

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/347005145>

Cryoprotective Effect of Vitamin E Supplementation of Different Extenders on Quality and Fertilizing Ability of Frozen–Thawed Brown Trout Sperm

Article in *Biopreservation and Biobanking* · June 2021

DOI: 10.1089/bio.2020.0062

CITATIONS

0

READS

20

5 authors, including:



Yusuf Bozkurt

İskenderun Technical University

100 PUBLICATIONS 771 CITATIONS

[SEE PROFILE](#)



İlker Yavaş

Mustafa Kemal University, Antakya, Hatay, Türkiye

54 PUBLICATIONS 318 CITATIONS

[SEE PROFILE](#)



Tugba Raika Kıran

2 PUBLICATIONS 0 CITATIONS

[SEE PROFILE](#)



Aziz Gül

Mustafa Kemal University

37 PUBLICATIONS 323 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



Effect of Different Antioxidants on Post-Thaw Quality, DNA Damage and Fertilization Ability of African Catfish Sperm [View project](#)



Heavy Metals, Trace Elements and Biochemical Composition of Different Honey Produce in Turkey [View project](#)

Cryoprotective Effect of Vitamin E Supplementation of Different Extenders on Quality and Fertilizing Ability of Frozen-Thawed Brown Trout Sperm

Yusuf Bozkurt,¹ İlker Yavaş,² Mustafa Numan Bucak,³ Tuğba Raika Kıran,^{4,*} and Aziz Gül⁵

Vitamin E is one of the most powerful antioxidants for prevention of cell damage resulting from cryopreservation, but its efficacy for cryopreserving brown trout sperm is still unclear. In this work, the protective effect of vitamin E on quality, fertilizing capacity, and DNA damage of brown trout (*Salmo trutta macrostigma*) sperm after cryopreservation was evaluated. Sperm samples were diluted at the ratio of 1:10 with three different extenders (E): (E-I): 300 mM glucose, 10% egg yolk; (E-II): 33.3 mM glucose, 5.1 mM NaCl, 0.5 mM NaHCO₃, 15% DMA; and (E-III): 61.6 mM NaCl, 134.2 mM KCl, 1.9 mM CaCl₂, 0.8 mM MgCl₂, 2.3 mM NaHCO₃ in distilled water. Each extender was supplemented with 10% DMSO and different concentrations of vitamin E at 0.1, 0.5, and 1.0 mM. Spermatozoa frozen without vitamin E (0 mM, control) and fresh sperm were also used. After dilution, the sperm was aspirated into 0.25 mL straws, frozen 3 cm above the liquid nitrogen (LN₂) surface, and plunged into the LN₂. Cell motility, viability, fertilization, and eyeing were determined in post-thawed samples. DNA damage was determined by the comet assay after cryopreservation. Supplementation of 1 mM vitamin E to all extenders exhibited the best cryoprotective effect in terms of sperm motility, duration of motility, viability, fertility, and DNA integrity against cryopreservation damage, compared with 0.1, 0.5, and control group (0 mM) ($p < 0.05$). The highest post-thaw motility ($62.4\% \pm 0.36\%$), fertilization (48.2 ± 0.84), and the lowest DNA damage (7.245%) were obtained with the extender-II including 1.0 mM vitamin E ($p < 0.05$). Consequently, vitamin E positively affected the motility parameters, fertility, and DNA integrity, and the results suggest the addition of extenders with vitamin E as an antioxidant for the cryopreservation of brown trout sperm.

Keywords: vitamin E, antioxidant, brown trout, sperm quality, DNA damage, cryopreservation

Introduction

CRYOPRESERVATION IS A LONG-TERM storage technique that is efficiently used as an important biotechnological tool for the aquaculture industry. Its benefits can be summarized as follows: synchronization of the availability of the gamete year-round, reduction of the nursing cost of broodstock, promotion of breeding techniques for broodstock management, and development of the hatchery management by protecting sperm of the selected individuals in cryobanks.^{1,2} Thus, the improvement of effective cryopreservation methods

is very important to facilitate the use of cryopreserved sperm for large-scale larvae production.

Salmo trutta macrostigma is one of the important freshwater fish species in terms of biodiversity and its economical value. Unfortunately, its population declines day by day because of illegal practices and heavy fishing pressure, degradation of spawning habitats, and river damming.^{3,4}

Cryopreservation of salmonid sperm has been widely investigated because of its high commercial and recreational value in terms of the food sector and fishing. On the contrary, the sperm cryopreservation process, despite its various

¹Department of Aquaculture, Faculty of Marine Sciences and Technology, İskenderun Technical University, İskenderun, Turkey.

²Department of Reproduction and Insemination, Faculty of Veterinary Science, Mustafa Kemal University, Antakya, Turkey.

³Department of Reproduction and Artificial Insemination, Faculty of Veterinary Science, Selçuk University, Konya, Turkey.

⁴Department of Biomedical Engineering, Faculty of Engineering and Natural Sciences, İskenderun Technical University, İskenderun, Turkey.

⁵Department of Animal Science, Faculty of Agriculture, Mustafa Kemal University, Antakya, Turkey.

*Present affiliation: Malatya Turgut Özal University, Health Services Vocational School, Medical Laboratory Techniques Programme, Malatya, Turkey.

advantages, causes cellular damage that can reduce semen quality such as motility, membrane stability, DNA integrity, and also it impairs its fertilization capacity and embryo development.³⁻⁶ The main cause of the damage in sperm during the cryopreservation process is oxidative stress owing to the inconsistency between reactive oxygen species (ROS) and antioxidant activity of sperm cells.

Naturally, sperm cells are protected against oxidative stress reactions with antioxidant components of the seminal plasma. On the contrary, dilution of sperm causes a reduction of seminal plasma ingredients and an increase in the susceptibility of sperm cells to oxidative stress during the cryopreservation process.⁷ It has been proven that by reducing the oxidative damage it is possible through supplementation of some substances, which have antioxidant properties, to the cryosolutions, the sperm cells can be protected against damaging effects of ROS.⁸ However, antioxidant effectiveness can vary among species and is dependent upon its concentration and structure.

Cabrita et al.⁷ reported that cryosolutions containing vitamins with antioxidant properties decreased DNA fragmentation in gilthead seabream (*Sparus aurata*) when compared with that in European sea bass (*Dicentrarchus labrax*). From these results it is known that vitamin E (α -tocopherol) is one of the natural antioxidants, which has the ability to penetrate the sperm membrane and protect the sperm cells against the detrimental influence of free radicals.⁹ In addition, it can neutralize lipid peroxidation and also decrease oxidative damage.¹⁰

However, to the best of our knowledge, there is a lack of information concerning whether vitamin E could improve the quality and decrease DNA damage of sperm after the cryopreservation process in brown trout. In this framework, the objective of this study was to evaluate the influence of three different extenders (ionic and sugar based) with different vitamin E concentrations (0.1, 0.5, and 1.0 mM) on post-thaw quality, fertilization ability, and DNA damage after cryopreservation of brown trout semen.

Materials and Methods

Ethical approval

This study was approved by the Local Animal Experiment Ethics Committee of the Mustafa Kemal University (1005-M-0111) in Antakya, Hatay.

Animals

Brown trout gametes were obtained from 2- to 3-year-old broodstock at the Kılıç Bafa Aquaculture Farm (Kahramanmaraş, Turkey) in January. Mature male (386.2 ± 5.7 g, $n=20$) and female (427.5 ± 4.2 g, $n=10$) brown trout were kept in 500 L indoor tanks, which were supplied with constant hatchery water ($8.2^\circ\text{C} \pm 1.24^\circ\text{C}$; 10.6 ± 1.5 mg/L O_2) flow at rate of 2.0 L/s. Before sampling, individuals were anesthetized with 15 mg/L quinaldine (Sigma) for a few minutes.

Collection of gametes

For sperm collection, the external urogenital pore of the mature males was wiped dry with a towel to avoid contamination with water, urine, and feces. Then sperm was

collected by gentle abdominal massage into 50 mL sterile glass tubes separately. The sperm-containing tubes were placed into a styrofoam box including crushed ice ($4^\circ\text{C} \pm 1^\circ\text{C}$) until evaluation for quality in terms of motility, density, pH, and color. For egg collection, mature females were wiped dry and stripped by gentle abdominal massage. The eggs were collected into a 1000 mL round-bottomed bowl. The collected eggs were evaluated for homogenous shape, color, and size, and used for fertilization within 30 minutes of stripping.

Sperm cryopreservation

The motility of selected sperm samples were evaluated with the aid of an activation solution (AS) (45 mM NaCl, 5 mM KCl, and 30 mM Tris-HCl, pH 8.2).¹¹ For this aim, 1 μL of sperm suspension was placed on a glass slide and activated by adding 100 μL AS, and sperm motility was determined using a CCD video camera (model SPC-2000P; Japan) mounted on a phase-contrast microscope at $100\times$ magnification (BX43; Olympus, Tokyo, Japan). The observed video records were scanned to evaluate the percentages (%) and duration (s) of motility at least three times for each sample. Sperm motility was evaluated as the percentage of cells exhibiting progressive forward movement, whereas the duration of motility was evaluated until forward movement stopped.

Spermatozoa density was determined according to the hemocytometric method. For this aim, sperm were diluted at a ratio of 1:1000 with Hayem solution (35.2 mM Na_2SO_4 , 17.1 mM NaCl, 1.8 mM HgCl_2 , 200 mL bicine) and density was determined using a 100 μm deep Thoma hemocytometer (TH-100; Hecht-Assistent, Sondheim, Germany) at $400\times$ magnification with an Olympus BX50 phase contrast microscope (Olympus) and expressed as spermatozoa $\times 10^9$ mL^{-1} (three replicates).¹² Standard pH electrodes were used to measure sperm pH, and semen color was evaluated visually within 30 minutes after sperm collection.

Sperm obtained from 18 males exhibiting $>80\%$ motility were individually split into three subsamples and each of them diluted at a ratio of 1:10 (v:v) with three different extenders (glucose and ionic based) composed of (E-I)¹²: 300 mM glucose, 10% egg yolk; (E-II)¹³: 33.3 mM glucose, 5.1 mM NaCl, 0.5 mM NaHCO_3 , 15% DMA; and (E-III)¹⁴: 61.6 mM NaCl, 134.2 mM KCl, 1.9 mM CaCl_2 , 0.8 mM MgCl_2 , 2.3 mM NaHCO_3 in distilled water. After dissolving vitamin E in 50 mL ethanol,¹⁵ each extender was supplemented with 10% DMSO and vitamin E at 0.1, 0.5, and 1.0 mM concentrations separately. The selected concentrations of vitamin E were based on previous studies [7, 24]. Control groups for each extender were supplemented only with 10% DMSO.

Diluted sperm was equilibrated at 4°C for 10 minutes and then drawn into 0.25 mL straws (IMV, France) and sealed with polyvinyl alcohol (PVA). Sperm concentration was $\sim 12.5 \times 10^6$ in each 0.25 mL straw. Before freezing, diluted sperm were maintained in a cool chamber at 4°C to obtain isothermal conditions. Sperm samples were frozen 3 cm above the liquid nitrogen (LN_2) surface in a styrofoam box for 10 minutes and then the frozen samples in each experiment were plunged into LN_2 . Nine straws per sperm sample were frozen. After 1 week storing of samples in LN_2 , re-warming was performed in a water bath at 30°C for 20

seconds. After this, samples were examined in terms of motility, viability, fertilizing capacity, and DNA integrity.

Sperm analysis

To evaluate the frozen–thawed sperm samples, a 10 μ L drop of the sperm dilution was placed on a microscope slide, 20 μ L (AS) was added and then the sperm suspensions were thoroughly mixed for 2 seconds. The motility and motility duration of spermatozoa were immediately recorded for 1 minute after activation using a CCD video camera (CMEX-5, the Netherlands) mounted on a phase-contrast microscope (100 \times ; BX43; Olympus). Sperm motility was evaluated from sperm performing progressive forward movement, whereas motility duration was determined by counting the time from activation until it stopped moving.¹¹ At least five straws were used for evaluation of each parameter and it was performed three times for each treatment.

To assess the percentage of viable sperm, eosin-nigrosin preparations were formed according to the method defined by Björndahl et al.¹⁶ and a total of 300 sperm cells were counted on each slide at a 1000 \times magnification. Sperm showing partial or complete purple colorization were considered nonviable and only sperm showing strict exclusion of the stain were considered viable.

Fertilization ability of cryopreserved sperm

The good quality eggs pooled from four females were divided into batches of about 100 eggs and fertilized with 100 μ L frozen–thawed sperm or 25 μ L fresh sperm (sperm-to-egg ratio: $\sim 5 \times 10^6$:1) in 500 mL round-bottomed bowls. Next, the fertilization solution (125 mM NaCl, 20 mM Tris-HCl, 30 mM glycine, pH 9) was added onto the eggs in a ratio of 1:2 (fertilization solution/eggs).^{17,18} The eggs were slightly stirred for 30 minutes and rinsed with hatchery water (8.2 \pm 1.2 $^\circ$ C; 10.6 \pm 1.5 mg/L O₂) at the end of stirring, and finally transferred into the labeled vertical incubators supplied with flowthrough hatchery water. Eggs were incubated \sim 40 days until the eyed stage. The fertilization (number of 4-cell stage embryos/number of total eggs) rates were determined under a stereomicroscope at 20-fold magnification. Three replicates were performed for each treatment with frozen–thawed and fresh sperm.

Evaluation of sperm DNA damage

DNA damage of sperm cells was explored using the single cell gel electrophoresis (COMET) assay that was generally carried out under neutral conditions. The performed method was similar to several protocols with few modifications that were applied to previous studies.^{19,20} First, diluted sperm samples were centrifuged at 300 g for 10 minutes at 4 $^\circ$ C. Seminal plasma was removed and remaining sperm cells were washed with PBS, then adjusted to a concentration of 1×10^5 spermatozoa/mL. For this aim, each microscope slide was precoated with a layer of 1% normal melting point agarose in

PBS and thoroughly dried at room temperature. Next, 100 μ L of 0.7% low melting point agarose at 37 $^\circ$ C was mixed with 10 μ L of the sperm samples and dropped on top of the first layer. Slides were allowed to solidify for 5 minutes at 4 $^\circ$ C in a moist box.

The coverslips were removed and the slides were immersed in freshly prepared cold lysis buffer with 2.5 M NaCl, 100 mM Na₂-EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, and 40 mM dithiothreitol (pH 10) for 1 hour at 4 $^\circ$ C. Then, the slides were incubated overnight at 37 $^\circ$ C in 100 μ g/mL proteinase K and added to the lysis buffer. The slides were removed from the lysis buffer, drained, and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA (pH 13), for 20 minutes to unwind the DNA. Electrophoresis was performed for 20 minutes at room temperature at 25 V and was adjusted to 300 mA. Then, the slides were washed with a neutralizing solution of 0.4 M Tris, pH 7.5. After neutralization, the slides were stained with 50 μ L of 2 μ L/mL ethidium bromide and covered with a coverslip. The whole process was performed under dim light to prevent further DNA damage.^{21,22}

The images of 100 randomly chosen nuclei were analyzed visually. Observations were made at a magnification of 400 \times using a fluorescent microscope (BX51; Olympus). Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a “comet” pattern, whereas whole sperm heads, without a comet, were not considered as damaged. All data were expressed as a mean percentage undamaged sperm heads \pm SEM.

Statistical analysis

All analyses were repeated three times for each treatment. Results were presented as mean \pm standard deviation. Motility values were normalized through arcsine transformation and differences among the parameters were tested with two-way analysis of variance (extender \times vitamin E). Correlation analyses were performed to determine the interactions between tested extenders and vitamin E concentrations. Duncan *post hoc* test was implemented for all comparisons among the treatments at level of $p < 0.05$. All statistical analyses were carried out using SPSS 17.0 software package program.

Results

Sperm quality parameters

In fresh sperm, the mean percentage (%) and duration (s) of motile spermatozoa were 80.55% \pm 13.81% and 47.22 \pm 13.15 seconds, respectively. Mean spermatological properties of fresh sperm are given in Table 1. The effect of different extenders containing different concentration of vitamin E on post-thaw progressive motility, motility duration, and viability parameters of frozen–thawed sperm are given in Figures 1–3. Increasing the vitamin E concentration in all extender types caused an increase in all mentioned post-thaw quality parameter values in comparison with those that not containing vitamin E (0 mM)

TABLE 1. MEAN SPERMATOLOGICAL PROPERTIES OF FRESH BROWN TROUT (*SALMO TRUTTA MACROSTIGMA*) SPERM (N=20)

Volume (mL)	Motility (%)	Motility Duration (s)	Density ($\times 10^9$ /mL)	Total density ($\times 10^9$)	pH	Color
5.81 \pm 3.14	80.55 \pm 13.81	47.22 \pm 13.15	19.65 \pm 1.92	114.167 \pm 6.02	7.34 \pm 0.11	Milky White

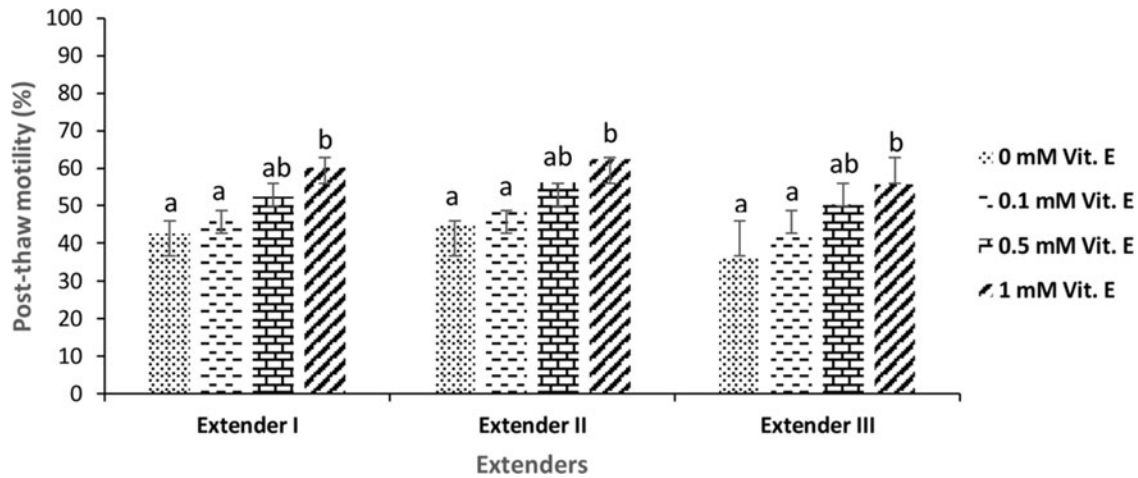


FIG. 1. The mean post-thaw motility (%) of frozen–thawed brown trout (*Salmo trutta macrostigma*) sperm. Different letters indicate differences among treatments (ANOVA, $p < 0.05$, $n = 9$). ANOVA, analysis of variance.

(control group) ($p < 0.05$). The highest post-thaw average motility, motility duration, and viability values were attained with the extender-II containing 1 mM vitamin E concentration. Statistical differences were significant among the treatments in terms of motility, duration, and viability of frozen–thawed sperm ($p < 0.05$).

Fertility

Increasing the vitamin E concentration in all extender types caused an increase in post-thaw fertility values in comparison with those that not containing vitamin E (0 mM) ($p < 0.05$). In addition, higher values were obtained with the extender-II in terms of all vitamin E concentrations. Among the cryopreserved samples, the highest average fertilization rate ($48.2\% \pm 1.2\%$) was obtained using the extender-II containing 1 mM vitamin E (Fig. 4). Statistical differences were significant among the treatments in terms of fertility of frozen–thawed sperm ($p < 0.05$).

DNA damage

DNA damage of brown trout (*Salmo trutta macrostigma*) sperm cryopreserved with four different vitamin E

concentrations and three different extenders are presented in Table 2. There were significant differences among the treatment groups ($p < 0.05$). Sperm cells frozen–thawed in the absence of vitamin E (0 mM) showed the highest DNA damage among all extenders. The extenders supplemented with vitamin E decreased the DNA damage. In the comet test, all extender groups containing vitamin E ranging from 0.1 to 1.0 mM produced lower DNA damages than that of the extenders not containing vitamin E ($p < 0.05$).

Discussion

Vitamin E (α -tocopherol) is a fat-soluble vitamin and also one of the most common antioxidants. Although the importance of vitamin E supplementation is addressed in many other studies, the majority of these studies have indicated the nutritional value of vitamin E supplementation.²³ However, several studies have reported its protective effect on sperm quality in domestic animals in recent years.^{24–26} On the contrary, studies about its protective effect on post-thaw sperm quality and DNA integrity are limited in aquatic species.

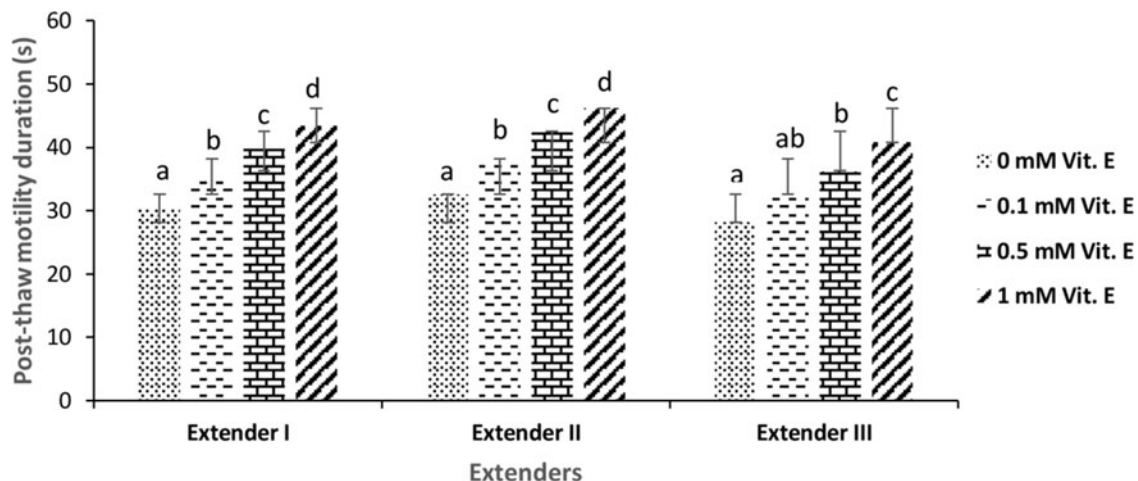


FIG. 2. The mean post-thaw motility duration (s) of frozen–thawed brown trout (*Salmo trutta macrostigma*) sperm. Different letters indicate differences among treatments (ANOVA, $p < 0.05$, $n = 9$).

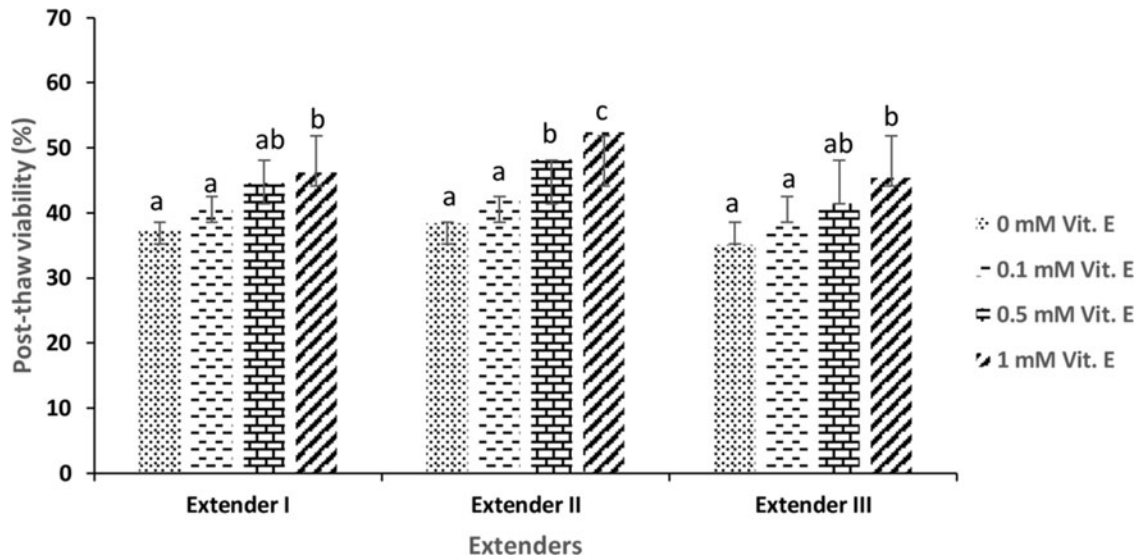


FIG. 3. The mean post-thaw viability (%) of frozen-thawed brown trout (*Salmo trutta macrostigma*) sperm. Different letters indicate differences among treatments (ANOVA, $p < 0.05$, $n = 9$).

In this study, the effect of different extenders and vitamin E concentrations on post-thaw motility and fertilizing ability of cryopreserved brown trout sperm were compared. It was demonstrated that supplementation of the extenders with vitamin E increased the post-thaw motility, viability, duration, and fertilization rates depending on the extender type. In contrast, other researchers have shown that supplementation of vitamin E to the freezing medium caused a decrease in the motility rate in *Pagrus major*.²⁷ In addition, this vitamin did not affect the sperm motility rates of *Prochilodus lineatus*,²⁸ *Sparus aurata*, or *Dicentrarchus labrax*.^{7,29} These results differ from that of this study, suggesting that vitamin E is more effective for brown trout. These differences could be owing to the fish species or even to other conditions used such as different concentrations of vitamin E, cryoprotectants, and their interactions with the vitamin E extender.

It is well known that motility is a significant feature in evaluating fresh and post-thaw sperm quality. Consistent with our results, Ubilla and Valdebenito³⁰ found that post-thaw motility, fertilizing capability, and storage period increased when using vitamin C in extenders in rainbow trout sperm cryopreservation. According to Martinez-Paramo et al.²⁹ supplementation of cryosolutions with α -tocopherol and ascorbic acid improved sea bass post-thaw sperm motility. On the contrary, Cabrita et al.⁷ indicated that post-thaw motility did not significantly increase with the addition of ascorbic acid and tocopherol to the cryosolution in gilthead seabream and European seabass sperm. The findings of this study indicated that there were significant improvements in the quality of frozen-thawed brown trout sperm resulting in higher fertilization rates in response to vitamin E additions to the extenders.

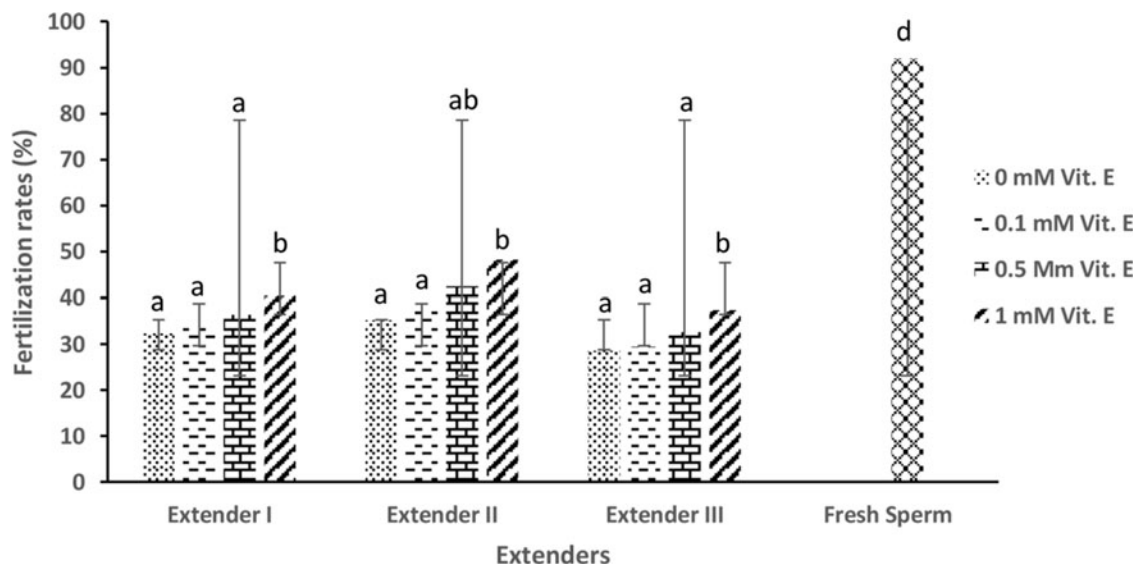


FIG. 4. The mean post-thaw fertilization rates (%) of frozen-thawed brown trout (*Salmo trutta macrostigma*) sperm. Different letters indicate differences among treatments (ANOVA, $p < 0.05$, $n = 9$).

TABLE 2. DNA DAMAGE OF FROZEN–THAWED BROWN TROUT (*SALMO TRUTTA MACROSTIGMA*) SPERM

	DNA damage levels (%)
Extender I	
Vit.E-0 mM	12.083 ± 0.454 ^a
Vit.E-0.1 mM	11.750 ± 0.727 ^a
Vit.E-0.5 mM	10.142 ± 0.404 ^{ab}
Vit.E-1.0 mM	9.272 ± 0.272 ^b
Extender II	
Vit.E-0 mM	11.612 ± 0.551 ^a
Vit.E-0.1 mM	8.555 ± 0.256 ^{ab}
Vit.E-0.5 mM	8.142 ± 0.404 ^{ab}
Vit.E-1.0 mM	7.245 ± 1.124 ^b
Extender III	
Vit.E-0 mM	13.925 ± 0.484 ^a
Vit.E-0.1 mM	13.681 ± 0.310 ^a
Vit.E-0.5 mM	11.611 ± 0.498 ^{ab}
Vit.E-1.0 mM	10.875 ± 0.739 ^b

Different letters indicate differences among treatments (ANOVA, $p < 0.05$, $n = 9$). ANOVA, analysis of variance.

The findings of this study also revealed a lower motility of frozen–thawed brown trout sperm than fresh ones, which might be owing to a decrease in the ATP stores resulting in low mitochondrial activity, damage in cell membrane integrity, or serious cryogenic DNA damage occurring during the cryopreservation process. The assessment of the DNA situation of frozen–thawed sperm is particularly important in terms of commercial-scale usage or for cryobanking. After cryopreservation, DNA fragmentation has also been reported in rainbow trout,³¹ seabream,⁵ sea bass,³² Nile tilapia,³³ and paddlefish.³⁴ The probable physiological reason for this situation may be the insufficient natural antioxidants in seminal plasma, which can promote the creation of free radicals. The positive effect of Vitamin E is that it deactivates the negative effects of ROS with a consequent improvement of the thawed semen quality.

Furthermore, a decline in TAS (total antioxidant potential) in frozen–thawed samples, which shows a decrease in antioxidants, naturally exists in the seminal plasma of gilthead seabream, as was reported by Martínez-Paramo et al.³⁵ This situation may have occurred because of reducing the capacity of sperm to withstand further cryodamage in the absence of antioxidants such as vitamin E in the cryosolution. The results of this study reveal decreasing DNA damage in cryopreserved brown trout sperm with an increasing concentration of vitamin E in the extenders used. This situation can be explained by the observed reduction in DNA damage, which may be owing to the decrease in ROS activity when vitamin E is added as an antioxidant.

The findings of this study are in agreement with the results of Massaeli et al.³⁶ who indicated that vitamin E was rather effective in the prevention of lipid peroxidation reactions in the cell membranes. In addition, according to some studies of vitamin E supplementation, it improved post-thawed sperm motility, viability, membrane integrity of goat, rooster,^{37,38} and human sperm DNA integrity,²⁴ which is in agreement with our findings.

Conclusion

In conclusion, the findings of this study demonstrated that supplementation of vitamin E to extenders significantly

improved sperm quality in terms of motility, motility duration, viability, DNA damage, and fertility in brown trout. Overall, the addition of vitamin E to the freezing extenders is advisable to optimize brown trout sperm cryopreservation. However, further research is required to select the best concentration for this purpose.

Acknowledgments

The authors thank the staff of the Kılıç Bafa Aquaculture Farm.

Author Disclosure Statement

No conflicting financial interests exist.

Funding Information

This research was funded by İskenderun Technical University Scientific Research Fund (Project No: ISTE BAP-2017-YP-04).

References

- Di Iorio M, Esposito S, Rusco G, et al. Semen cryopreservation for the Mediterranean brown trout of the Biferno River (Molise-Italy): Comparative study on the effects of basic extenders and cryoprotectants. *Sci Rep* 2019;9:9703.
- Nynca J, Dietrich GJ, Dobosz S, Grudniewska J, Ciereszko A. Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen. *Aquaculture* 2014;433:62–65.
- Bozkurt Y, Yavaş İ, Karaca F. Cryopreservation of brown trout (*Salmo trutta macrostigma*) and ornamental koi carp (*Cyprinus carpio*) sperm. In: Katkov, I. (ed). *Current Frontiers in Cryopreservation*. Rijeka, Croatia: InTech; 2012:293–304.
- Bozkurt Y, Yavaş İ, Gül A, Bucak MN, Yeni D, Avdatek F. Effect of extender supplemented with boron on post-thaw motility, viability, DNA damage and fertilization ability of cryopreserved brown trout (*Salmo trutta macrostigma*) spermatozoa. *CryoLetters* 2019;40:275–283.
- Cabrera E, Robles V, Rebordinos L, Sarasquete C, Herráez MP. Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. *Cryobiology* 2005;50:144–153.
- Sarvi K, Niksirat H, Amiri BM, Mirtorabi SM, Rafiee GR, Bakhtiyari M. Cryopreservation of semen from the endangered Caspian brown trout (*Salmo trutta caspius*). *Aquaculture* 2006;256:564–569.
- Cabrera E, Ma S, Diogo P, Martínez-Paramo S, Sarasquete C, Dinis MT. The influence of certain aminoacids and vitamins on post-thaw fish sperm motility, viability and DNA fragmentation. *Anim Reprod Sci* 2011;125:189–195.
- Beconi MT, Francia CR, Mora NG, Affranchino MA. Effect of natural antioxidants on frozen bovine semen preservation. *Theriogenology* 1993;40:841–851.
- Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of spermatozoa preparation techniques. *J Androl* 1988;9:367–376.
- Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. In: Halliwell B, & Gutteridge JMC, (eds). *Free Radicals in Biology and Medicine*, 3rd Edition. Oxford: Oxford University Press; 1999:1–25.
- Horvath A, Miskolczi E, Urbanyi B. Cryopreservation of common carp sperm. *Aquat Living Resour* 2003;16:457–460.

12. Tekin N, Seçer S, Akçay E, Bozkurt Y. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) semen. *Isr J Aquacult Bamid* 2003;55:208–212.
13. Zhang X, Liu Y. Study of cryopreservation of fish spermatozoa. *Acta Sci Nat Univ Norm Hunan* 1991;14:255–259.
14. Kurokura H, Hirano R, Tomita M, Iwashashi M. Cryopreservation of carp sperm. *Aquaculture* 1984;37:267–273.
15. Nabi I, Fatmi Sh, Zerrouki-Daoudi N, Iguer-Ouada M. Interest of vitamin E and cholesterol preloaded in cyclodextrins on motility of cryopreserved rabbit semen. *Rev Med Vet* 2017;168:87–92.
16. Björndahl L, Söderlund I, Kvist U. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Hum Reprod* 2003;18:813–816.
17. Lahnsteiner F. Semen cryopreservation in the *Salmonidae* and in the Northern pike. *Aquacult Res* 2000;31:245–258.
18. Lahnsteiner F, Weismann T, Patzner RA. A uniform method for cryopreservation of semen of the salmonid fishes *Oncorhynchus mykiss* (Walbaum), *Salmo trutta f. fario* L., *Salmo trutta f. lacustris* L., *Coregonus* sp. *Aquacult Res* 1995;26:801–807.
19. Cavas T. *In vivo* genotoxicity evaluation of atrazine and atrazine-based herbicide on fish *Carassius auratus* using the micronucleus test and the comet assay. *Food Chem Toxicol* 2011;49:1431–1435.
20. Ku WW, Chapin RE, Wine RN, Gladen BC. Testicular toxicity of boric acid (BA): Relationship of dose to lesion development and recovery in the F344 rat. *Reprod Toxicol* 1993;7:305–319.
21. Haines G, Marples B, Daniel P, Morris I. DNA damage in human and mouse spermatozoa after *in vitro* irradiation assessed by the comet assay. *Adv Exp Med Biol* 1998;444:79–93.
22. Singh NP, Stephens RE. X-ray induced DNA double-strand breaks in human sperm. *Mutagenesis* 1998;13:75–79.
23. Brewer MS. Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. *Compr Rev Food Sci Food Saf* 2011;10:221–247.
24. Kalthur G, Raj S, Thiyagarajan A, Kumar S, Kumar P, Adiga SK. Vitamin E supplementation in semen-freezing medium improves the motility and protects sperm from freeze-thaw-induced DNA damage. *Fertil Steril* 2011;95:1149–1151.
25. Silva SV, Soares AT, Batista AM, et al. Vitamin E (Trolox) addition to Tris-egg yolk extender preserves ram spermatozoon structure and kinematics after cryopreservation. *Anim Reprod Sci* 2013;137:37–44.
26. Zhu Z, Fan X, Lv Y, et al. Vitamin E analogue improves rabbit sperm quality during the process of cryopreservation through its antioxidative action. *PLoS One* 2015;23:e0145383.
27. Liu Q, Wang X, Wang W, et al. Effect of the addition of six antioxidants on sperm motility, membrane integrity and mitochondrial function in red seabream (*Pargus major*) sperm cryopreservation. *Fish Physiol Biochem* 2015;41:413–422.
28. Navarro RD, Navarro FKSP, Felizardo VO, Murgas LDS, Andrade SS. Semen quality of curimba (*Prochilodus lineatus*) cryopreserved with vitamins. *Acta Sci Mar* 2014;36:55–60.
29. Martinez-Paramo S, Diogo P, Dinis MT, Herraes MP, Sarasquete C, Cabrita E. Incorporation of ascorbic acid and a-tocopherol to the extender media to enhance antioxidant system of cryopreserved sea bass sperm. *Theriogenology* 2012;77:1129–1136.
30. Ubilla A, Valdebenito I. Use of antioxidants on rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) sperm diluent: Effects on motility and fertilizing capability. *Lat Am J Aquat Res* 2011;39:338–343.
31. Cabrita E, Robles V, Alvarez R, Herraes MP. Cryopreservation of rainbow trout sperm in large volume straws: Application to large scale fertilization. *Aquaculture* 2001;201:301–314.
32. Zilli L, Schiavone R, Zonno V, Storelli C, Vilella S. Evaluation of DNA damage in *Dicentrarchus labrax* sperm following cryopreservation. *Cryobiology* 2003;47:227–235.
33. Bozkurt Y, Yavaş İ, Bucak MN, Yeni D. Effect of different cryoprotectants (Glycerol, Methanol and Dimethyl Sulfoxide) on post-thaw quality, viability, fertilization ability and DNA damage of cryopreserved Nile tilapia (*Oreochromis niloticus*) spermatozoa. *CryoLetters* 2019;40:11–17.
34. Li P, Wei Q, Liu L. DNA integrity of *Polyodon spathula* cryopreserved sperm. *J Appl Ichthyol* 2008;24:121–125.
35. Martinez-Paramo S, Perez-Cereales S, Gomez-Romano F, Sanchez JA, Herraes MP. Cryobanking as tool for conservation of biodiversity: Effect of brown trout sperm cryopreservation on the male genetic potential. *Theriogenology* 2009;71:594–604.
36. Massaeli H, Sobrattee S, Pierce GN. The importance of lipid solubility in antioxidants and free radical generating systems for determining lipoprotein peroxidation. *Free Radical Bio Med* 1999;26:1524–1530.
37. Kargar R, Mohsen F, Gholamreza G, Mohammad HNE. Dietary flax seed oil and/or Vitamin E improve sperm parameters of cloned goats following freezing-thawing. *Cryobiology* 2017;74:110–114.
38. Moghbeli M, Kohram H, Zare-Shahaneh A, Zhandi M, Sharideh H. Are the optimum levels of the catalase and vitamin E in rooster semen extender after freezing-thawing influenced by sperm concentration? *Cryobiology* 2016;72:264–268.

Address correspondence to:

Yusuf Bozkurt, PhD

Department of Aquaculture

Faculty of Marine Sciences and Technology

İskenderun Technical University

İskenderun 31200

Turkey

E-mail: yusuf.bozkurt@iste.edu.tr