

The effects of vitamin E supplementation on sperm parameters, chromatin integrity, and gene expression before and after freezing in aged mice

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Objective: Some age-related testicular changes, such as Sertoli cell vacuolization and blood–testis barrier breakdown, reduce total sperm production and male fertility. Therefore, this study investigated the effect of vitamin E on restoring testicular function in aged mice. Sperm cryo-resistance was also assessed.

Methods: Twenty-eight 48-week-old male Naval Medical Research Institute mice were divided into four groups for a daily gavage of vitamin E: the control group received distilled water, while the three treatment groups were administered 100, 200, and 400 mg/kg, respectively, for 4 weeks. Subsequently, semen analyses, DNA fragmentation index (DFI), and protamine deficiency tests were conducted. Testicular histology, tissue antioxidant enzyme activity, and gene expression levels were also assessed.

Results: The two higher dosages of vitamin E were associated with a higher sperm count, greater progressive motility, and improved sperm morphology ($p < 0.05$). These benefits were also evident after sperm freezing ($p < 0.05$). Although chromatin abnormalities increased following vitrification, the treatment groups showed better outcomes ($p < 0.05$). The tubular diameter, epithelium height, and luminal diameters remained unchanged with age. The tissue antioxidant capacity was greater in the groups receiving the high doses of vitamin E. Additionally, significant increases in inhibitor of DNA binding protein-4 (*Id4*) and GDNF family receptor alpha-1 (*Gfra1*) expression were observed in the higher vitamin E dosage groups, and promyelocytic leukemia zinc finger protein (*Plzf*) expression was notably present in the 400 mg/kg treatment group compared to the control group ($p < 0.05$).

Conclusion: Antioxidant supplementation might enhance reproductive outcomes in aging males. The observed effects included improved sperm cryo-resistance, which is advantageous for future applications such as sperm freezing or fertility preservation.

Keywords: Antioxidants; Gene expression; Male fertility; Oxidative stress; Spermatogonia; Vitamin E

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Introduction

Recently, there has been a trend among working couples to delay childbearing [1]. This, coupled with declining fertility rates, has increased the demand for reproductive technologies [2,3]. There was a misconception that men, unlike women, would maintain strong fertility indefinitely [2]. While men do continue to produce sperm and maintain fertility potential throughout their lives, both the quantity and quality of sperm decline with age [1,4]. Previous studies have examined the age-related changes in spermatogonial stem cells (SSCs) and their microenvironment, revealing that a diminished capacity to support the stem cell niche—critical for self-renewal and maintenance

nance—may play a significant role in age-related male infertility [3].

One of the key indicators of aging is oxidative stress, which arises when there is an imbalance between the production of free radicals and the body's antioxidant defenses. As we age, oxidative stress becomes more prevalent, leading to oxidative damage to lipids, proteins, DNA, and ultimately resulting in cell death [5].

In addition to the challenges associated with spermatogenesis in older men, these issues become particularly significant when such individuals require assisted reproductive technology and sperm cryopreservation. These procedures can lead to cellular damage and impaired sperm function. Sperm freezing has been a valuable method for the long-term storage of sperm cells and the preservation of male fertility, and it has been in widespread use since the 1970s [6]. However, it has been observed that the production of reactive oxygen species (ROS) may increase, or antioxidant activity may decrease during the freeze-thaw cycle, which can result in sperm membrane lipid peroxidation [7,8]. Consequently, some researchers have investigated the potential benefits of antioxidant supplementation during the freezing process to protect sperm from oxidative DNA damage. Vitamin E, a hydrophobic antioxidant and peroxy radical scavenger, is one such supplement that has been studied. It helps to halt the chain reaction of lipid peroxidation in the sperm plasma membrane and preserves the integrity of sperm DNA [9].

Although there have been some successes in sperm freezing following antioxidant supplementation, the ideal antioxidant treatment method has yet to be fully identified. This is due to the wide range of antioxidant regimens and varying concentrations used in previous studies [10]. The trend of marrying later in life, postponing childbearing, the increased frequency of remarriage, and the longer life expectancy of men—who theoretically can father children throughout their lives—has led to age-related infertility becoming a significant issue for modern society. However, there is a notable lack of detailed studies on fertility preservation in older men for future use, even though sperm quantity and quality are known to decline with age. Therefore, further research is necessary to identify the appropriate antioxidants, including dosage, combination, and method of administration, for human sperm cryopreservation to potentially improve the sperm recovery rate.

We investigated the rehabilitation of spermatogenesis, as well as the maintenance and differentiation of SSCs in aged male mice treated with vitamin E. This was indirectly assessed by comparing sperm parameters, evaluating chromatin quality, examining testicular histopathological changes, measuring tissue antioxidant capacity, and analyzing gene expression of SSC markers, including GDNF family receptor alpha-1 (Gfra1), promyelocytic leukemia zinc finger protein (Plzf), and inhibitor of DNA binding protein-4 (ID4). Additionally, we will explore the impact of this antioxidant, administered as a food supplement,

on the resilience of mouse sperm to the freezing process.

Methods

1. Ethical approval

This study received approval from the Research Ethics Committee of Rafsanjan University of Medical Sciences, Rafsanjan, Iran (IR.RUMS.AEC.1402.003). All protocols were carried out in accordance with the approved procedures outlined in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

2. Animals and treatment

Twenty-eight male Naval Medical Research Institute mice, 48 weeks old and weighing 30 to 40 g, were divided into four groups of seven for this study. Throughout the experiment, the animals were maintained under standard conditions with a temperature of 22 ± 2 °C, a relative humidity of 60%, and a 12-hour light-dark cycle. They had ad libitum access to food and water, with plates provided by the Pars Dam Company of Iran. The four study groups were as follows: (1) the control group, which received distilled water by gavage daily without any drug administration; (2) a group that received 100 mg/kg body weight (BW) of vitamin E by gavage for 4 weeks; (3) a group that received 200 mg/kg BW of vitamin E for the same duration; and (4) a group that received 400 mg/kg BW of vitamin E by gavage for 4 weeks. These dosages were chosen based on the treatment doses reported in previous studies [11-14].

3. Sperm parameter evaluation

At the end of the treatment period, which lasted 35 days (equivalent to one cycle of spermatogenesis in mice) [15], the animal was euthanized. This was done after inducing deep anesthesia through an intraperitoneal injection of ketamine and xylazine. The testicular tissues and cauda epididymis were removed by surgery. One testis was preserved in 7% formalin for histological analysis using hematoxylin and eosin staining. Half of the contralateral testis was allocated for gene extraction, while the remaining portion was used for antioxidant assays. Of the two cauda epididymides, one was designated for sperm analysis, and the other was prepared for sperm cryopreservation.

For the conventional semen analysis, cauda epididymal tissue was incubated in a 100 μ L drop of Ham's F-10 medium (Sigma), supplemented with 5% human serum albumin (HSA; LifeGlobal). Testicular tissues were gently disrupted using two fine needles. Following a 10-minute incubation period, spermatozoa were collected from the surface of the drop. An embryologist then evaluated sperm concentration, motility, and morphology.

Sperm analysis was conducted using a Neubauer counting chamber (Marienfeld Equipment). The evaluation of sperm parameters adhered to the standards established by the World Health Organization [16]. Sperm concentration was measured, and motility was classified into three categories: progressive (A and B), non-progressive (C), and immotile (D) spermatozoa. For assessing sperm cell morphology, Diff-Quik staining was employed, and the head, neck, mid-piece, and tail structures were examined under light microscopy at 100× magnification (Figure 1). Various head abnormalities were identified, including hammer-shaped, collapsed, and triangular heads (Figure 1A-1C). Common tail defects observed were hairpin neck, proximal and distal bent tails (Figure 1E), as well as heavy-type cytoplasmic droplets (CD) and light-type CDs (Figure 1F, 1G) [17,18].

4. Sperm freezing and thawing

Sperm cryopreservation was performed in accordance with the protocol described by Nakagata [19], with some modifications. The cryoprotectant agents (CPAs) used were composed of 18% raffinose pentahydrate (1.8 g), 3% skim milk (0.3 g), and 100 mmol/L L-glutamine. These components were dissolved in 10 mL of distilled water and incubated for 90 minutes at 30 °C in a water bath. Following incubation, the solution was centrifuged at 10,000 rpm for 60 minutes. The supernatant was then removed, and the remaining liquid was filtered through a 0.2 µm filter. The prepared CPA was stored at room temperature until needed, in 1.5 mL aliquots within 2 mL cryotubes.

The cauda epididymis was placed in 100 µL of CPA medium, which was then covered with mineral oil in a Petri dish. The cauda was punctured at several points using a fine needle syringe to facilitate the release of sperm into the freezing medium. After a 3-minute interval, 10 µL of a cryoprotectant solution was combined with the sperm and added to 90 µL drops of human tubal fluid (HTF) solution

(Irvine Scientific). Sperm analysis was conducted using 10 µL of the HTF. Subsequently, plastic straws were utilized for sperm freezing. Both ends of each straw were sealed with a thermal sealer. The straws were placed in a 50 mL conical tube, which was then positioned on the surface of liquid nitrogen for 10 minutes. Following this, the tube was fully immersed in the nitrogen (Figure 2).

For the thawing process, the straws were placed in a 37 °C water bath for 15 minutes. Upon removal, both ends of the straws were cut, and the warmed sperm suspension was transferred into 100 µL of HTF medium, which was then overlaid with mineral oil in a 60 mm Petri dish. The drops were incubated for 30 minutes in a 37 °C incubator. Ten microliters of the suspension were then placed in a Neubauer counting chamber for analysis of sperm parameters [7].

5. Sperm DNA fragmentation assessment

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to evaluate sperm DNA fragmentation, utilizing the In-Situ Cell Death Detection Kit (Roche Diagnostics GmbH) in accordance with the manufacturer's instructions. Spermatozoa that exhibited a spectrum of green fluorescence were considered TUNEL-positive [20].

6. Sperm chromatin assessment (protamine deficiency)

Chromomycin A3 (CMA3) staining is a technique used to detect sperm protamine deficiency. To this end, sperm samples were washed twice with phosphate-buffered saline (PBS; Ca²⁺ and Mg²⁺-free). Thin smears were prepared, immediately fixed in an ethanol:acetone (1:1) solution (Merck), and then air-dried at room temperature for 30 minutes. Each slide was treated with 100 µL of CMA3 solution (Sigma-Aldrich) for 20 minutes in the dark and analyzed using fluorescence microscopy [21]. Cells that exhibited bright yellow staining

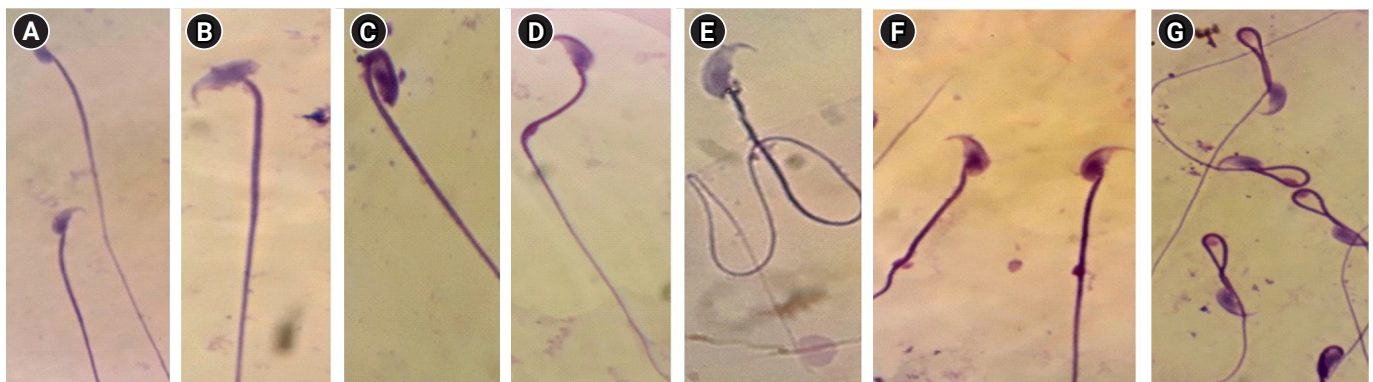


Figure 1. Mouse epididymal sperm morphology (Diff-Quik staining). (A) Two sperm with normal morphology, (B) sperm with an abnormal head and neck junction, (C) sperm lacking a hook shape and with a bent head, (D) sperm with a distally bent tail and a cytoplasmic droplet, (E) sperm with an abnormal head and neck junction, a distally bent tail, and a coiled tail, (F) sperm with a heavy-type cytoplasmic droplet, and (G) sperm with a distally bent tail and a light-type cytoplasmic droplet (magnification ×1,000).

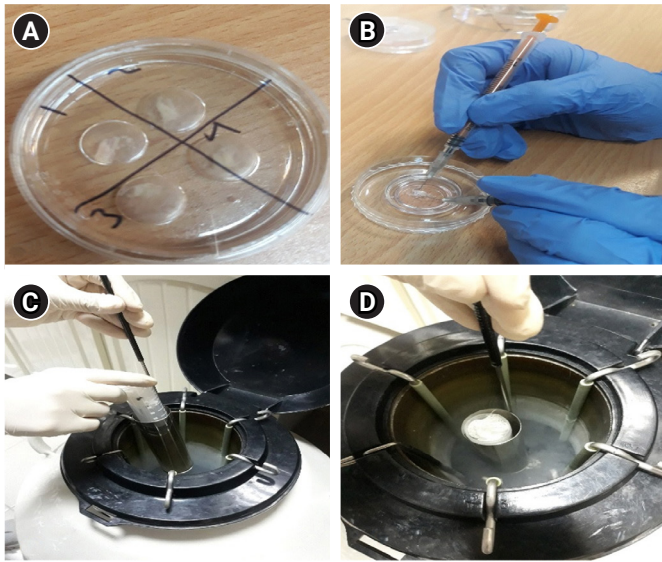


Figure 2. (A) Incubation of the cauda epididymis in cryoprotectant medium, (B) traumatization of the cauda epididymis with a fine needle, (C) holding the conical tube above the surface of the liquid nitrogen, and (D) immersion of the tube in the liquid nitrogen.

(indicative of abnormal chromatin packaging) were classified as CMA3-positive, while those with yellowish-green staining (representing normal chromatin packaging) were classified as CMA3-negative.

7. Testicular histology study

Testicular histology was performed to determine whether vitamin E could mitigate age-related abnormalities in testicular structure. To this end, testicular sections of 5- μm thickness, spaced at 50- μm intervals, were prepared and stained with hematoxylin and eosin [22]. Five sections from each of the four different groups were examined for histological analysis. The objectives of the stereological analysis included Sertoli cells, spermatogonia, primary spermatocytes, spermatids, and the diameter of seminiferous tubules, as well as the thickness of the germinal epithelium layer. These analyses were conducted using an optical microscope (Olympus) at $\times 400$ magnification (Figure 3).

The stereological study was conducted using Image J software (<http://rsb.info.nih.gov/nihimage/>). Tubular and luminal diameters were measured by determining the cross-sectional area of the tubule or lumen, followed by calculating the radius of an equivalent circle. An equivalent circle is defined as one having an area that matches the measured value. This approach is more precise than measuring two diameters of an oval-shaped lumen (Figure 4).

In the same sections, the thickness of the spermatogenic epithelium (SET) was measured. This measurement was taken as the distance between the basement membrane and the tubular lumen,

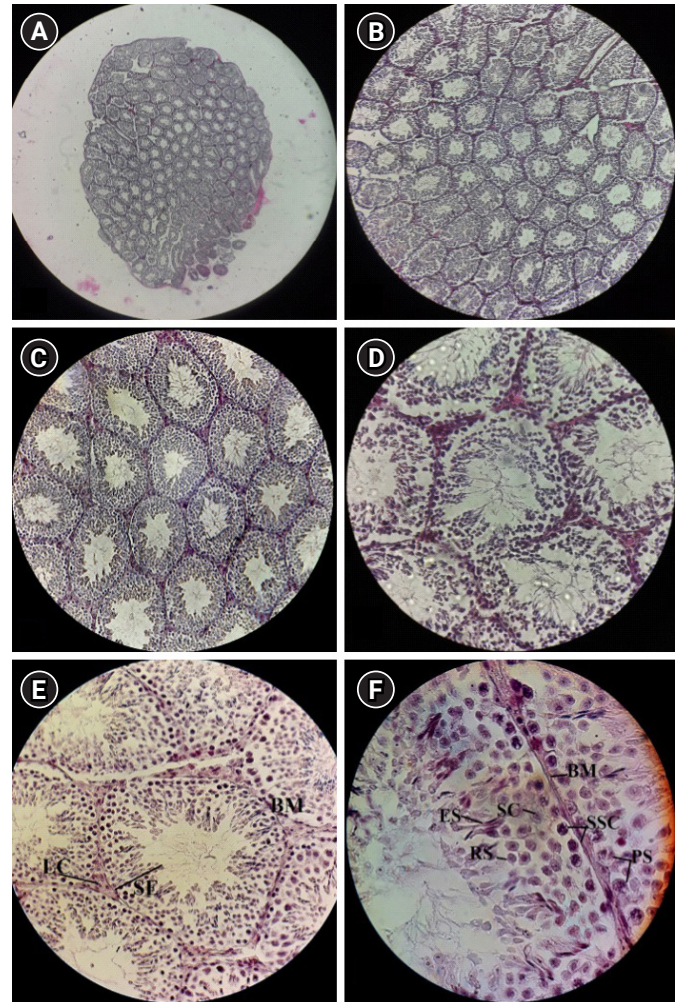


Figure 3. A cross-section of the seminiferous tubules from a normal mouse testis, showing a well-organized and intact epithelium. The seminiferous tubules have a well-defined lumen, and the germ cells are attached to the basement membrane: (A) $\times 4$ magnification (B) $\times 10$ magnification, (C) seminiferous tubules at $\times 20$ magnification. (D) The lumen contains cytoplasm, round spermatids, and elongated spermatids, (E) seminiferous tubules, basal membrane (BM), Leydig cells (LC), and seminiferous epithelium (SE) at $\times 40$ magnification. (F) Spermatogonial stem cell (SSC), primary spermatocyte (PS), Sertoli cells (SC), round spermatid (RS), elongated spermatid (ES).

with an average of four measurements recorded at four quadrants of the tubule (90° , 180° , 270° , and 360°).

Testicular histological changes in the germinal epithelium caused by aging include thickening of the basement membrane, increased tunica propria thickness, decreased tubular diameter, fibrosis, sclerosis, thinning of the SET, and eventual obliteration of the tubules [23]. In this study, we evaluated the seminiferous tubules' contours, the general appearance of the epithelial layer, the structure of the basement membrane, and the condition of Sertoli and Leydig cells during

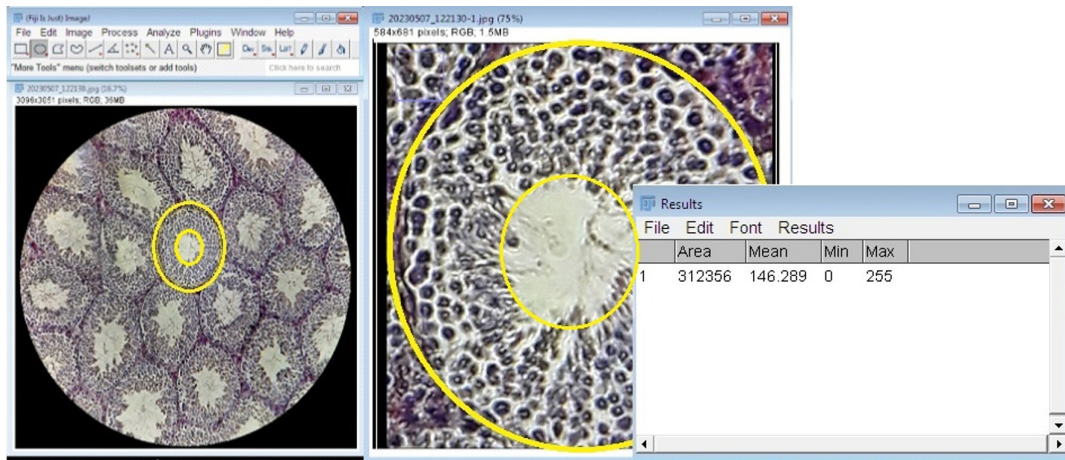


Figure 4. The tubular and luminal diameters were measured using the ImageJ software.

microscopic examination.

8. Tissue antioxidant enzyme activity measurement

To assess the antioxidant capacity of testis tissue, we measured the levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx). For this purpose, the testicular tissue was finely minced and homogenized following the protocol provided by the kit (Zelbio). The samples were homogenized in ice-cold PBS (pH 4.7) using a homogenizer. The resulting suspension was then centrifuged at 6,000 rpm for 20 minutes at 4 °C. After centrifugation, the supernatant was carefully collected and stored in sterile microtubes at -20 °C. Subsequently, the activities of the antioxidant enzymes SOD and GPx, as well as the concentration of MDA, were determined in the testis tissue of the various experimental groups using a microplate reader.

9. Molecular analysis

After the treatment period of 4 weeks, the testicular tissue was assessed for the expression levels of the *Gfra1*, *Id4*, and *Plzf* genes using real-time polymerase chain reaction. Total RNA extraction and cDNA synthesis from the tissue samples were performed with the Pars Tous kit, following the manufacturer's recommended protocol (Mashhad). Five microliters of the synthesized cDNA were combined with SYBR Green master mix and the respective forward and reverse primers for the target genes. Gene expression was compared between groups after normalization to β -actin, which served as the housekeeping gene. Primers were designed with the aid of Primer3 software (<http://bioinfo.ut.ee/primer3/>).

10. Statistical analysis

SPSS ver. 20 (IBM) was utilized for the analysis of the collected

data. The data were presented as mean \pm standard deviation (n=7) for parametric variables, and as median (interquartile range) for non-parametric variables. Group comparisons were conducted using one-way analysis of variance (ANOVA) when the normality assumption was met, which included sperm parameters, DNA fragmentation index (DFI), tissue antioxidant enzyme activities, and histological characterization. However, the Kruskal-Wallis test was employed in instances where the assumption of homogeneity of variance was not met, specifically for epithelium height and luminal diameter measurements. The paired-sample *t*-test was applied to compare sperm parameters before and after freezing. The chi-square test was used for categorical data. The statistical significance threshold was set at $p \leq 0.05$.

11. Availability of data and materials

Derived data supporting the findings of this study are available from the corresponding author on request.

Results

1. Sperm parameters

The results of sperm parameters in the control and treatment groups (vitamin E) are presented in Table 1. All treatment groups exhibited a higher sperm count than the control group ($p < 0.05$), with the highest count observed at the highest dose of vitamin E (400 mg/kg) compared to the lowest dose (100 mg/kg). Additionally, vitamin E significantly increased progressive motility at doses of 200 and 400 mg/kg (38.85% \pm 18.77% and 37.14% \pm 7.10%, respectively) compared to the control group (17.57% \pm 5.06%). Improved sperm morphology was noted at the 400 mg/kg dose compared to the control group ($p < 0.05$). However, no significant differences in sperm mor-

Table 1. Mouse epididymal sperm parameters before freezing

Groups	Sperm count	Progressive motility (A+B)	Non-progressive (C)	Immotile sperm	Morphology
Control	15.00 ± 4.04	17.57 ± 5.06	54.42 ± 12.98	33.71 ± 11.07	41.42 ± 9.25
Vitamin E 100 mg/kg	21.71 ± 20.00	27.85 ± 12.48	40.00 ± 6.21	32.14 ± 35.00	50.42 ± 9.77
Vitamin E 200 mg/kg	25.50 ± 5.01	38.85 ± 18.77	32.42 ± 13.56	37.71 ± 10.60	55.28 ± 12.72
Vitamin E 400 mg/kg	27.42 ± 2.50	37.14 ± 7.10	36.28 ± 9.56	28.00 ± 4.96	57.71 ± 7.69
<i>p</i> -value ^{a)}	0.00	0.01	0.008	0.51	0.028

Values are presented as mean ± standard deviation.

^{a)}One-way analysis of variance and the *post hoc* Tukey test showed a significantly higher sperm count in all treatment groups than in the control group, and in the 400 mg/kg group than in the 100 mg/kg group. There was also higher progressive motility and lower non-progressive sperm in the 200 and 400 mg/kg groups than in the control group. Better morphology was recorded in the 400 mg/kg group than in the control group.

Table 2. Mouse epididymal sperm parameters after freezing

Groups	Sperm count	Progressive motility (A+B)	Non-progressive (C)	Immotile sperm	Morphology
Control	11.57 ± 4.31	8.57 ± 1.61	37.71 ± 13.86	48.28 ± 12.72	25.85 ± 7.17
Paired-sample <i>t</i> -test	0.176	0.005	0.013	0.011	0.000
Vitamin E 100 mg/kg	21.14 ± 5.14	13.28 ± 8.01	45.57 ± 9.03	41.14 ± 13.30	35.14 ± 7.73
Paired-sample <i>t</i> -test	0.779	0.035	0.126	0.145	0.024
Vitamin E 200 mg/kg	23.28 ± 7.43	24.42 ± 9.89	48.28 ± 11.32	27.28 ± 9.96	50.28 ± 6.96
Paired-sample <i>t</i> -test	0.455	0.128	0.115	0.899	0.485
Vitamin E 400 mg/kg	25.57 ± 7.52	27.71 ± 6.96	43.14 ± 3.62	29.14 ± 8.11	47.71 ± 13.86
Paired-sample <i>t</i> -test	0.467	0.115	0.089	0.702	0.182
<i>p</i> -value ^{a)}	0.002	0.00	0.27	0.005	0.001

Values are presented as mean ± standard deviation.

^{a)}One-way analysis of variance and the *post hoc* Tukey test showed a significantly higher sperm count after freezing in all treatment groups than in the control group. There was a higher percentage of progressive motile sperm in the 200 and 400 mg/kg groups than in the control group. A higher percentage of immotile sperm was seen in the control group than in the 200 and 400 mg/kg treatment groups. A better morphological score was recorded in the 200 and 400 mg/kg groups than in the control group, and the result was significant in the 200 mg/kg group compared to the 100 mg/kg group.

phology were observed between the 100 and 200 mg/kg doses when compared to the control group ($p > 0.05$).

The effects of oral supplementation with vitamin E on sperm parameters after cryopreservation are shown in Table 2. Sperm samples cryopreserved in the treatment groups exhibited a higher sperm count than those in the control group ($p < 0.05$). Additionally, a significant difference was observed between the higher and lower doses of vitamin E (27.42 ± 2.50 vs. 21.71 ± 20.00, respectively). Apart from sperm count, no significant differences in sperm parameters were noted between the 100 mg/kg treatment group and the control group after freezing ($p > 0.05$). The 200 and 400 mg/kg treatment groups demonstrated a higher number of progressively motile sperm than the control group ($p < 0.05$).

As shown in Table 2, the addition of vitamin E following sperm freezing resulted in a significantly higher percentage of normal sperm morphology at the two higher doses of the vitamin (200 and 400 mg/kg) than in the control group ($p < 0.05$). Furthermore, the 200 mg/kg dose showed a greater protective effect than the 100 mg/kg dose ($p < 0.05$).

The results for the paired-samples *t*-test are presented in Table 2. There was a significant reduction in progressive motility and morphology scores in both the control and the 100 mg/kg treatment groups ($p < 0.05$). No significant differences were observed in the sperm parameters between the 200 and 400 mg/kg groups.

2. Sperm DNA integrity

To determine the impact of vitamin E treatment on sperm DNA integrity during cryopreservation, the total DFI of sperm was evaluated in all groups, both before and after the cryopreservation process. The results indicated no significant difference in mean DFI levels before freezing between the control and treatment groups ($p < 0.05$). However, post-freezing results were significant, with higher DFI levels observed in the control group compared to those treated with 200 and 400 mg/kg of vitamin E ($p > 0.05$) (Table 3).

3. Sperm protamine deficiency

The chromatin integrity results (CMA3+) indicated an equivalent level of protamine deficiency prior to freezing. However, post-freez-

ing measurements revealed a significant difference. Notably, lower protamine deficiency was observed in the groups receiving the two higher doses of vitamin E compared to the control group ($p < 0.05$). The paired-sample *t*-test revealed a reduction in the normal intact chromatin structure following vitrification across all groups. Conversely, significant chromatin damage was observed after freezing in the control groups, which exhibited a higher mean DFI and more positive CMA3 reactions (Table 3).

The characteristics of the seminiferous tubules, the composition of different cells, and the common types of histological abnormalities are presented in Table 3. Notably, there was a significantly higher count of spermatogonial cells in the groups receiving the two higher doses of vitamin E compared to both the lower dose (100 mg) and the control groups. However, this increase was not significant for spermatocyte cells across the three treatment groups ($p > 0.05$). Since the spermatid cell count did not follow a normal distribution, the Kruskal-Wallis test was employed. The results indicated a significantly higher number of spermatids in the groups treated with the high doses (200 and 400 mg/kg) compared to the lower dose and control groups (Mann-Whitney test, $p < 0.05$).

Table 4 presents the results of two common structural abnormalities associated with aging: vacuolization and epithelial detachment. An irregular contour, characterized by the presence of immature ger-

minal epithelial cells in the lumen, was observed in one case within the control group. Both vacuolization and detachment were more prevalent in the control group, although the difference was not statistically significant ($p > 0.05$). Only one instance of irregular contour was noted in the control group (Figure 5). The Pearson chi-square test, supplemented by the Fisher exact test when appropriate, revealed no significant differences in the occurrence of these abnormalities between the control and the three treatment groups ($p > 0.05$).

As shown in Figure 6, there were no significant differences in tubular diameter (one-way ANOVA, *post hoc* Tukey), epithelial height, or luminal diameter (Kruskal-Wallis) among the study groups.

4. Biochemical parameter evaluation

The results for the measurement of tissue antioxidant enzyme activities—specifically, SOD and GPx—and MDA content are presented in Figure 7. As indicated, at the end of the 4th week of treatment, there was a significant increase in GPx activity in the 400 mg/kg treatment group compared to both the control group and the 100 mg/kg treatment group. Additionally, SOD activity was significantly higher in the groups treated with 200 and 400 mg/kg than in the control group.

At the end of treatment, the levels of MDA in the testicular tissues

Table 3. DNA fragmentation and protamine deficiency, before and after freezing

Variable	DNA fragmentation		Protamine deficiency	
	Before freezing	After freezing	Before freezing	After freezing
Control	46.57 ± 13.01	63.00 ± 11.48	35.00 ± 5.53	48.00 ± 8.18
Vitamin E 100 mg/kg	39.57 ± 8.18	49.57 ± 12.60	28.00 ± 7.65	38.14 ± 7.98
Vitamin E 200 mg/kg	35.00 ± 8.56	40.57 ± 11.31	29.42 ± 11.47	35.57 ± 8.30
Vitamin E 400 mg/kg	35.14 ± 8.39	42.28 ± 8.09	25.42 ± 9.88	33.14 ± 8.02
<i>p</i> -value ^{a)}	0.11	0.003	0.25	0.012

Values are presented as mean ± standard deviation.

^{a)}One-way analysis of variance did not show a significantly higher chromatin abnormality before freezing, but the results were significant after freezing in the control group than in the 200 and 400 mg/kg groups. A higher level of chromatin abnormalities was seen after freezing/thawing in all study groups (paired-sample *t*-test).

Table 4. Characteristics of seminiferous tubules, composition of different cells, and common types of histological deformities

Groups	Spermatogonia	Spermatocytes	Spermatids	Vacuoles (%)	Detachment (%)	Irregular contours
Control	14.71 ± 5.73	26.85 ± 8.59	135.71 ± 10.99	57.1	28.6	14.3
Vitamin E 100 mg/kg	11.85 ± 6.41	40.42 ± 13.89	128.42 ± 11.75	28.6	14.3	0
Vitamin E 200 mg/kg	27.00 ± 9.05	47.57 ± 8.97	190.42 ± 34.40	28.6	0	0
Vitamin E 400 mg/kg	30.57 ± 8.34	45.42 ± 14.31	173.00 ± 31.54	0	14.3	0
<i>p</i> -value ^{a)}	0.000	0.013	0.000	0.166	0.883	1.000

Values are presented as mean ± standard deviation.

^{a)}One-way analysis of variance and the *post hoc* Tukey test showed significantly higher spermatogonial cell counts in the 200 and 400 mg/kg groups than in the control and 100 mg/kg groups. The results were also significantly higher for spermatocytes in the two higher-dose groups than in the control group.

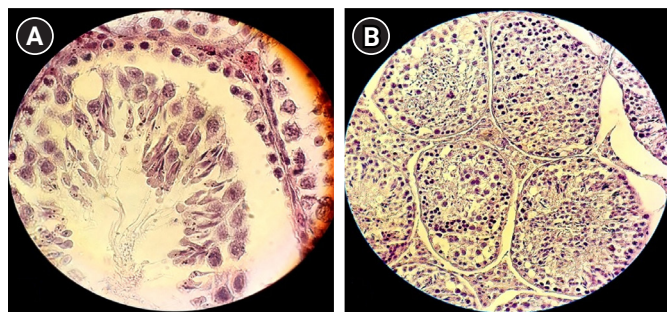


Figure 5. Three common seminiferous abnormalities with age: (A) epithelium detachment and vacuoles ($\times 100$), (B) seminiferous epithelium disorganized, irregular contours, with germ cells located in abnormal positions. It is difficult to identify the different cell types of cells, in the epithelium. The seminiferous epithelium resembles a dense mass ($\times 40$).

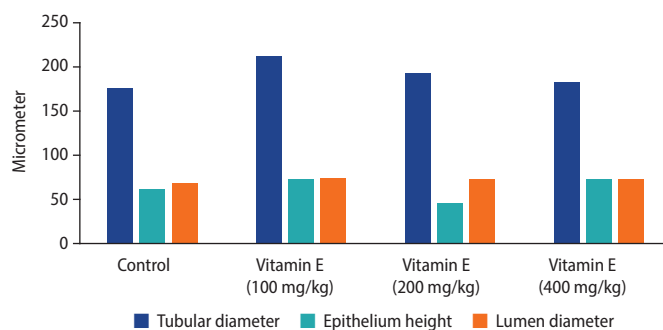


Figure 6. Comparison of seminiferous tubule diameter, epithelium thickness, and lumen diameter in different groups. One-way analysis of variance and the *post hoc* Tukey test showed no significant differences in the tubular diameter; likewise, the epithelium height and luminal diameter did not show significant differences in the study groups (Kruskal-Wallis test, $p > 0.05$).

of mice were significantly lower in the groups receiving higher doses of 200 and 400 mg/kg (16.9 ± 3.21 and 18.15 ± 4.13 nmol/mg protein, respectively) than in the control group (26.79 ± 8.09 nmol/mg protein) ($p < 0.05$).

5. Gene expression

After treatment, a higher level of expression of *Id4* and *Gfra1* was observed in the 200 mg/kg, and the results were significant in the 200 and 400 mg/kg compared to the control group ($p < 0.05$). The highest level of *Plzf* was seen in the 400 mg/kg treatment group, and the difference was significant only compared with the control group ($p < 0.05$) (Figure 8).

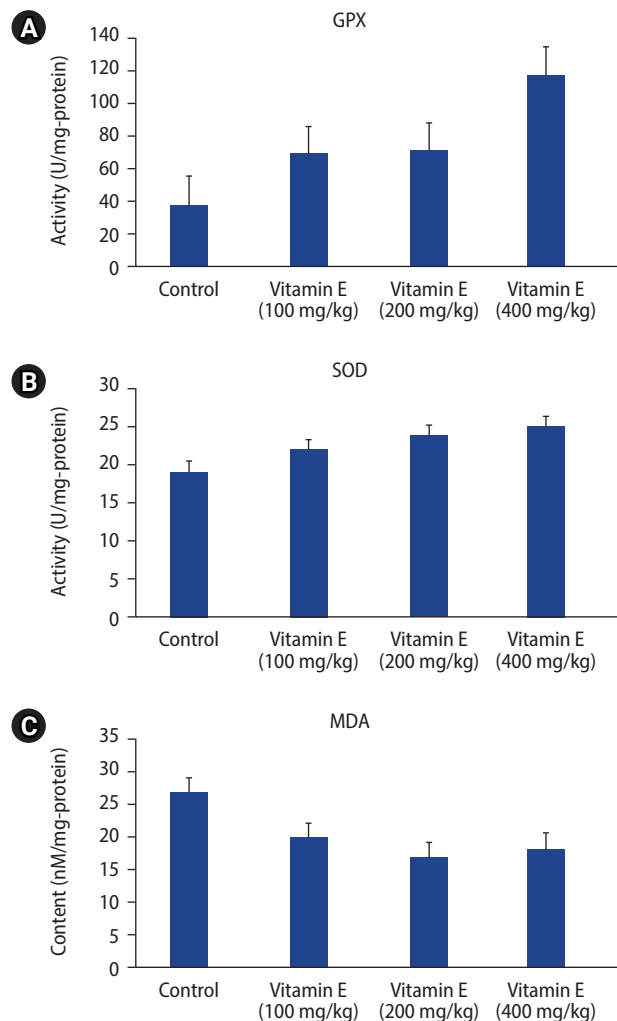


Figure 7. The results for tissue antioxidant capacity measurement (A: glutathione peroxidase [GPX]; B: superoxide dismutase [SOD] activities, and C: malonaldehyde [MDA] content). There was a significantly higher GPX activity in the 400 mg/kg compared to the control and 100 mg/kg groups. Also, a higher SOD activity was shown in the two groups of 200 and 400 mg/kg compared to the control group. Lower MDA content was seen in the two higher dosages than the control group (one-way analysis of variance, *post hoc*, Tukey $p < 0.05$).

Discussion

In the present study, we found that dietary vitamin E supplementation in older mice was significantly associated with improved sperm cryo-resistance. This improvement was evident in sperm parameters, chromatin health, testicular tissue antioxidant activities, and gene expression. The gene expression pathways suggested a tendency to preserve the SSC pool in the treated male mice. However, the data are insufficient to confirm changes in male sperm parameters with age due to a lack of longitudinal studies [1]. Some studies have reported decreases in semen volume [2,3], sperm motility

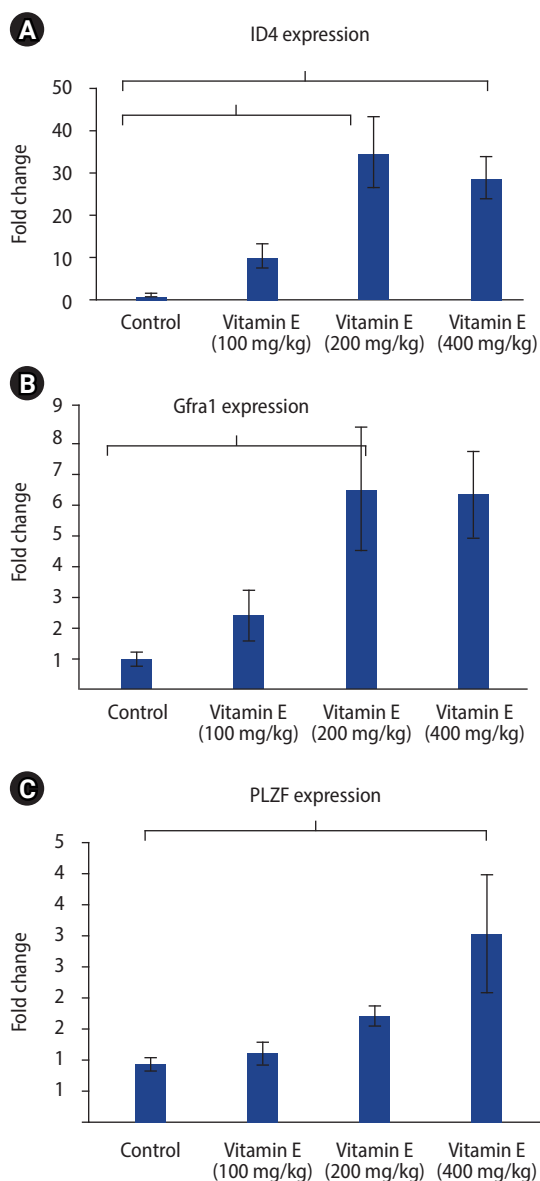


Figure 8. Gene expression pattern in the control and treatment groups. (A) Inhibitor of DNA binding protein-4 (ID4), (B) GDNF family receptor alpha-1 (Gfra1), and (C) promyelocytic leukemia zinc finger (PLZF) protein expressions.

[2,4,7], sperm count [4,7,24], viability [8,9], morphology [2,3], and histopathological changes [7]. Our results indicated that among the various sperm parameters that change with age, vitamin E supplementation, acting as an antioxidant, improved sperm count, motility, and morphology in the treatment groups compared to the control group. However, a higher dosage (400 mg/kg) was necessary to improve morphology. In another study by Liu et al. [14], results demonstrated better progressive motility and higher total antioxidant capacity in both seminal plasma and serum with 400 mg/kg of vitamin E compared to 200 mg/kg. Additionally, a study by Yarube et al. [13]

found that vitamin E (400 mg/kg), when combined with vitamin C, could mitigate the toxic effects of sodium nitrate. It was also observed that co-administration of 200 mg/kg vitamin E with mancozeb reduced the harmful effects of mancozeb on sperm characteristics and testicular parameters [12].

One hypothesis that explains the age-related decline in tissue and organ function is the oxidative stress theory, which involves the accumulation of ROS [5]. Spermatozoa are especially vulnerable to the harmful effects of ROS due to their cell membranes being rich in unsaturated fatty acids, which are susceptible to oxidation (lipid peroxidation), and their limited cytoplasmic enzymes available for neutralizing ROS [25]. Consequently, the potential benefits of antioxidant therapy for male infertility have been extensively studied [26-28].

The results also demonstrated the efficacy of oral vitamin E supplementation in sperm cryopreservation. Supplementation with higher doses of vitamin E (100, 200, and 400 mg/kg) resulted in increased sperm counts, a greater number of progressively motile sperm, and improved sperm morphology, particularly at the 200 and 400 mg/kg dosages. Therefore, the improvement of sperm parameters observed with *in vivo* vitamin E supplementation led to the expectation of improved outcomes following sperm cryopreservation.

Similar to our findings, researchers have demonstrated that live sperm count, sperm motility, and mitochondrial activity decreased following freezing, whereas lipid peroxidation experienced an increase. They also found that the addition of vitamin E to the sperm cryopreservation mixture led to a decrease in lipid peroxidation and an enhancement in fertility rates [29].

An evaluation of sperm parameters before and after freezing, using the paired-sample *t*-test, also confirmed the role of antioxidant supplementation in enhancing sperm resistance, particularly in terms of chromatin health. Higher levels of protamine deficiency and DNA fragmentation were observed after the freeze-thaw process in the control group. The destructive effect of cryopreservation on sperm chromatin has been previously demonstrated [10,30].

It was observed that spermatogonial cell counts were significantly higher in the groups receiving the two higher doses of vitamin E compared to the control groups. The lower dose of vitamin E (100 mg) did not prove to be as effective as the higher doses. Additionally, an increase in spermatid cells was noted in groups treated with higher doses of vitamin E. One histomorphological alteration observed in the testes is the disturbance of spermatogonia genesis [1]. SSCs in adult men undergo self-renewal and differentiation to maintain spermatogenesis throughout adulthood [11]. Studies have shown an age-related decrease in the number of germ cells and Sertoli cells [7]. Several underlying mechanisms have been proposed, with oxidative stress being a prominent hypothesis described in the literature [31-33]. Other studies have attempted to reverse age-related

testicular changes in mice using antioxidants such as catalase [31], or vitamin E (hazelnut) [7].

Of all the histopathologic changes observed, three abnormalities were insignificantly more prevalent in the control group: vacuoles, epithelial detachments, and irregular contours. Similarly, another study identified age-related histologic alterations in the testis, including vacuole formation in Sertoli cells at 12 months [22], epithelium detachment [34], and immature germinal epithelial cells in the lumen [7]. Consistent with our findings, an additional study demonstrated histopathological improvements following hazelnut supplementation.

The results showed that there were no significant differences in tubular diameter, epithelium height, or luminal diameter between the control and treatment groups. Similarly, our findings revealed no change in the mean seminiferous tubule diameter following vitamin E treatment in aged mice [35]. Two possible hypotheses might explain this: firstly, the tubular and luminal diameters, as well as epithelium height, may not undergo changes by the age of 12 months in mice. The minimal effect of vitamin E on testicular histology and morphology could be due to the fact that the imbalance between ROS production and antioxidant systems is not substantial enough to cause significant histological alterations by 12 months. Secondly, vitamin E may not be effective in reversing age-related alterations. Some studies have concluded that the basic structure of mouse seminiferous tubules does not change with age up to 18 months [35]. This finding contrasts with another study that reported a reduction in seminiferous tubule volume with age [36].

SOD is likely the first antioxidant agent that helps maintain the balance between the antioxidant and oxidant systems [11]. Within the endogenous antioxidant system, the enzymatic components include SOD, glutathione, and GPx, while the non-enzymatic components consist of vitamin E, uric acid, and bilirubin [35,37]. However, one study indicated that the antioxidant capacity of the testis decreases with age [35]. In contrast, other studies have concluded that the age-related decline in male reproductive function is associated with an imbalance between ROS production and antioxidant systems [38]. Our results demonstrated increased SOD and GPx activities at the two higher dosages of vitamin E. This was also accompanied by a significant decrease in MDA equivalent values, which are indicative of oxidation levels. These findings align with another study that reported an improvement in the seminal plasma oxidant-antioxidant balance following hazelnut diet supplementation—a rich source of vitamin E and phenolic compounds—while simultaneously observing a reduction in MDA levels [7].

Several studies have investigated gene expression patterns in the reproductive system of aged male mice [39-41], with results that are sometimes contradictory. This inconsistency may stem from the se-

lection of different ages of mice. The impact of vitamin E on gonad gene expression has been the subject of a limited number of studies. One such study examined the effect of vitamin E supplementation on the *CatSper 1* and *CatSper 2* genes, finding that gene expression was upregulated following treatment with vitamin E [39]. Additionally, long-term vitamin E treatment was found to increase the expression of genes related to oxidative stress in aged rats [42].

Plzf, which can function as both a transcriptional activator and a transcriptional repressor, regulates various signaling pathways, as well as differentiation and growth-regulatory processes [43]. This factor is expressed in numerous tissues, including the testes, and plays a critical role in maintaining the quiescence of SSCs [44]. It has been shown that *Plzf* naturally becomes upregulated with age, leading to the hypermaintenance of spermatogonial cells. However, it is unclear whether overexpression of *Plzf* negatively impacts the stemness of spermatogonial cells [45]. Inactivation of *Plzf* has been demonstrated to result in age-dependent germ cell loss, with testicular degeneration becoming inevitable due to reduced self-renewal capabilities of SSCs [46]. In this study, a higher level of *Plzf* gene expression correlates with an increased number of spermatogonial cells in the vitamin E-treated group. Additionally, elevated expression levels of *Id4* and *Gfra1* were observed in the treatment groups. Previous research has indicated that the genetic loss of *Gfra1* significantly reduced the self-renewal capacity of SSCs, and *in vitro*, a decrease in *Gfra1* transcript levels adversely affected SSC proliferation. *Gfra1*-positive single spermatogonia are considered the “actual stem cells” of the testes, essential for normal spermatogenesis [47]. It has been demonstrated that the male germline stem cell pool is contained within the *Id4*⁺ spermatogonial population. Several cell populations are identified as assigning stem cell function (SSCs) using lineage tracing or transplantation approaches, such as *Gfra1*, paired box 7 (*Pax7*), *Bmi1*, or neurogenin 3 (*Neurog3*), but the *Id4* population is the only type that acts throughout both tracing or transplantation [48]. Previous studies have demonstrated the significant role of vitamin E in promoting the proliferation of spermatogonial cells and primary spermatocytes, as well as in the production of spermatids and spermatozoa [49,50].

In summary, we found that although structural destruction was not prominent until 12 months, sperm parameters were reduced. Antioxidant supplementation for the improvement of aged male reproduction not only aids current fertility but also has implications for future applications, such as sperm freezing or fertility preservation. Furthermore, the effect of vitamin E on gene expression levels presents a promising approach for the regenerative efforts of aged testes in the future.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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