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Assessment of Vitamin A versus Vitamin E effect on Motility and DNA Integrity of Human Cryopreserved Sperms

Samir A. Al-Anbari[1] *MSc*, **Mustafa M. Ibraheem**[2] *PhD*, **Thaer M. Farhan**[2] *FIBMS*

[1]High Health Institute, Medical City Directorate, Ministry of Health, Baghdad, Iraq, [2]Dept. of Human Anatomy, College of Medicine, University of Al-Mustansiriyah, Baghdad, Iraq, [3]Dept. of Human Anatomy, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Background It is well known that cryopreservation procedure may result in negative impact on spermatozoa function throughout relative overproduction of reactive oxygen species (ROS). However, ROS overproduction can be corrected by antioxidants.

Objective To find out the effect of vitamin A on cryopreserved spermatozoa throughout measuring their motility rate and DNA fragmentation and compare it with vitamin E effect.

Methods Forty seminal fluid specimens were individually collected from forty healthy, non-drug and non-alcohol consumers, normozoospermic males in Um Al-Baneen Center for Infertility Management and In Vitro Fertilization in Baghdad. To facilitate comparing between used vitamins and experimenting each one alone, those specimens were distributed into 4 groups of 10 specimens each. Following deriving their own controls without any vitamin treatment, specimens of these groups, in general, were treated with two concentrations of vitamin A (20 µg/dl and 30 µg/dl), and two concentrations of vitamin E (10 µmol/l and 20 µmol/l). Then, each specimen was incubated for 1 hour before being cryopreserved in liquid nitrogen for 14 days. Motility percentage and DNA fragmentation were assessed following cryopreservation and thawing of spermatozoa.

Results Results revealed that there were significant statistically differences in post-thawing motility and DNA fragmentation means between specimens treated with vitamin A and their relevant control; between specimens treated with vitamin E and their relevant control; and between specimens treated with vitamin E and those treated with vitamin A among all groups of the study.

Conclusion These results lead to the conclusion that both of vitamin A and E play an important role in improving and protecting sperm motility and DNA integrity following cryopreservation, and vitamin E is more effective than vitamin A.

Keywords Seminal fluid analysis, vitamin A, vitamin E, reactive oxygen species, sperm cryopreservation, sperm DNA fragmentation, sperm motility assessment

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List of abbreviations: ART = Assisted reproductive technology, dUTP = deoxyuridine triphosphate, ROS = Reactive oxygen species, SCSA = Sperm Chromatin Structure Assay, SCD = Sperm chromatin dispersion; SFA = Seminal fluid analysis TdT= Terminal deoxynucleotidyl transferase, TUNEL = Terminal deoxynucleotidyl transferase – Mediated deoxyuridine triphosphate Nick-End Labeling

Introduction

In spite of the advances in the field of assisted reproductive technology (ART) nowadays [1-7], infertility still constitutes a big challenge in medicine since it affects about 48 million couples and about 186 million individuals live with infertility all over the world

[8]. It has many variable classifications [8-13], and has multiple etiological factors [14-17]. One of the most common etiological factors of male infertility is relative overproduction of reactive oxygen species (ROS) in relation to antioxidant activity since it accounts for 30-80% of male infertility cases [18-20]. ROS overproduction leads to increased oxidative stress, which can adversely affect various types of molecules within spermatozoa such as nucleic acids, lipids, and proteins resulting in DNA fragmentation, which subsequently lead to their apoptosis, lipid peroxidation in sperm plasma membrane, which accounts for motility defect represented by asthenozoospermia, and denaturation of the enzymes that finally lead to abnormal spermatogenesis represented by teratozoospermia [19,21,22]. Cryopreservation is considered as one of the extrinsic physical stress factors that account for overproduction of ROS [23-25]. In the same time, cryopreservation constitutes an important procedure used in ART since it keeps family chance to acquire children when the male partner requires chemotherapy, radiotherapy, or radical surgery; or when the male partner is azoospermic and exposed to high risk of testicular damage following testicular sperm extraction [5,25]. This urges researchers to experiment adding antioxidants into seminal fluid and follow up its effect on sperm parameters specifically following cryopreservation. It has been found that vitamin E and D improve and protect motility and DNA integrity of sperms following cryopreservation [26-28].

Regarding to vitamin A, it has been found that sperms of human ejaculate retain high levels of retinyl palmitate and stearate besides retaining retinyl hydrolase enzyme [29,30]. It has been shown that seminal plasma level of vitamin A in normozoospermic men constitutes about 19.1 (±2.5) µg/dl, while level of vitamin A in seminal plasma of oligozoospermic men constitutes about 10.4 (±1.8) µg/dl [31]. These collectively indicates to the role of vitamin A in sperm function. However, little studies were done to investigate the role of vitamin A in improving sperm parameters. These studies, in general, depended the oral route to supplement this vitamin to seminal fluid to improve sperm parameters and did not experiment the possibility of being added directly into it that facilitates its usage in ART. Besides what is preceded, these studies did not assess the role of vitamin A in improving sperm parameters following cryopreservation [32-34].

Methods of DNA fragmentation assessment can be categorized into: direct assessment methods such as TUNEL test and COMET test, and indirect assessment methods such as Sperm Chromatin Structure Assay (SCSA) and Sperm Chromatin Dispersion (SCD) [35,36]. However, TUNEL test is widely used because of its high valuable indication to DNA fragmentation and, in turn, to male infertility [36]. The word 'TUNEL' represents the abbreviation of Terminal deoxynucleotidyl transferase (TdT) – Mediated deoxyuridine triphosphate (dUTP) Nick-End Labeling [35]. Its principle is based on activation of endonuclease enzymes between nucleosomes when cells begin to undergo apoptosis. This results in genomic DNA cleavage which, in turn, exposes the cleaved site (3'-OH) to react with a fluorescein deoxyuridine triphosphate as a result of the catalytic effect of TdT. Then, cells are examined by fluorescence microscopy [37].

Undergoing seminal fluid analysis (SFA), motility can be assessed using light microscope with magnification power of (X200) or (X400) (38). The four – grade system is considered as the most convenient system to classify sperms according to velocity of their motility. It consists of four grades of sperm movement. Grade (A) represents rapidly progressive spermatozoa, which move actively either linearly or in a large circle, covering a distance, from the starting point to the end point, of at least 25 µm (equal to ½ tail length) in one second. Grade (B) represents slowly progressive spermatozoa, which move actively either linearly or in a large circle, covering a distance, from the starting point to the end

point, of 5 to <25 µm (or at least one head length to less than ½ tail length) in one second. Grade (C) represents non-progressive spermatozoa that include all other patterns of active tail movements that are associated with an absence of progression; i.e., swimming in small circles, such as the flagellar force displacing the head less than 5 µm (one head length), from the starting point to the end point, in one second. While, grade (D) represents immotile spermatozoa, which reveal no active tail movement. Summation of grade (A), (B), and (C) represents the motility rate [38-41].

The of objective of this study was trying to find a clinical basis to overcome stressful effect of cryopreservation and, thus, improve ART in cases requiring sperm cryopreservation, objectives of this study aim to find out the effect of vitamin A on cryopreserved spermatozoa throughout measuring their motility rate and DNA fragmentation and compare it with vitamin E effect.

Methods

This study was intended to be a prospective experimental analytic study. Its plan was determined to collect a number of specimens of seminal fluid from a number of randomly selected participants, in which each participant gave only one specimen following an abstinence of 2-5 days. Forty participants in this study were randomly selected from Um Al-Baneen Center for Infertility Management and In Vitro Fertilization, Al-Imamein Al-Kadhimein Medical City in Baghdad. Inclusion criteria of participants in this study included being less than 50 years old, healthy, non-smokers, non-drug consumers, and non-alcohol consumers, and having normozoospermic seminal fluid criteria with volume more than 2 ml for each specimen.

All seminal fluid specimens were subjected to same conditions. Following assessing their first SFA, all specimens then were incubated at room temperature (22-27˚C) preparing for experimental groups derivation. The collected specimens were subdivided according to their volumes into two categories. Using vitamin A (Central Drug House (P) Ltd.; India) and vitamin E (HIMedia Laboratories Pvt. Ltd.; India), each category was managed according to possibility to derive the main experimental groups of this study from it.

Representing the first category, the collected specimens with volume ranging between >2 ml and 2.5 ml were subjected to procedures by which vitamin A treated group (Group I) and vitamin E treated group (Group II) were derived. These procedures included dissolving a quantity of vitamin A or vitamin E in about 500 µl seminal plasma derived from centrifugation of seminal fluid to form vitamin A solution with a concentration approximately equal to 0.1 µg/µl or vitamin E solution with a concentration approximately equal to 4 µg/µl respectively. Then, 1 µl and 1.5 µl of vitamin A solution from each specimen were well-mixed with two partitions of about 500 µl of seminal fluid each preparing for two specimens with 20 µg/dl and 30 µg/dl concentrations of vitamin A respectively; while, 0.5 µl and 1 µl of vitamin E solution from each specimen were well-mixed with two partitions of about 500 µl seminal fluid each preparing for two specimens with 10 µmol/l and 20 µmol/l concentrations of vitamin E respectively.

Representing the second category, the collected specimens with volume >2.5 ml were subjected to procedures by which low concentrations vitamin A and E treated group (Group III) and high concentrations vitamin A and E treated group (Group IV) were derived. These procedures were similar to those deriving Group (I) and group (II) except that preparation of vitamin solution in each of group III and IV required derivation of two partitions of seminal plasma of about 500 µl each, which were derived from each specimen in the group in order to dissolve each vitamin individually.

Following vitamin supplemented subgroup derivation, each experimental group would be consisted of three subgroups: one of them representing the control, which was not supplemented with vitamins and the other two were supplemented with vitamins.

Table (1) briefly demonstrates vitamin treated subgroups preparation, while table (2) demonstrates the finally formed experimental groups.

Table 1. Preparation of vitamin supplemented subgroups

Procedure			Vitamin type	
			Vitamin A	**Vitamin E**
Concentration of vitamin used in the beginning of preparation			100 µg/µl	400 µg/µl (approximately equivalent to 1000 µmol/ml)
Preparation of vitamin solutions	Volume of solute (vitamin) taken		0.4 – 0.5 µl	4 – 5 µl
	Volume of solvent (seminal plasma) taken		399.6 – 499.5 µl	396 – 495 µl
	Concentration of vitamin solution formed		0.1 µg/µl (approximately)	4 µg/µl (approximately equivalent to 10 µmol/ml)
Preparation of vitamin treated subgroups	Lower vitamin concentration subgroups	Recommended volume of vitamin solution	1 µl	0.5 µl
		Recommended volume of seminal fluid	499 µl	499.5 µl
		Final vitamin concentration	0.0002 µg/µl (approximately equal to 20 µg/dl)	0.004 µg/µl (approximately equivalent to 10 µmol/l)
	Higher vitamin concentration subgroups	Recommended volume of vitamin solution	1.5 µl	1 µl
		Recommended volume of seminal fluid	498.5 µl	499 µl
		Final vitamin concentration	0.0003 µg/µl (approximately equal to 30 µg/dl)	0.008 µg/µl (approximately equivalent to 20 µmol/l)

Table 2. Finally formed experimental groups

Experimental group	**Group I: vitamin A treated group**	**Group II: vitamin E treated group**	**Group III: Low concentrations vitamin A and vitamin E treated group**	**Group IV: High concentrations vitamin A and vitamin E treated group**
Its constituting subgroups	Control (500 µl)	Control (500 µl)	Control (500 µl)	Control (500 µl)
	Low vitamin A concentration (500 µl)	Low vitamin E concentration (500 µl)	Low vitamin A concentration (500 µl)	High vitamin A concentration (500 µl)
	High vitamin A concentration (500 µl)	High vitamin E concentration (500 µl)	Low vitamin E concentration (500 µl)	High vitamin E concentration (500 µl)

Following their derivation, these experimental groups were subjected to 1 hour incubation at room temperature (22-27°C) before being cryopreserved for 14 days. Following cryopreservation and thawing of sperms, assessment of sperm motility rate, and DNA fragmentation were done for all specimens in all experimental groups.

Motility was assessed by undergoing seminal fluid analysis, and examining spermatozoa by light microscope at (X400) magnification power. While DNA fragmentation was assessed by TUNEL test depending fluorescein isothiocyanate (FITC) stain as the main fluorescent stain, which stains fragmented DNA green, and 4,6-diamidino-2-phenylindole (DAPI) as the contrast stain [37].

Statistical analysis was done using GraphPad Prism 9.3.1 released on 2021. ANOVA and Multiple Comparisons Tuckey's tests were

applied to statistically evaluate results. Significant P value was specified to be <0.05 [28].

Results

Applying ANOVA test and Tukey's multiple comparisons test, statistical studies revealed the following according to each experimental group.

In group (I) as shown in tables (3) and (4), in addition to graphs (1) and (2), it has been shown that there are significant statistical differences in motility rate and DNA fragmentation means between vitamin A treated subgroups and their relevant controls. However, there is no significant statistical difference between lower and higher vitamin A treated subgroups (adjusted P value < 0.05).

This means that motility and DNA preservation is significantly more in vitamin A treated subgroups than in control; while, the difference in motility and DNA preservation between low and high vitamin A subgroups is of no significance in spite of being higher in high vitamin A subgroup.

Table 3. Multiple comparison of post-thawing motility of vitamin A group

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control (post) vs. low vit. A (post)	-13.5	-20.94 to -6.062	Yes	***	0.0003
Control (post) vs. high vit. A (post)	-18.5	-25.94 to -11.06	Yes	****	<0.0001
Low vit. A (post) vs. high vit. A (post)	-5	-12.44 to 2.438	No	ns	0.2362

(***) refers to P value <0.001; while, (****) refers to P value <0.0001

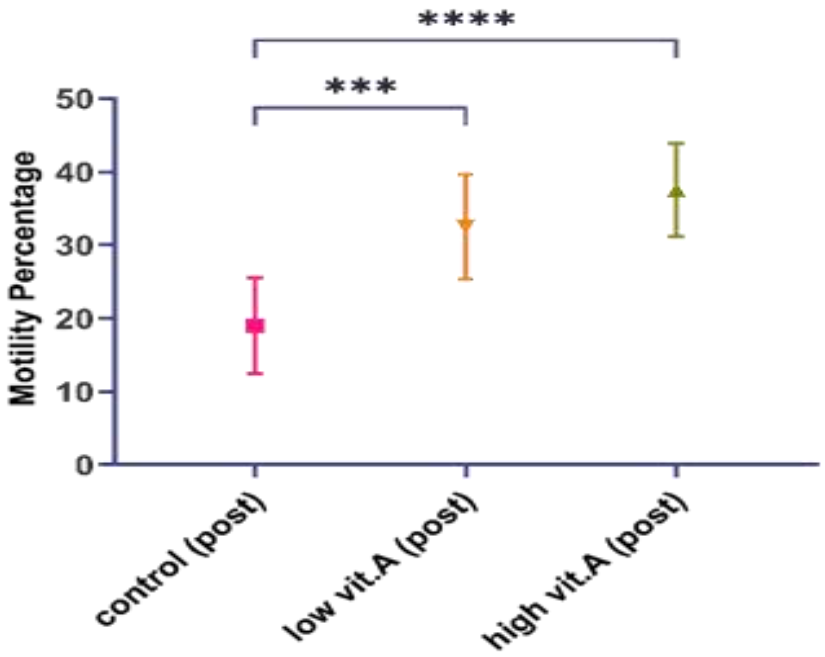

Figure 1. Post-thawing motility percentage assessment and statistical analysis of vitamin A experimental group. It reveals that there are significant differences among its subgroups. The symbol (***) refers to P value <0.001; while, (****) refers to P value <0.0001

Table 4. Multiple comparison test of post-thawing DNA fragmentation of vitamin A group

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control (post) vs. low vit. A (post)	16.6	1.783 to 31.42	Yes	*	0.0258
Control (post) vs. high vit. A (post)	15.4	0.5825 to 30.22	Yes	*	0.0404
Low vit. A (post) vs. high vit. A (post)	-1.2	-16.02 to 13.62	No	ns	0.978

(*) refers to P value <0.05

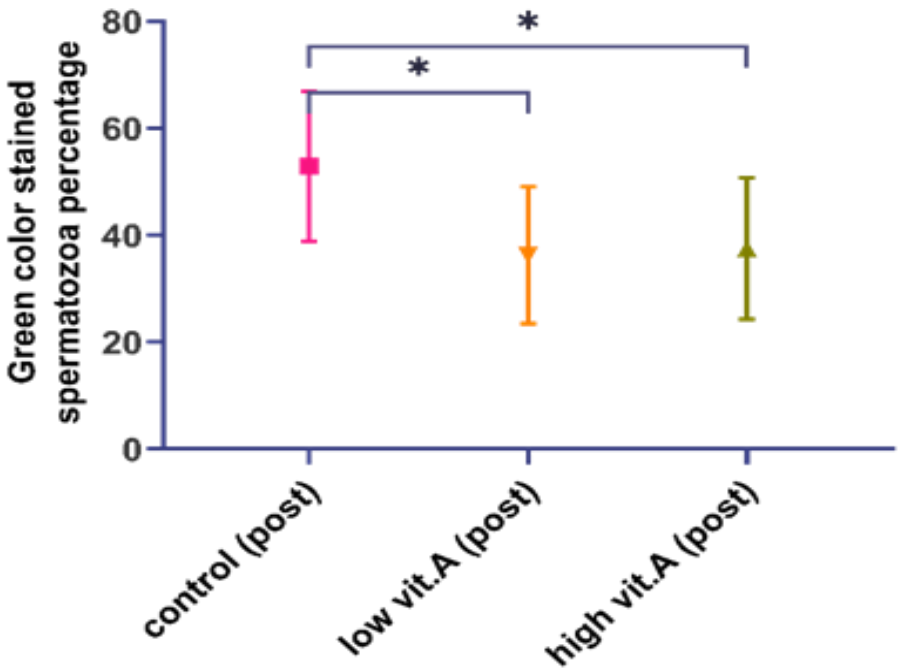

Figure 2. Post-thawing DNA fragmentation assessment and statistical analysis of vitamin A experimental group. It reveals that there are significant differences among its subgroups. The symbol (*) refers to P value <0.05

In group (II) as shown in table (5) and (6), in addition to graph (3) and (4), it has been shown that there are significant statistical differences in motility rate and DNA fragmentation means between vitamin E treated subgroups and their relevant controls (adjusted P value <0.05). However, there is no significant statistical difference between lower and higher vitamin E treated subgroups.

This means that motility and DNA preservation is significantly more in vitamin E treated subgroups than in control; while, the difference in motility and DNA preservation between low and high vitamin E subgroups is of no significance in spite of being higher in high vitamin E subgroup.

Table 5. Multiple comparison test of post-thawing vitamin E group motility

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control (post) vs. low vit. E (post)	-13.5	-20.13 to -6.871	Yes	****	<0.0001
Control (post) vs. high vit. E (post)	-15.5	-22.13 to -8.871	Yes	****	<0.0001
Low vit. E (post) vs. high vit. E (post)	-2	-8.629 to 4.629	No	ns	0.7374

(***) refers to P value <0.001; while, (****) refers to P value <0.0001

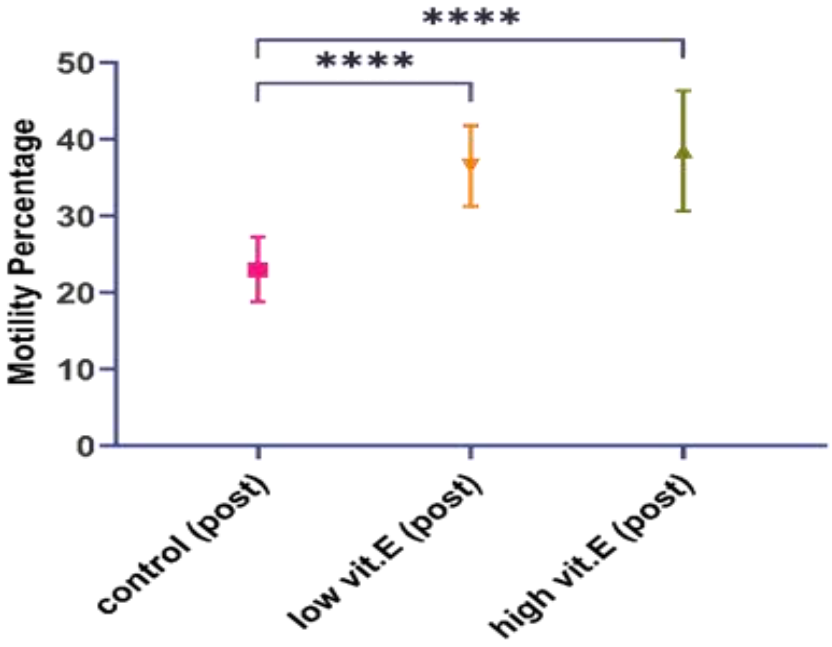

Figure 3. Post-thawing motility assessment and statistical analysis of vitamin E experimental group. It reveals that there are significant differences among its subgroups. The symbol (****) refers to P value <0.0001

Table 6. Multiple comparison test of post-thawing vitamin E group DNA fragmentation assessment

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control (post) vs. low vit. E (post)	22.5	13.25 to 31.75	Yes	****	<0.0001
Control (post) vs. high vit. E (post)	23	13.75 to 32.25	Yes	****	<0.0001
Low vit. E (post) vs. high vit. E (post)	0.5	-8.746 to 9.746	No	ns	0.9901

(***) refers to P value <0.001; while, (****) refers to P value <0.0001

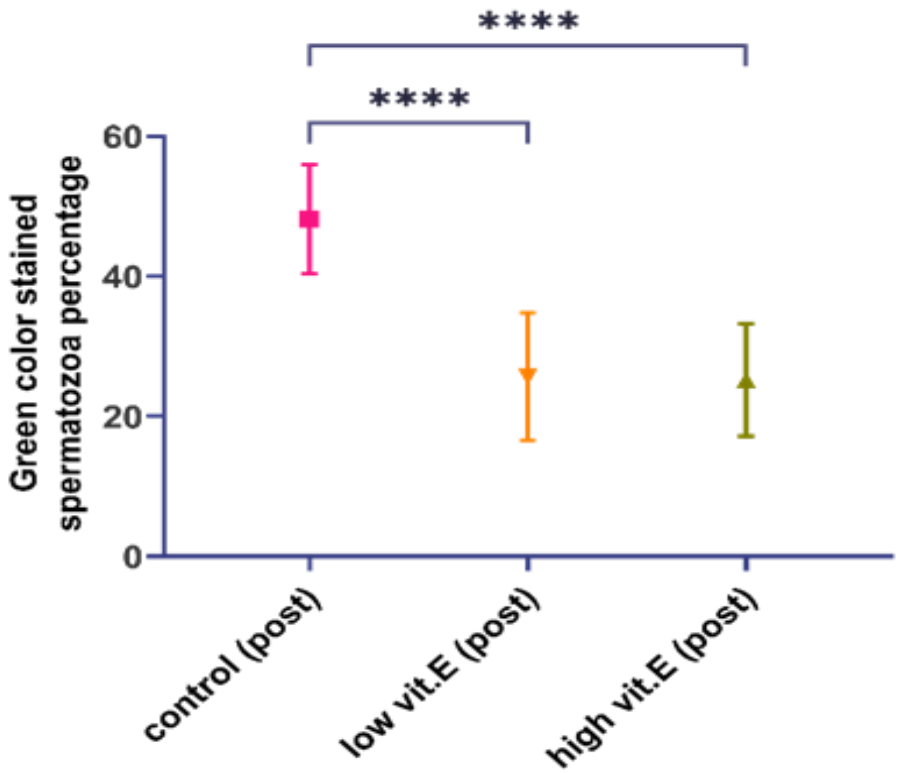

Figure 4. Post-thawing DNA fragmentation assessment and statistical analysis of vitamin E experimental group. It reveals that there are significant differences among its subgroups. The symbol (****) refers to P value <0.0001

In group (III) as shown in table (7) and (8), in addition to graph (5) and (6), it has been shown that there are significant statistical differences in motility rate and DNA fragmentation means between lower concentrations vitamin A and E treated subgroups and their relevant control, and between motility rate means of vitamin A treated subgroup and vitamin E treated subgroup (adjusted P value <0.05). However, there is no significant statistical difference between DNA fragmentation means of vitamin A treated subgroup and vitamin E treated subgroup.

This means that motility and DNA preservation is significantly more in low vit. E and low vit. A treated subgroups than in control, and significantly more in low vit. E treated subgroups than in low vitamin A treated subgroups.

Table 7. Multiple comparison of post-thawing low concentration vitamin A and E treated group motility assessment

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control (post) vs. low vit. A (post)	-7.8	-14.74 to -0.8635	Yes	*	0.0252
Control (post) vs. low vit. E (post)	-16.5	-23.44 to -9.564	Yes	****	<0.0001
Low vit. A (post) vs. low vit. E (post)	-8.7	-15.64 to -1.764	Yes	*	0.0118

(***) refers to P value <0.001; while, (****) refers to P value <0.0001

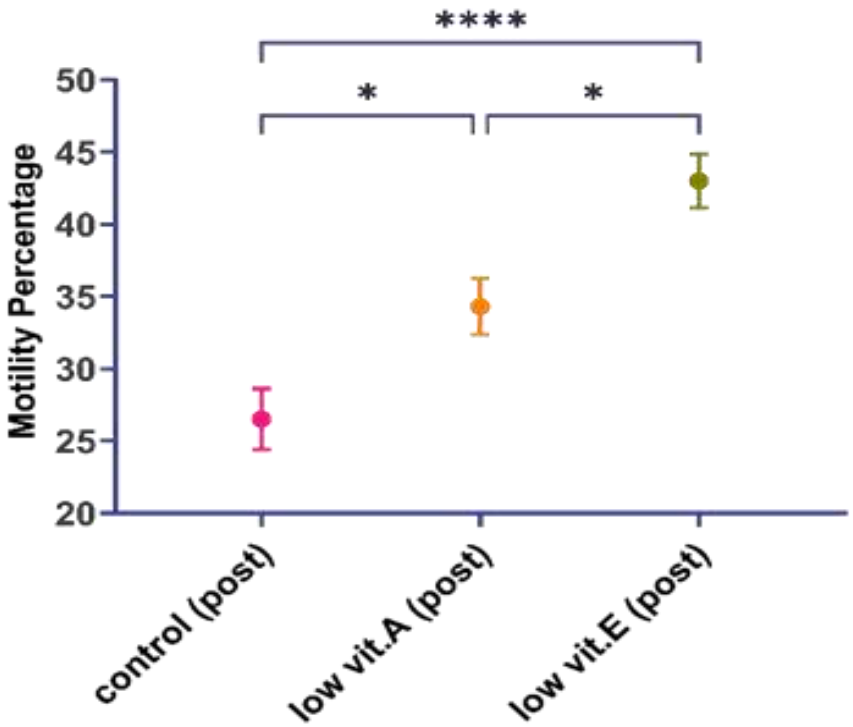

Figure 5. Post-thawing motility assessment and statistical analysis of low concentration vitamin A and E treated experimental group. It reveals that there are significant differences among its subgroups. The symbol (*) refers to P value <0.05; while, (****) refers to P value <0.0001

Table 8. Multiple comparison test of post-thawing low concentration vitamin A and E treated group DNA fragmentation

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control (post) vs. low vit. A (post)	12.1	1.382 to 22.82	Yes	*	0.0245
Control (post) vs. low vit. E (post)	22.7	11.98 to 33.42	Yes	****	<0.0001
Low vit. A (post) vs. low vit. E (post)	10.6	-0.1178 to 21.32	No	ns	0.053

The symbol (*) refers to P value <0.05; while, (****) refers to P value <0.0001

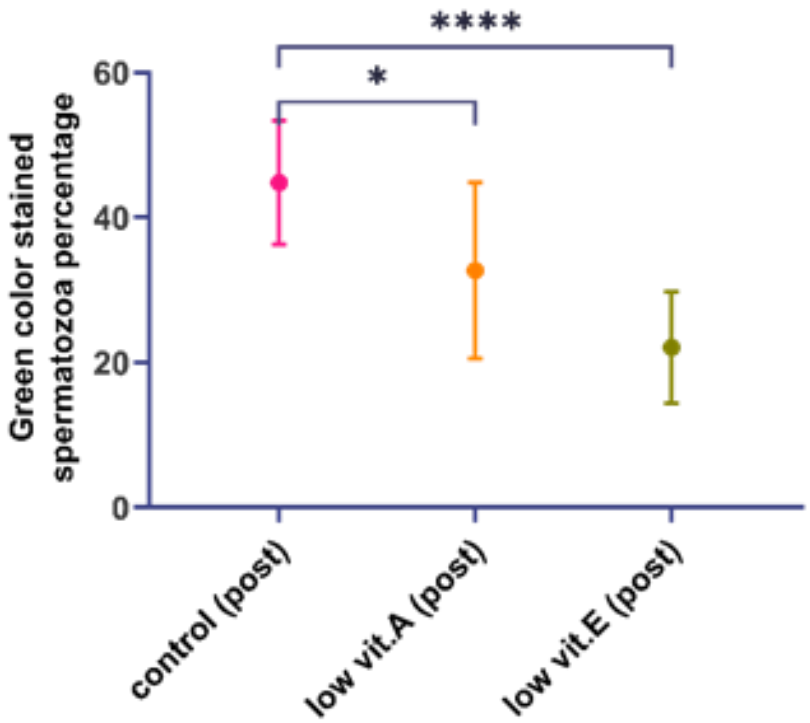

Figure 6. Post-thawing DNA fragmentation assessment and statistical analysis of low concentration vitamin A and E treated group. It reveals that there are significant differences among its subgroups. The symbol (*) refers to P value <0.05; while, (****) refers to P value <0.0001

In group (IV) as shown in tables (9) and (10) in addition to graphs (7) and (8), it has been shown that there are statistically significant differences in motility rate and DNA fragmentation means between higher concentrations vitamin E treated subgroup and their relevant control (adjusted P value < 0.05); and between motility means of vitamin A treated subgroup and its relevant control (adjusted P value <0.05). However, there is no statistically significant differences in motility rate and DNA fragmentation means between vitamin A treated subgroup and vitamin E treated subgroup, and between DNA fragmentation means of vitamin A treated subgroup and its relevant control.

This means that motility and DNA preservation is significantly more in high vitamin E and high

vitamin A treated subgroups than in control, and significantly more in low vitamin E treated subgroups than in high vit A treated subgroups.

Table 9. Multiple comparison test of post-thawing motility of high concentration vitamin A and E group

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control (post) vs. high vit. A (post)	-8.8	-16.09 to -1.513	Yes	*	0.0156
Control (post) vs. high vit. E (post)	-15.5	-22.79 to -8.213	Yes	****	<0.0001
High vit. A (post) vs. high vit. E (post)	-6.7	-13.99 to 0.5870	No	ns	0.076

The symbol (*) refers to P value <0.05; while, (****) refers to P value <0.0001

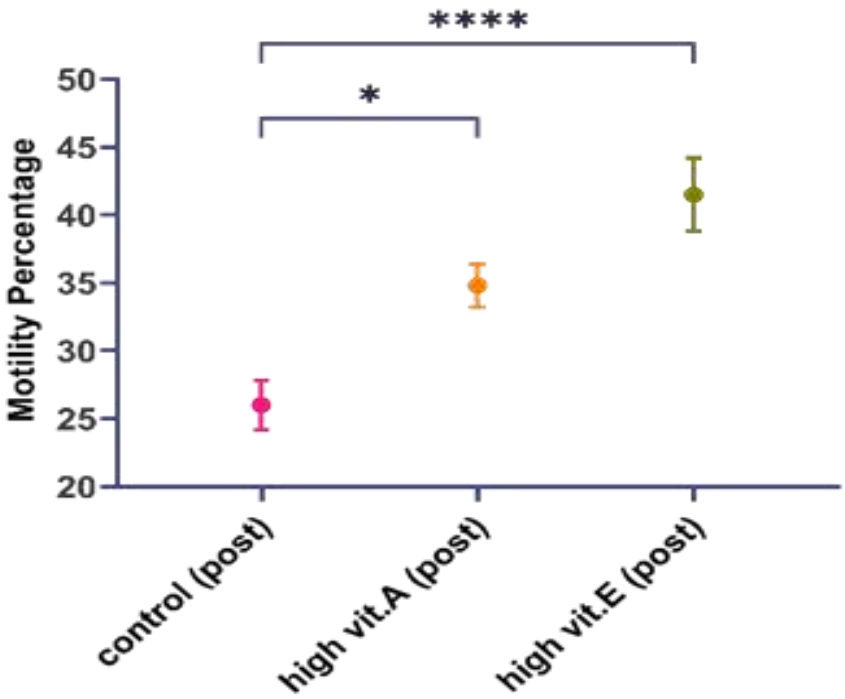

Figure 7. Post-thawing motility assessment and statistical analysis of high concentration vitamin A and E treated experimental group. It reveals that there are significant differences among its subgroups. The symbol (*) refers to P value <0.05; while, (****) refers to P value <0.0001

Table 10. Multiple comparison test of post thawing high concentration vitamin A and E treated group DNA fragmentation

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control (post) vs. high vit. A (post)	9.6	-4.494 to 23.69	No	ns	0.2277
Control (post) vs. high vit. E (post)	16.1	2.006 to 30.19	Yes	*	0.0227
High vit. A (post) vs. high vit. E (post)	6.5	-7.594 to 20.59	No	ns	0.4964

The symbol (*) refers to P value < 0.05

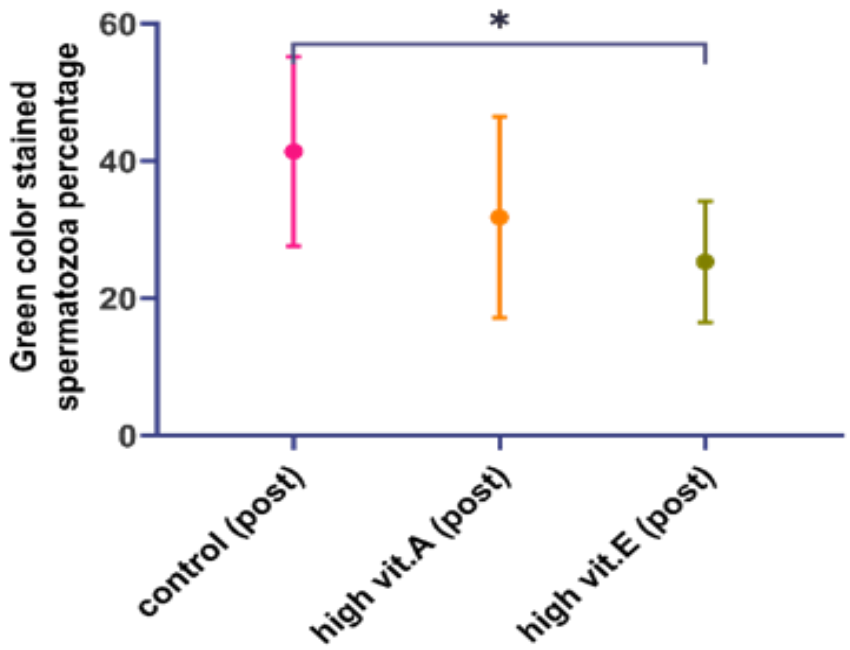

Figure 8. Post-thawing DNA fragmentation assessment and statistical analysis of high concentration vitamin A and E treated group. It reveals that there is significant difference among its subgroups. The symbol (*) refers to P value < 0.05

Figures (9), (10), (11), (12), (13), (14), and (15) reveal TUNEL test showing DNA fragmentation of spermatozoa of different subgroups.

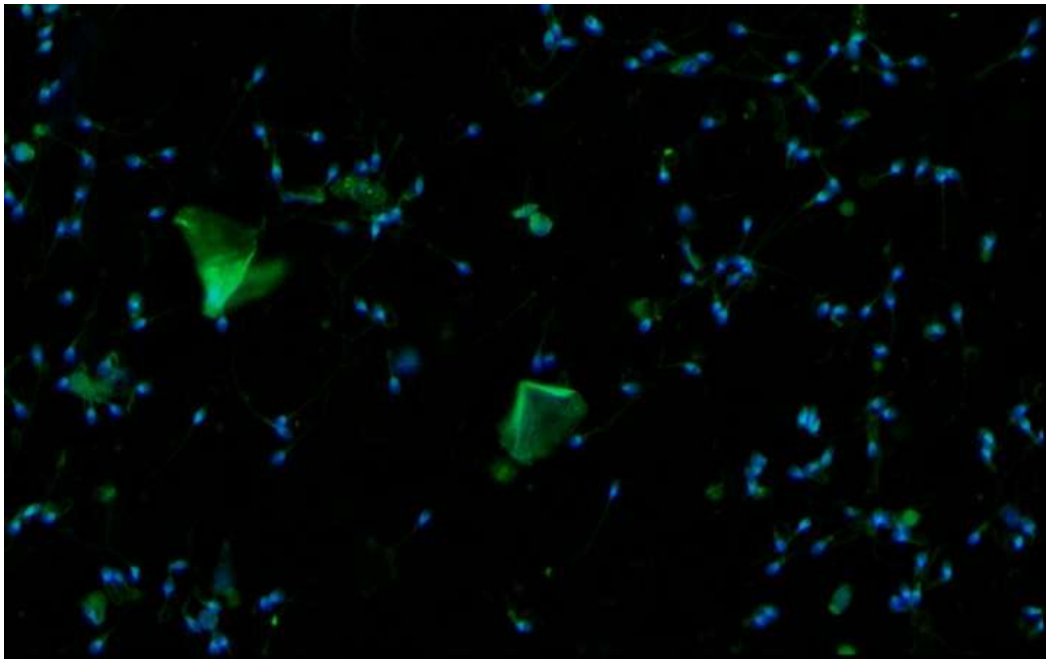

Figure 9. This figure represents demonstration for TUNEL test, in which the seminal fluid sample was stained with the main fluorescent DNA stain (FITC) and the counter stain (DAPI); and reveals the presence of spermatozoa, round cells and epithelial cells. Magnification power was (X200)

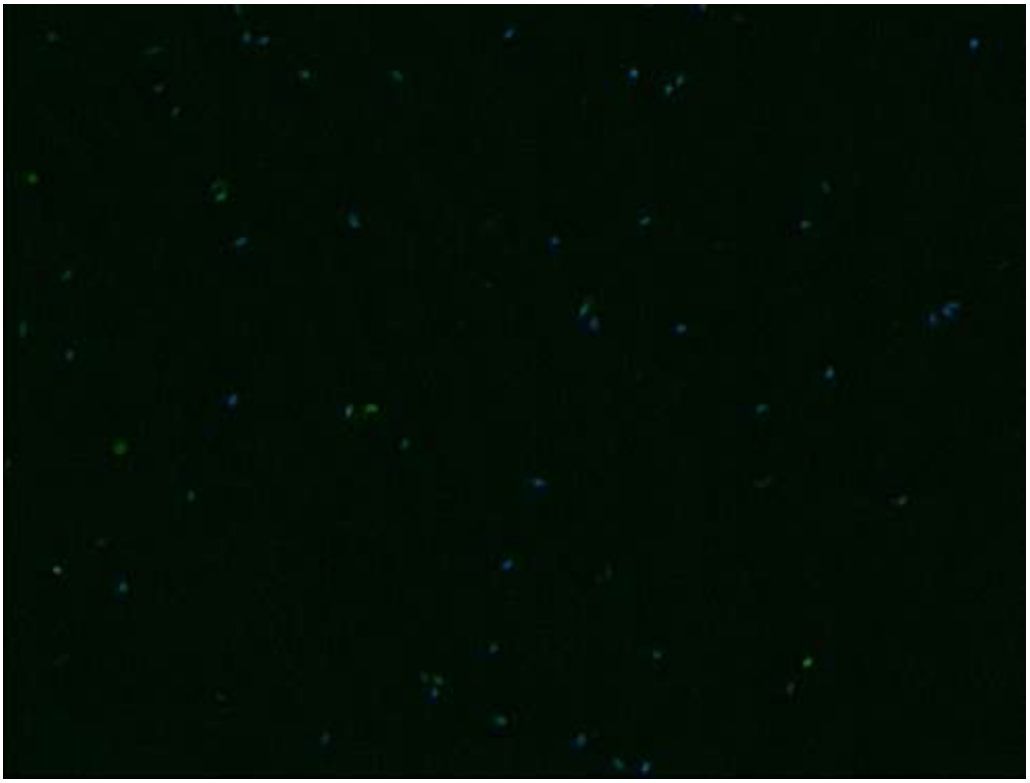

Figure 10. This figure demonstrates TUNEL test undergone for a post-thawing control sample. Magnification power was (X100)

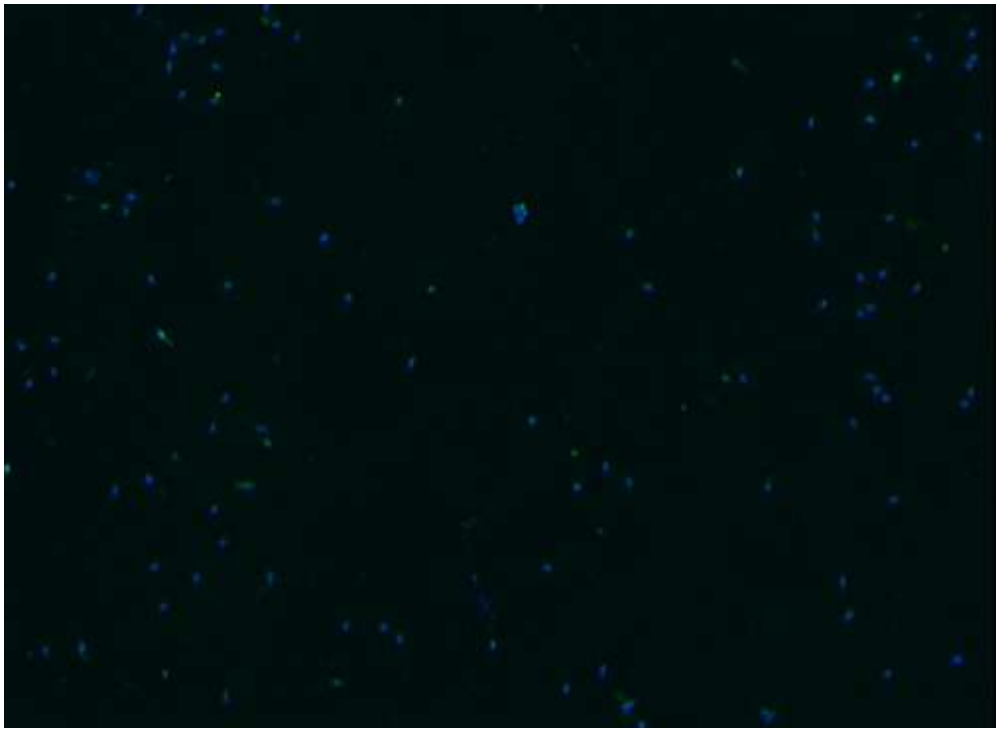

Figure 11. This figure demonstrates TUNEL test undergone for a sample from post-thawing low concentration vitamin A treated group. Magnification power was (X100)

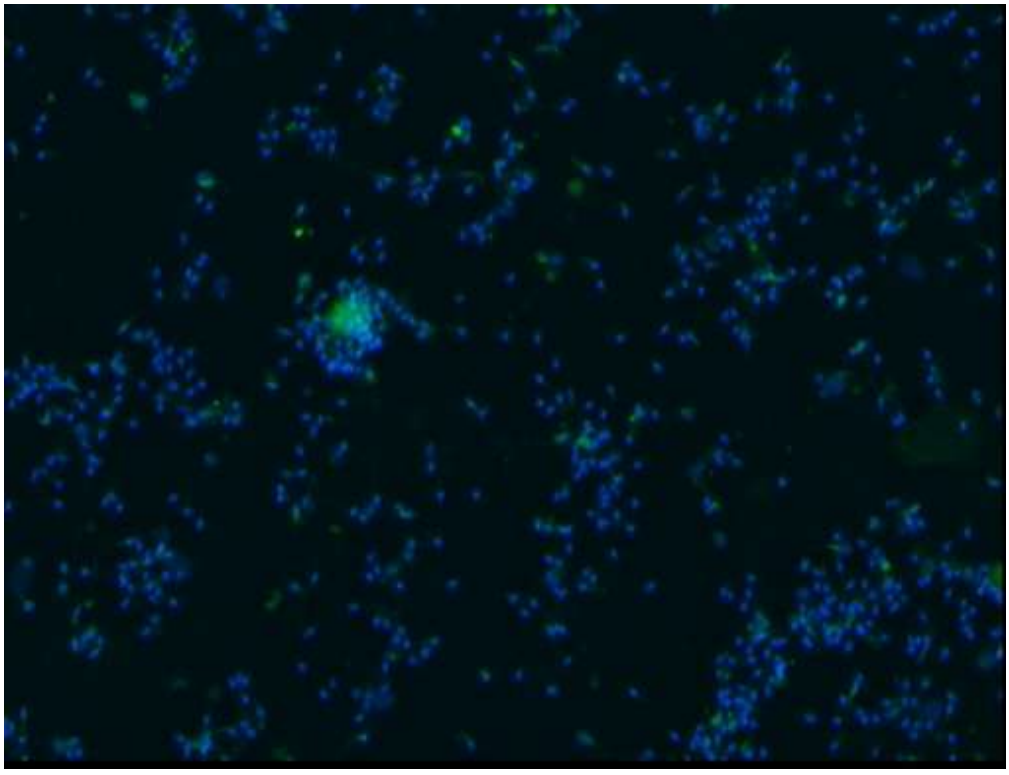

Figure 12. This figure demonstrates TUNEL test undergone for a sample from post-thawing low concentration vitamin E treated group. This sample reveals high number of spermatozoa. Magnification power was (X100)

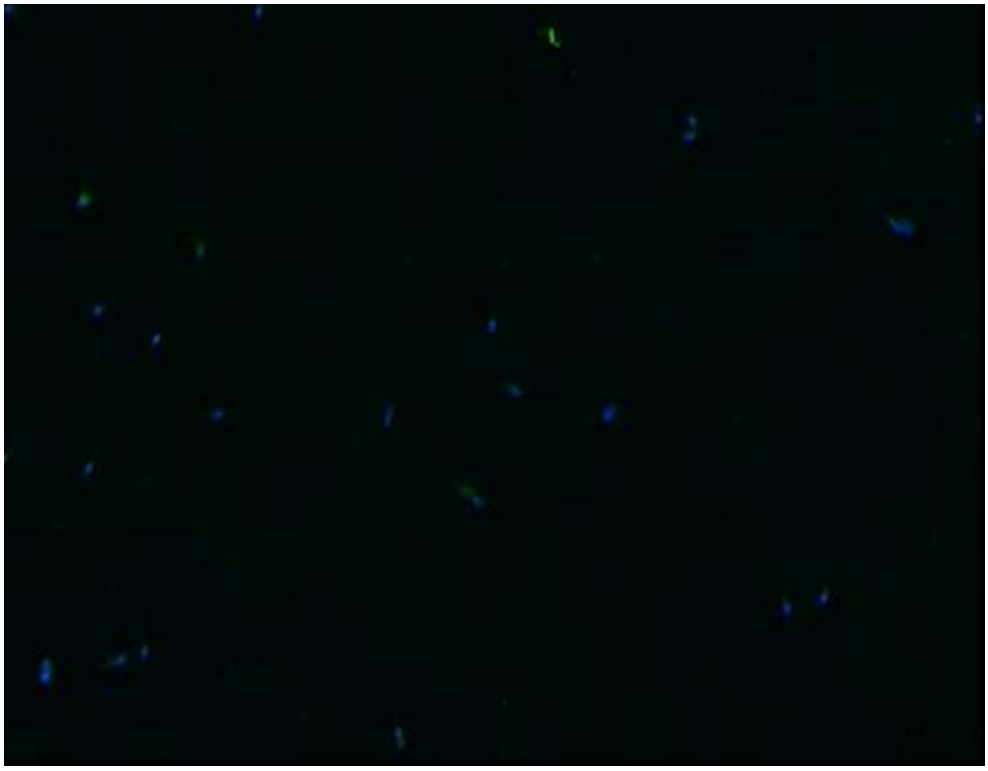

Figure 13. This figure demonstrates TUNEL test undergone for a sample from post-thawing high concentration vitamin A treated group. Magnification power was (X100)

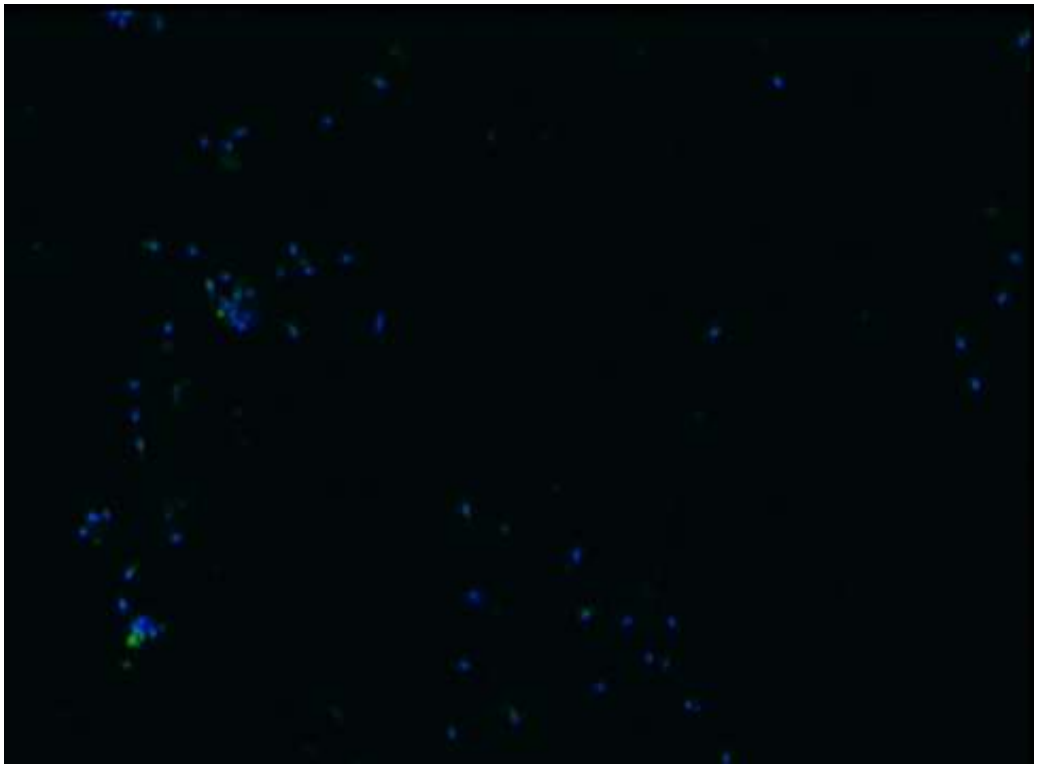

Figure 14. This figure demonstrates TUNEL test undergone for a sample from post-thawing high concentration vitamin E treated group. Magnification power was (X100)

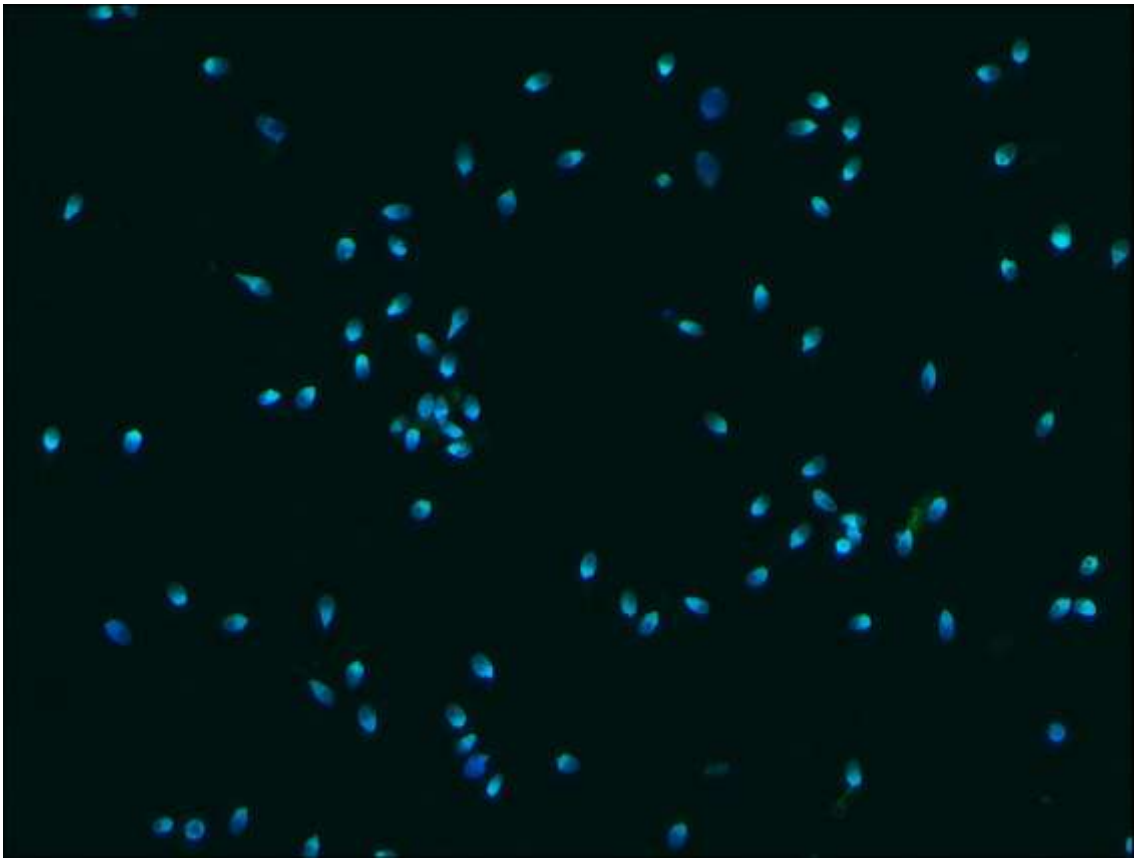

Figure 15. This figure demonstrates TUNEL test undergone for a sample of post-thawing lower concentration vitamin A group, in which spermatozoa were stained by FITC as the main fluorescent stain and DAPI as the counter stain. Magnification power was (X400)

Discussion

In spite of being more possible to be conducted on animal models, this study was not intended to be done on animal models but on human-being since animal model experiments depend on prediction to meet reality while studies done on human-being reflect the real effect of antioxidants on human sperm quality. Depending normozoospermic men in this study, this is in order to minimize the effect of any factor other than ROS to properly assess antioxidant role [28].

This study was designed to investigate the role of vitamin A in maintaining and improving sperm function and to compare it with the role of one of the previously experimented antioxidants such as vitamin E in maintenance of sperm activity, specifically following thawing of cryopreserved sperms since cryopreservation has a negative impact on spermatozoa.

Regarding to their concentrations used in this study, each of vitamin A and vitamin E was experienced with two concentrations, which are in close relation to their normal seminal plasma levels since the concentrations of 20 µg/dl and 10 µmol/L, which represented the lower concentrations used in this study, were approximately equal to the normal upper limits of vitamin A and vitamin E concentrations in seminal plasma of normozoospermic men according to Singer et al. (1982), and Omu et al. (1999) respectively [31,42]. This was intended in order to study the effect of normal upper limits of these vitamins and their slight increment on sperm motility, and DNA fragmentation before and after cryopreservation.

Comparing results of vitamin A treated subgroups with those of their relevant controls,

statistical differences in means of motility rate and DNA fragmentation between them, in general, reveal the effect of vitamin A in improving motility rate and reducing DNA fragmentation of sperms. This agrees with Singer et al. (1982), Pappas et al. (1993), Schreiber et al. (2012), and Ghyasvand et al. (2015), who collectively indicate the role of vitamin A in maintaining and improving sperm function [29-31,34]. However, these significant differences show variable extent among all groups taking their P values in consideration. This is convenient with Nallella et al. (2004), whose findings revealed that the process of cryopreservation could produce significant increment in inter-sample variability in post-thawing sperm parameters in comparison to pre-cryo parameters [43]. Besides that, small sample size could be an additional reason behind this increased variability since it is associated with increased impact of random error, which is in accordance with what is stated by Lee et al. (2015), Thiese et al. (2016) and Andrade et al. (2020) [44-46]. Added to what is preceded, the findings of Le et al. (2019), which revealed that DNA fragmentation index is not strongly correlated with other conventional semen parameters, may also bring for increased variability in statistical differences among groups [47].

Comparing results of vitamin E treated subgroups with those of their relevant controls, statistical differences in means of motility rate and DNA fragmentation between them, in general, reveal the effect of vitamin E in improving motility rate and reducing DNA fragmentation of sperms. This agrees with the findings of Maruoka et al. (2008), Zhu et al. (2011), and Howard et al. (2015), which revealed that vitamin E has a potent antioxidant that scavenges ROS, repairs and protects plasma membrane, and stabilizes it [26,48,49].

Comparing between vitamin A treated subgroups and vitamin E treated subgroups, statistical differences in means of motility rate and DNA fragmentation between them, in general, reveal that vitamin E has more effect in improving sperm function than that of vitamin A. This could be due to the potent dual action of vitamin E as both an ROS scavenger and plasma membrane stabilizer, which agrees with Maruoka et al (2008), and Howard et al. (2011) [48,49].

Comparing between lower concentration vitamin A and higher concentration vitamin A treated subgroups, statistical results revealed that there are no significant differences in motility rate and DNA fragmentation means between them. This may be due to individual baseline plasma level differences in their vitamin A contents as stated by singer et al. (1982) [31], which make variable responses toward addition of the high concentration of vitamin A that either result in improvement of spermatozoa function or suppression in their function due to the toxic effect of vitamin A. This, in turn, make the statistical differences between means of these parameters in the two subgroups small and not significant. This requires performing the study on larger size sample to reach the proper conclusion.

Comparing between lower concentration vitamin E and higher concentration vitamin E treated subgroups, statistical results reveal that there are no significant differences in motility rate and DNA fragmentation means between them. This may be due to individual baseline plasma level differences in their vitamin E contents as stated by Omu et al. (1999) [42], which make, upon addition of high concentration vitamin E, variable responses that either result in improvement of spermatozoa function or relative suppression in their function due to the toxic effect of vitamin E. This, in turn, make the statistical differences between means of the parameters in the two subgroups small and not significant. This requires performing the study on larger size sample to reach the proper conclusion.

Taking these results in consideration, it has been concluded that both concentrations of vitamin A and vitamin E have a vital role in improving and protecting spermatozoa motility and DNA integrity parameters following cryopreservation. However, differences between the low and high concentrations of each vitamin used in this study need to be further assessed; and it is recommended both in terms of safety and efficacy to use low levels

for both vitamin A and vitamin E since both vitamin A and E can be potentially toxic at high concentrations taking in consideration that there is no significant difference in motility and DNA fragmentation between low and high concentrations of these vitamins. In addition to that, vitamin E is shown to be more effective than vitamin A.

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Author contribution

The authors, altogether, conceived and planned the study. The experiment was done by Samir A. Al-Anbari under supervision of Dr. Ibraheem and Dr. Farhan.

Conflict of interest

There is no conflict of interest.

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Correspondence to Samir A. Al-Anbari
E-mail: samer_anbari@yahoo.com
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