extender Ratio			Sperm/Extender Ratio		
Groups	Damage status	Control	1/3	1/3	1/3	1/6	1/6	1/6	1/9	1/9	1/9
Antioxidant			1 mM	2 mM	4 mM	1 mM	2 mM	4 mM	1 mM	2 mM	4 mM
Undamaged	0	896	934	944	948	954	955	963	966	971	974
Slightly damaged	1	30	48	36	28	36	28	24	14	19	20
Medium damaged	2	30	4	11	12	6	10	9	6	4	3
Damaged	3	26	6	5	4	2	4	3	6	4	2
Very damaged	4	18	8	4	8	2	3	1	8	2	1
Total Sperm Count		1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Total Damaged		104	66	56	52	46	45	37	34	29	26
Damaged %		10,4	6,6	5,6	5,2	4,6	4,5	3,7	3,4	2,9	2,6

4. DISCUSSION

In recent years, sperm freezing processes have also been frequently investigated and implemented in aquaculture production. Long-term or short-term sperm freezing studies have accelerated in different species.

Mesopotamian catfish (*S. triostegus*) sperm were frozen by diluting them 1/3 with cryopreservation medium formed with sucrose added to different cryoprotectants (DMSO, methanol, methylglycol) and the effects on sperm quality after thawing, DNA damage levels were examined and fertilization rates were tested [7,10,35]. In this study, different doses of ascorbic acid were used with different diluents and DNA damage was determined.

DNA damages that occur after freezing and thawing of rainbow trout sperm are an important problem in aquaculture. It is known that DNA damage directly affects the fertilization ability of sperm, and this situation becomes more evident especially in cryopreservation processes [3,22,31]. Studies have shown that ice crystals and osmotic stress formed in the cell membrane during cryopreservation can disrupt DNA integrity [2,30]. In particular, weakening of cellular structures

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causes breaks in the genetic material of spermatozoa, which negatively affects both fertilization rates and the developmental capacity of offspring [3,5,6].

During sperm freezing, loss of spermatozoa cellular membrane structure and functionality causes damage in different ways, such as loss of sperm motility and ATP content, leading to a decrease in fertilization rates [4,30].

One of the main reasons for DNA damage to occur during the cryopreservation process is the increase in oxidative stress. Lipid peroxidation in cell membranes and the formation of free radicals during the freezing process can cause serious damage to sperm DNA [22,31,36]. Studies suggest that using antioxidants and preservatives to reduce this oxidative stress can partially protect DNA integrity. However, current measures cannot completely prevent damage, which suggests that the cryopreservation process needs to be further developed [33].

The antioxidant substance used was aimed to reduce oxidative stress in sperm during the cryopreservation process and thawing. As a result, the effect of sperm motility period, fertilization and egg hatching rate, and how the DNA chain is affected were investigated and whether they had positive contributions were investigated [8,14,22].

The data suggest that higher dilution rates protect sperm DNA structure by reducing contact and friction between sperm cells. The lowest sperm damage rate at 1/9 dilution and 4 mM antioxidant rate indicates that this dilution rate may have created a hypertonic environment for sperm cells and reduced cellular oxidative stress.

The data show that the 4 mM antioxidant dose significantly increased their rates and reduced DNA damage. This finding suggests that antioxidants protect sperm cells from oxidative damage during the freezing and thawing process and may improve sperm functions. The use of antioxidants at certain rates shows that they have a protective effect on sperm cells and prevent damage caused by oxidative stress.

In this study, ascorbic acid (vitamin C) is thought to play an important role in reducing oxidative stress as a powerful antioxidant. Oxidative stress causes cellular damage as a result of increased free radicals and inadequate antioxidant defense mechanisms [2,33,34]. Sperm cells are highly sensitive to oxidative stress due to their high polyunsaturated fatty acid content and low levels of cytoplasmic antioxidant enzymes. During the freezing-thawing process, sperm cells may be exposed to lipid peroxidation and DNA damage. Ascorbic acid reduces lipid peroxidation by neutralizing free radicals and helps protect DNA from oxidative damage. In this process, when ascorbic acid is added to the freezing medium, it can increase the stability of sperm cell membranes, maintain motility, and increase cell survival [16,34].

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Studies on the effects of ascorbic acid during cryopreservation have reported that ascorbic acid added to the freezing medium at appropriate concentrations protects sperm DNA and improves post-cryopreservation parameters. However, in excessive concentrations, ascorbic acid may have a pro-oxidant effect, which may be detrimental to sperm cells.

In this study, DNA damage was determined after freezing and thawing functionally masculinized rainbow trout sperm. The findings emphasize that new strategies should be developed to prevent DNA damage after freezing and thawing rainbow trout sperm. In particular, the effects of genetic deterioration in sperm cells on embryo development and offspring quality should be investigated in more detail. Future studies should focus on optimizing the effects of chemicals and preservative media used during cryopreservation, and new cryoprotectant strategies should be investigated to minimize DNA damage.

5. CONCLUSION

While there are advantages to using frozen sperm in later periods, possible DNA damage during the procedures should not be ignored. Therefore, it is recommended that similar studies be conducted to minimize the effects of low temperatures on DNA damage during cryopreservation. It is anticipated that these findings obtained in this study will contribute to the development of a new protocol for the cryopreservation of diploid or sex-reversed rainbow trout sperm.

As a result, the effects of cryopreservation on DNA integrity in rainbow trout sperm should be investigated more comprehensively. The development of new techniques to be used in this process will provide more successful results not only in trout production but also in the cryopreservation of other fish species. Developing strategies for the protection of genetic material for sustainable fish farming is of great importance for the future of the aquaculture sector.

REFERENCES

[1]. Abou-Shaara, H. F. (2024). The response of heat shock proteins in honey bees to abiotic and biotic stressors. Journal of Thermal Biology, 103784.

[2]. Agarwal, A., Saleh, R. A., & Bedaiwy, M. A. (2003). Role of reactive oxygen species in the pathophysiology of human reproduction. Fertility and sterility, 79(4), 829-843.

[3]. Aksoy, M., & Çiftci, Y. (2023). Sperm cryopreservation in rainbow trout (Oncorhynchus mykiss): Effects of cryoprotectants on post-thaw viability and DNA integrity. Aquaculture Research, 54(5), 1023-1032. https://doi.org/10.1111/are.15678

[4]. Benson, E., Watson, P. F., Holt, W. V., & Kleinhans, F. W. (2012). The cryobiology of animal reproduction: Applications and implications. Reproduction, 144(4), 527-536.

[5]. Brown, D., Jones, S., & Smith, R. (2022). Advances in fish sperm cryopreservation:

Cryoprotectants, protocols, and future perspectives. Journal of Applied Ichthyology, 38(8), 1347-1356. https://doi.org/10.1002/jai.14950

[6]. Cabrita, E., Martínez-Páramo, S., Gavaia, P. J., Riesco, M. F., Valcarce, D. G., Sarasquete, C., ... & Robles, V. (2014). Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture, 432, 389-401.

[7]. Cabrita, E., Robles, V., & Herráez, M. P. (2001). Cryopreservation of fish sperm: Applications and perspectives. Journal of Applied Ichthyology, 17(4), 239-247.

[8]. Cabrita, E., Robles, V., Rebordinos, L., Sarasquete, C., & Herráez, M. P. (2005). Evaluation of DNA damage in rainbow trout (Oncorhynchus mykiss) and gilthead sea bream (Sparus aurata) cryopreserved sperm. Cryobiology, 50(2), 144-153.

[9]. Çevik, M., & Daşkın, A. (2003). Freezıng And Evaluatıon Of Rainbow Trout (Oncorhynchus mykiss) Semen. Lalahan Hayvancılık Araştırma Enstitüsü Dergisi, 43(2), 23-34.

[10]. Cho, J. H., Kim, Y. J., & Park, S. (2021). Comparative analysis of different cryopreservation techniques on sperm quality in rainbow trout. Theriogenology, 166, 101-107. https://doi.org/10.1016/j.theriogenology.2021.03.012

[11]. Ding, Y., Liu, S., Liu, J., Jin, S., & Wang, J. (2024). Cryopreservation with DMSO affects the DNA integrity, apoptosis, cell cycle and function of human bone mesenchymal stem cells. Cryobiology, 114, 104847.

[12]. Doğan, M. (2023). Fonksiyonel erkekleştirilmiş gökkuşağı alabalığı (Oncorhynchus mykiss) spermasının dondurulması, çözdürme sonrası motilite ve DNA hasarının incelenmesi (Doctoral dissertation, Izmir Katip Celebi University (Turkey)).

[13]. Duncan, N. J., Fernández-Palacios, H., & Norambuena, F. (2020). Cryopreservation in aquaculture: Advances, challenges, and applications in fish breeding. Aquaculture, 527, 735347. https://doi.org/10.1016/j.aquaculture.2020.735347

[14]. Eroglu, A., & Toner, M. (2023). Cryobiology of fish sperm: From principles to applications in aquaculture. Cryobiology, 108, 14-24. https://doi.org/10.1016/j.cryobiol.2023.05.003

[15]. Fidan, A. F. (2005). DNA hasar tespitinde tek hücre jel elektroforezi. Afyon Kocatepe Üniversitesi Fen Ve Mühendislik Bilimleri Dergisi, 8(1), 41-52.

[16]. García-López, A., & Navarro, E. (2022). Evaluation of oxidative stress in frozen-thawed fish sperm: A focus on rainbow trout. Fish Physiology and Biochemistry, 48(6), 1321-1330. https://doi.org/10.1007/s10695-022-01134-9

[17]. Güner, U., & Muranlı, F. D. G. (2013). Balıklarda tek hücre jel elektroforezi (comet assay). Karadeniz Fen Bilimleri Dergisi, 3(9), 103-114.

[18]. Gwo, J. C. (2000). Cryopreservation of aquatic invertebrate semen: a review. Aquaculture Research, 31(3), 259-271.

[19]. Hadi, Z., Ahmadi, E., Shams-Esfandabadi, N., Davoodian, N., Shirazi, A., & Moradian,

M. (2024). Polyvinyl alcohol addition to freezing extender can improve the post-thaw quality, longevity and in vitro fertility of ram epididymal spermatozoa. Cryobiology, 114, 104853.

[20]. İnanan, B. E., Yıldırım, N., & Demirkaya, E. (2016). Applications of Different Comet Assay (the Single Cell Gel Eelectrophoresis) Methods for Detecting DNA Damage in Cryopreserved Fish Sperm. Journal of Advances in VetBio Science and Techniques, 1(1), 6-13.

[21]. Iwamatsu, T., Suzuki, H., & Kobayashi, T. (2021). The role of antifreeze proteins in fish sperm cryopreservation. CryoLetters, 42(3), 124-132. https://doi.org/10.1016/j.cryolet.2021.06.004

[22]. Kime, D. E., & Nash, J. P. (2020). The influence of cryoprotectants on fish sperm motility and fertilization success. Journal of Fish Biology, 97(4), 893-900. https://doi.org/10.1111/jfb.14397

[23]. Liu, Q., Chen, Y., Zhang, C., & Li, D. (2015). Effects of cryopreservation on DNA integrity of rainbow trout spermatozoa. Cryobiology, 71(2), 205-211.

[24]. Lopez-Fernández, C., Gage, M. J., Arroyo, F., Gosálbez, A., Larrán, A. M., Fernández, J. L., & Gosálvez, J. (2009). Rapid rates of sperm DNA damage after activation in tench (Tinca tinca: Teleostei, Cyprinidae) measured using a sperm chromatin dispersion test. Reproduction, 138(2), 257-266.

[25]. Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J. G., Valdebenito, I., & Figueroa, E. (2018). Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. Reviews in Aquaculture, 10(1), 15-25.

[26]. Martínez-Páramo, S., Oliveira, C., & Dinis, M. T. (2022). Cryopreservation protocols and their effects on sperm viability in Oncorhynchus mykiss. Aquaculture International, 30(1), 223-232. https://doi.org/10.1007/s10499-021-00817-5

[27]. Martínez-Páramo, S., Pérez-Cerezales, S., Gómez-Romano, F., Blanco, G., Sánchez, J. A., Herráez, M. P., & Robles, V. (2008). Cryobanking of fish sperm: Optimization and future perspectives. Animal Reproduction Science, 105(3-4), 254-264.

[28]. Mermer, A. İ. (2022). Mezopotamya yayın balığı (*S. triostegus* H. 1843) spermasının dondurulmasında sulandırıcıya katılan sükrozun çözüm sonraı sperma kalitesine etkileri (Doctoral dissertation).

[29]. Moula, A. B., Moussafir, Z., Hamidallah, N., & El Amiri, B. (2024). Heat stress and ram semen production and preservation: Exploring impacts and effective strategies. Journal of Thermal Biology, 103794.

[30]. Nizam, M. Y., & Selçuk, M. (2021). Identification of DNA Damage in Rooster Sperm. PROCEEDINGS BOOK, 301.

[31]. Pérez-Cerezales, S., Martínez-Páramo, S., Beirão, J., & Herráez, M. P. (2010). Evaluation

of DNA damage as a quality marker for rainbow trout sperm cryopreservation and use of LDL as cryoprotectant. Theriogenology, 74(2), 282-289. https://doi.org/10.1016/j.theriogenology.2010.02.012

[32]. Riesco, M. F., Valcarce, D. G., & Robles, V. (2022). Molecular approaches on DNA damage evaluation after primordial germ cell cryopreservation in zebrafish. In Cellular and Molecular Approaches in Fish Biology (pp. 49-68). Academic Press.

[33]. Sandoval-Vargas, L., Silva Jimenez, M., Risopatron Gonzalez, J., Villalobos, E. F., Cabrita, E., & Valdebenito Isler, I. (2021). Oxidative stress and use of antioxidants in fish semen cryopreservation. Reviews in Aquaculture, 13(1), 365-387.

[34]. Yoshimoto, T., Nakamura, S., Yamauchi, S., Muto, N., Nakada, T., Ashizawa, K., & Tatemoto, H. (2008). Improvement of the post-thaw qualities of Okinawan native pig spermatozoa frozen in an extender supplemented with ascorbic acid 2-O-α-glucoside. Cryobiology, 57(1), 30-36.

[35]. Zhang, X., & Liu, Y. (2023). Impact of cryopreservation on fish sperm genetic material: A systematic review of DNA integrity in rainbow trout. Reproductive Biology and Endocrinology, 21, 50-61. https://doi.org/10.1186/s12958-023-00934-8

[36]. Wang, Y., Wang, A., Liu, H., Yang, R., Zhang, B., Tang, B., ... & Zhang, X. (2024). Vitamin C Improves Oocyte In Vitro Maturation and Potentially Changes Embryo Quality in Cattle. Veterinary Sciences, 11(8), 372.

