Asian Journal of Healthy and Science
p-ISSN: 2980-4302
e-ISSN: 2980-4310
Vol. 5 No. 1 January, 2026

Alpha-Tocopherol Stimulates Motility, Capacitation and Survival of Human Sperm

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Abstract

Human sperm produce reactive oxygen species (ROS) during mitochondrial respiration in low amounts required for various signaling pathways. The production of physiological ROS in sperm cells can regulate important functional characteristics such as motility, capacitation, acrosome reactions, hyperactivation, and sperm-oocyte fusion. However, excess ROS can have detrimental effects. In previous studies, it has been reported that α-tocopherol can improve motility and protect sperm from the harmful effects of oxidative stress. However, the molecular mechanism of this effect is still unclear. This study aimed to analyze the effects of α-tocopherol on sperm lipid peroxidation, motility, capacitation, and cell survival. After incubation for 2 hours with various concentrations of α-tocopherol, sperm motility was checked using a computer-assisted sperm analyzer (CASA), ROS was measured with MDA levels, sperm membrane integrity was checked using a hypo-osmotic swelling test, while capacitation, cell survival, and apoptosis were checked using western immunoblotting. Results showed that α-tocopherol does not affect MDA levels in sperm cells. However, in other parameters, the addition of α-tocopherol improves sperm motility, membrane integrity, and tyrosine phosphorylation. α-Tocopherol also has a positive effect on sperm survival, as demonstrated by increased phosphorylation of Akt and decreased expression of caspase-3 in sperm cells. α-Tocopherol does not affect the lipid peroxidation of the sperm cell membrane, but it does improve membrane integrity and stimulate the motility, capacitation, and survival of human sperm cells.

Keywords: AKT; Alpha-tocopherol; capacity; motility; Squirting

INTRODUCTION

Infertility is a common clinical issue worldwide. Around 45-50% of infertility cases are caused by male factors.(Evans et al., 2021) According to the World Health Organization (WHO), approximately 15% of couples of reproductive age experience infertility, with male factor infertility contributing to about 186 million cases globally (Agarwal et al., 2021). The prevalence of male infertility has shown an increasing trend over recent decades, partly attributed to environmental factors, lifestyle changes, and oxidative stress. Factors contributing to male fertility decline such as oxidative stress can occur due to common pathways such as varicocele, urinary tract infections, and genitourinary inflammation.(Moretti et al., 2023)

Oxidative stress occurs when there is an imbalance between free radicals (pro-oxidants) and antioxidants in the body, with free radical levels exceeding those of antioxidants. Approximately 30-40% of infertile men have elevated levels of free radicals in their semen.(Sabeti PhDCandidate et al., 2016) One example of free radicals that contribute to male infertility is reactive oxygen species (ROS). ROS are highly reactive oxygen-derived free radicals.(Zou et al., 2017) ROS are generated through the reduction of oxygen by a single electron, resulting from metabolic processes and chemical reactions in the body.(Sarniak et al., 2016) Excessive ROS can lead to male infertility by damaging the sperm cell plasma membrane, which can result in reduced sperm motility.(Barik et al., 2019) This occurs because the sperm cell membrane contains high levels of polyunsaturated fatty acids (PUFAs), which can react with ROS to form lipid peroxidation.(Aitken, 2017a; Gualtieri et al., 2021)

Other studies have shown that increased ROS can lead to sperm cell apoptosis through the activation of caspase proteins.(Aitken et al., 2015) However, despite its harmful effects, a physiological level of ROS is necessary for sperm capacitation.(Barik et al., 2019) Capacitation

is a physiological and biochemical modification of sperm that occurs in the female reproductive tract before the acrosome reaction.(Ickowicz et al., 2012) Therefore maintaining a balanced level of ROS is crucial for the survival of sperm cells, as sperm are naturally more vulnerable to ROS than other body cells.(Gualtieri et al., 2021)

Given the rising global burden of male infertility and the established role of oxidative stress in compromising sperm function, there is an urgent need to identify effective antioxidant interventions that can protect and enhance sperm quality. While various antioxidants have been proposed, α-tocopherol (vitamin E) has shown promising results but requires further investigation into its specific molecular mechanisms.

Treatment using antioxidants such as α-tocopherol to protect cells have been reported in previous studies. α-Tocopherol, a form of vitamin E, plays a role in preventing lipid peroxidation and protecting sperm from damage caused by ROS.(Barik et al., 2019; Vasconcelos Franco et al., 2013) Based on previous studies α-tocopherol has been shown to reduce MDA levels in cryopreserved boar sperm[12] and enhance motility in human sperm cells.(Ghafarizadeh et al., 2021; Keshtgar et al., 2012) Ghafarizadeh et al. (2021) demonstrated that vitamin E improved sperm motility and viability in asthenoteratozoospermic men in vitro. Keshtgar et al. (2012) reported that α-tocopherol had positive effects on teratozoospermic semen samples. Breininger et al. (2005) found that α-tocopherol improved biochemical and dynamic parameters in cryopreserved boar semen by reducing MDA levels.

However, these studies primarily focused on general sperm parameters without investigating detailed kinetic parameters (VAP, VSL, VCL) or the underlying molecular mechanisms involving capacitation markers and cell survival pathways. However, its effect on sperm kinetics (VAP, VSL and VCL), capacitation and cell survival have not been studied before. Furthermore, most previous studies used animal models or examined only limited aspects of sperm function, leaving a significant gap in understanding the comprehensive molecular effects of α-tocopherol on human sperm.

In this study, we investigated the effects of α-tocopherol on VAP, VSL and VCL; tyrosine phosphorylation as indicator of capacitation, and prosurvival capacity indicated by AKT phosphorylation and caspase3 activation. This study is the first to comprehensively examine the molecular mechanisms of α-tocopherol action on human sperm by integrating detailed kinetic analysis (CASA parameters), capacitation markers (tyrosine phosphorylation), and survival signaling pathways (Akt phosphorylation and caspase-3 expression) in a single experimental framework. Unlike previous studies that focused on single parameters, our study provides a holistic understanding of how α-tocopherol influences multiple critical aspects of sperm function simultaneously.

The primary objective of this study is to elucidate the molecular mechanisms by which α-tocopherol affects human sperm function, specifically examining its effects on: (1) lipid peroxidation (MDA levels), (2) detailed sperm kinetics (VAP, VSL, VCL), (3) membrane integrity, (4) capacitation status (tyrosine phosphorylation), and (5) cell survival pathways (Akt phosphorylation and caspase-3 expression). The findings from this study are expected to provide valuable insights for developing evidence-based antioxidant therapies for male infertility treatment. Clinically, this research could inform the optimal dosage and application of α-tocopherol supplementation in assisted reproductive technologies. From a scientific perspective, this study contributes to the fundamental understanding of antioxidant mechanisms in male reproductive biology.

METHOD

This study was approved by Research Ethical Committee of the Faculty of Medicine Universitas Indonesia (No. KET-790/UN2. F1/ETIK/PPM.00.02/2023) for the use of human semen sample. A total number of 15 semen samples from different individuals with normal

criteria (concentration ≥20 million cells/ml) were obtained from volunteers with prior informed consent. Samples were collected in sterilized containers and left at room temperature for 45 min for liquefaction. The liquefied sperm sample was then washed using 50% percol. Then centrifuged at 2.000 rpm for 30 minutes, and the supernatant was discarded, leaving the sperm pellet at the bottom of the tube. The pellet was resuspended in 3 mL of Biggers, Whitten, and Whittingham (BWW) working stock medium to wash the sperm. Then centrifuged again at 2000 rpm for 15 minutes, and the supernatant was discarded, leaving the sperm pellet at the bottom of the tube. Pellet was resuspended in 1 ml of BWW, and sperm cell concentration was determined using a counting chamber. Data for all donors participating in this study are available.

Treatment of α-tocopherol

The washed sperm cells were divided into six groups, each containing approximately 10 million cells in 500 µL of BWW. α-tokoferol (Sigma Chemical Co., Saint Louis) was used to reduce ROS levels on human sperm. A stock solution of α-tocopherol at a concentration of 10 mM was prepared by dissolving α-tocopherol in 100% alcohol.(Li et al., 2010) The six groups were treated with different concentrations of α-tocopherol: control (no α-tocopherol), 20 µM, 40 µM, 60 µM, 80 µM, and 100 µM. The groups were then incubated at 37°C for 2 hours.

Malondialdehyde (MDA) evaluation

Measurement of ROS levels in sperm cells was performed using the TBARS assay to determine the MDA levels produced from the lipid peroxidation reaction by ROS. After α-tokoferol incubation, sample was centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 100 µL of Sodium Dodecyl Sulphate (SDS) extraction buffer and heated for 5 minutes to lyse the cells. Then, 200 µL of 20% trichloroacetic acid (TCA) was added, and the sample was centrifuged for 10 minutes. The supernatant was discarded, and the pellet was collected. Subsequently, 400 µL of 0.67% thiobarbituric acid (TBA) solution was added, and the mixture was heated for 10 minutes. After cooling, the absorbance was measured using a spectrophotometer at a wavelength of 532 nm.

Sperm motility (kinetic) examination

Kinetic of sperm was examined by using a computer-assisted sperm analyzer (CASA, Hamilton Thorne, USA). Three microliter from each group of sperm samples was added into CASA slide chamber (Leja, the Netherlands) and observed under the CASA microscope. The kinetic parameters of sperm that were examined were average path velocity (VAP), straight-line velocity (VSL), and curvy linear velocity (VCL).

Hypoosmotic Swelling (HOS) Test

Sperm membrane integrity was examined by hypo-osmotic swelling test (HOST). Sperm cells that had been diluted in BWW were divided into separated microtubes according to treatment groups. Cell suspensions were mixed with an equal amount (1:1) of hypo-osmotic solution (7.35 gr sodium citrate. 2H2 O; 13.51 gr fructose in 1-l ddH_2O) and then incubated for 30 min at 37°C. Swollen (indicating intact cell membrane) and un-swollen (indicating leaky cell membrane) cells were counted under a light microscope. The percentage of sperm cells with intact membranes was determined by counting the number of swollen cells, dividing it by the total number of cells counted, and multiplying by 100%. Sperm swelling was identified as a change in tail shape according to WHO standards.

Sperm protein extraction

After treatment with α-tocopherol, sperm cells were centrifuged at 13,000 rpm for 5 min and pellet was dissolved in 50 µL SDS extraction buffer (2% SDS, 10% sucrose, and 0.1875 M Tris pH 6.8). Sample was boiled for 5 min and centrifuged again at 13,000 rpm for 10 min. Supernatant was transferred to a new tube for western blot analyses. Protein concentration was determined using a nanodrop spectrophotometer.

Western immunoblotting

Western immunoblotting was performed to examine tyrosine phosphorylation activity as an indicator of sperm capacitation, Akt protein phosphorylation, and caspase-3 analysis as a prosurvival indicator after sperm cells were treated with varying doses of α-tocopherol. Twenty-five microliters isolated protein, whose concentration was measured based on absorbance using a nanodrop spectrophotometer, was separated on a 10% SDS-PAGE. The gel was then transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Uppsala, Sweden) for 1 hour and 15 minutes. The membrane was blocked with 3% Bovine Serum Albumin (BSA) for 1 hour in a shaker. Membrane was then incubated with primary antibodies (Monoclonal Anti Phosphotyrosine mouse antibody (Sigma Aldrich) 1:2000; P-Akt1 mouse monoclonal IgG (Santa Cruz, USA) 1:100; Caspase 3 Monoclonal mouse antibody (Santa Cruz, USA) 1:400; and α-Tubulin Monoclonal mouse antibody (Santa Cruz, USA) 1:500) overnight at 4°C. The next day, the membrane was washed with tris-buffered saline, 0.1% Tween (TBST) 3 × 5 min and then incubated with secondary antibodies (Anti-mouse IgG HRP conjugated (R&D systems, USA) 1:1000 for 2 h at room temperature. The membrane was washed again with 1 × TBST for another 3 × 5 min and visualization was carried out using enhanced chemiluminescence (ECL) plus western blot detection system (GE Healthcare Life Science, UK). Signal was detected using Imagequanto LAS 4000 (GE Healthcare Life Science, Sweden). Band intensity was measured using ImageJ (NIH, USA).

Data analysis

The data obtained were analyzed using SPSS 26. Data normality and homogenity were performed using Shapiro-Wilk and Levene's Test, respectively. One-way ANOVA was used to analysed significant different between treatment groups and control. Differences between groups were further analyzed using post hoc tests. p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Malondialdehyde levels

MDA examination was conducted to confirm the occurrence of oxidative stress in sperm cells under physiological conditions, which were then treated with the antioxidant α-tocopherol. The average MDA levels in sperm cells showed that the control group had higher levels compared to the group treated with the antioxidant α-tocopherol. The average MDA in the control group was 1.51±1.30 nmol/ml. In the treatment groups, the average MDA levels were 1.40±1.21; 1.33±1.17; 1.48±1.31; 1.35±1.20; and 1.35±1.20 nmol/ml at α-tocopherol concentrations of 20 μM, 40 μM, 60 μM, 80 μM, and 100 μM, respectively [Figure 1]. Overall, there was a decrease in the average MDA levels after treatment with α-tocopherol. The group treated with 40 μM α-tocopherol showed the lowest reduction in MDA levels, with a value of 1.33±1.17 nmol/ml. However, this decrease was not significant (P > 0,05).

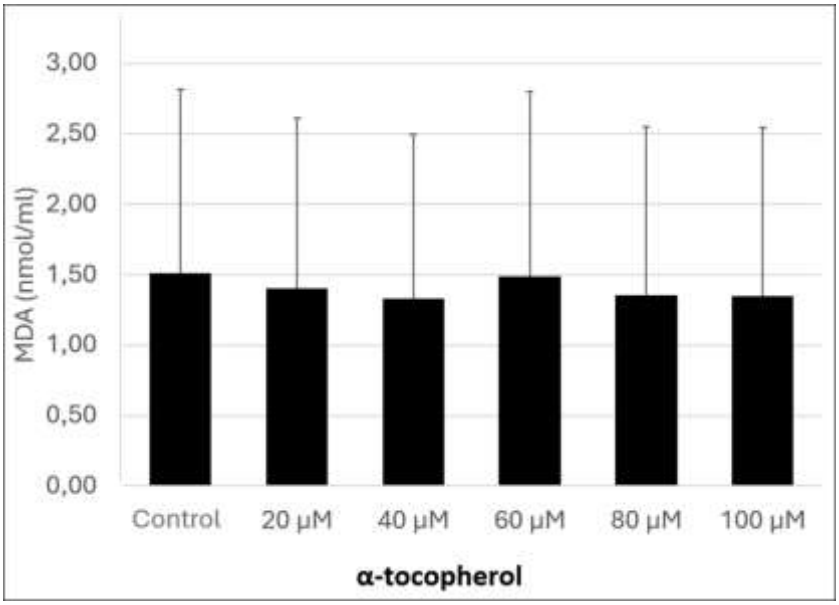

Figure 1: Analysis of malondialdehyde by spectrophotometer on sperm cells extract after treatment with α-tocopherol to confirm the occurrence of oxidative stress in sperm cells. Overall, there was a decrease in the average MDA levels after treatment with α-tocopherol. But, the decrease was not significant ($P > 0,05$).

Oxidative stress in male germ cells is believed to be a contributing factor to male subfertility and infertility. The addition of antioxidants, such as α-tocopherol, is expected to reduce ROS (Reactive Oxygen Species) levels, thereby minimizing oxidative stress in sperm. Although α-tocopherol has potential as an antioxidant, this study demonstrated that supplementation of α-tocopherol at concentrations ranging from 20 µM to 100 µM did not significantly reduce MDA levels in human spermatozoa. This finding contrasts with previous studies on cryopreserved boar sperm, (Breininger et al., 2005) where α-tocopherol significantly decreased MDA levels. The discrepancy may be attributed to species-specific differences or the unique physiological conditions of human sperm compared to boar sperm. Furthermore, this is also suspected to be due to the naturally low ROS levels present in the sperm cell treatment groups in this study, which are insufficient to induce lipid peroxidation in the cell membrane. As a result, the measured MDA levels were minimal, and the addition of α-tocopherol did not result in a significant decrease.

Sperm kinetics

Sperm kinetic was analyzed using CASA to observe sperm motility in detail. Three sperm kinetic parameters were selected in this study, namely VAP, VSL, and VCL. Our study showed that treatment with α-tocopherol increase all three parameters of CASA. Sperm VAP increased significantly from 30,51 ± 5,8 µm/sec at control group to 44,86 ± 8,37 µm/sec at concetration of 100 µM ($P < 0.05$) [Figure 2, black bars]. Sperm VSL increased significantly from 7,97 ± 3,85 µm/sec at control group to 28,61 ± 9,16 µm/sec at concetration of 100 µM ($P < 0.05$) [Figure 2, white bars]. Sperm VCL increased significantly from 28,83 ± 4,50 µm/sec to 45,60 ± 9,56 µm/sec at concetration of 100 µM ($P < 0.05$) [Figure 2, diagonally stripped bars].

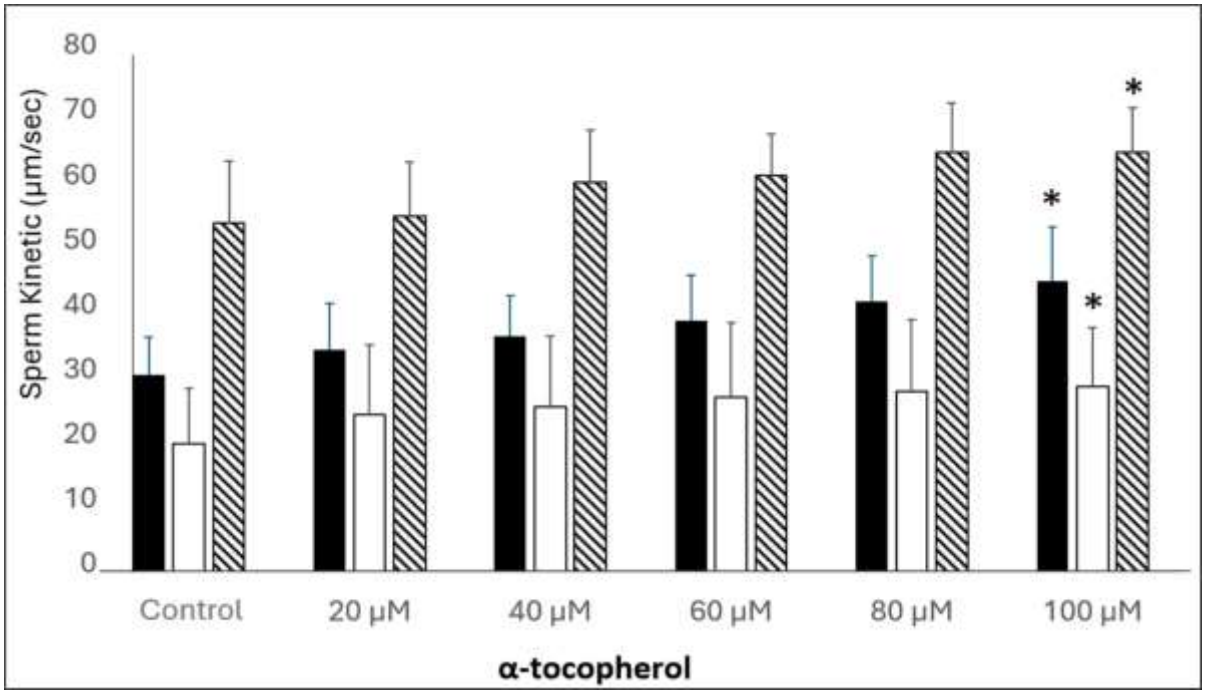

Figure 2: Analysis of sperm kinetics using computer-assisted sperm analyzer. Black bars represent average path velocity, white bars represent straight-line velocity, and diagonally-stripped bars represent curvilinear velocity. Significant changes were observed at α-tocopherol concentration of 100 µM ($P < 0.05$). Error sign on top of each bar represents SEM. *Statistically significant change. SEM: Standard error of the mean.

The concentration of 100 µM significantly improved motility parameters (VAP, VSL, and VCL) compared to the control group, suggesting that α-tocopherol enhances the motility mechanisms of sperm, an essential indicator of sperm quality and male fertility. This suggests that α-tocopherol reduces ROS levels, decreasing lipid peroxidation and maintaining the integrity of the sperm cell membrane. This is because sperm motility is greatly influenced by intracellular signaling which depends on the permeability of the plasma membrane. A plasma membrane that undergoes extensive lipid peroxidation will lose its integrity and its ability to selectively regulate substances entering the cell. As a result, membrane fluidity, ion gradients, receptor transduction, transport processes, and enzyme activities are disrupted, ultimately impairing sperm cell functions, including motility.(Agarwal et al., 2014; Kothari et al., 2010)Salinthone et al. demonstrated that administration of α-tocopherol at concentrations of 10, 25, 50, 100, 200, and 500 µg/ml effectively increased cAMP levels in peripheral blood mononuclear cells (PBMCs).(Salinthone et al., 2012)

Sperm membrane integrity

Sperm membrane integrity was analyzed using HOS test after treatment with α-tocopherol. Positive HOS indicates intact sperm plasma membranes (curved sperm tail), while negative HOS indicates damaged sperm plasma membranes (straight sperm tail). Our data showed increase in the sperm membran integrity after treatment with various concentrations of α-tocopherol. The percentage of sperm cells with curly tails, indicating intact membrane, went up from the control throughout concentrations of 20, 40, 60, 80, 100 µM with 56,35% (control), 61,94% 66,95%, 67,69 %,72,71%, and 72,41%, respectively. All the chages were significant compared to the control ($P < 0.05$) [Figure 3].

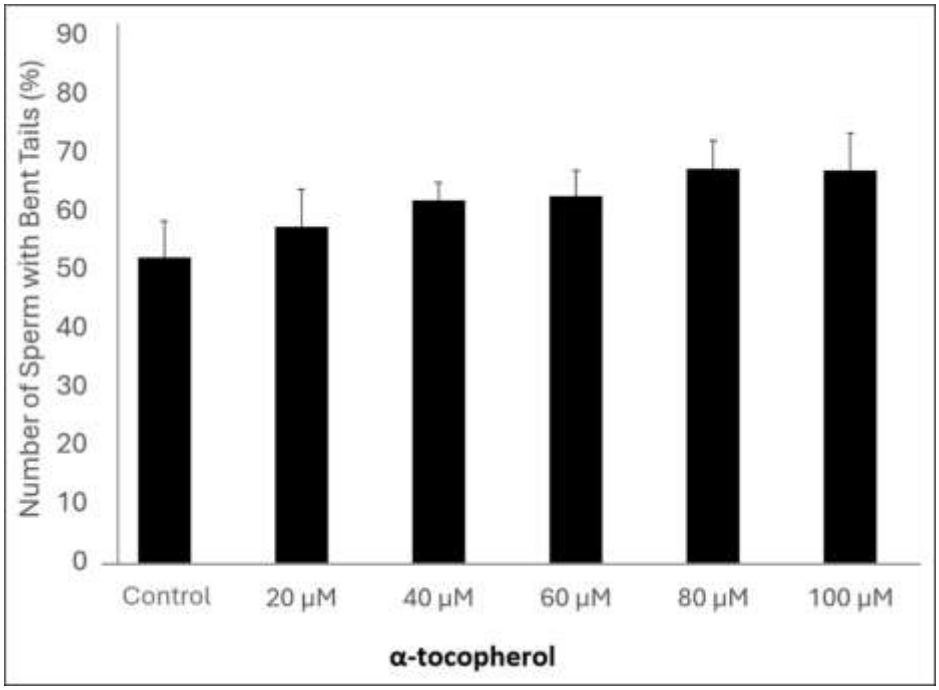

Figure 3: Analysis of hypo-osmotic swelling test to assess sperm membrane integrity after treatment with α-tocopherol. Sperm with swollen or bent tail, indicating intact membrane, were counted in at least three observation fields. All changes are significantly different ($P < 0.05$). Error sign represents SEM. SEM: Standard error of the mean.

Membrane integrity significantly improved across all α-tocopherol concentrations, demonstrating its protective role against oxidative stress-induced damage. The hypoosmotic swelling test results suggest that α-tocopherol stabilizes the plasma membrane, ensuring the sperm's structural integrity and functional viability. The antioxidant properties of α-tocopherol effectively mitigate membrane damage caused by lipid peroxidation resulting from ROS generated during sperm cell metabolism. Evaluating sperm membrane integrity is crucial as an indicator of both viability and motility. This is due to the fact that sperm cells are transcriptionally inactive and rely on external signals to undergo post-translational modifications required to achieve functional maturity. These signaling processes depend on the integrity of the cell membrane, which houses receptors essential for sperm cell function. Therefore, the addition of α-tocopherol in this study has been shown to enhance sperm cell viability and membrane integrity by suppressing lipid peroxidation in the sperm membrane.

Sperm tyrosine phosphorylation

One of the indicators of sperm capacitation is an increase in global tyrosine phosphorylation in many proteins located in the sperm tail. Our western immunoblotting data showed that exposure of sperm cells with increasing concentration of α-tocopherol from 20 to 100 µM gradually increased global tyrosine phosphorylation detected in the total sperm protein. Tyrosine phosphorylation can be detected at approximately ~32 kDa, ~43 kDa, ~55 kDa, ~70 kDa, and ~95 kDa. The highest band intensity was obtained at an α-tocopherol concentration of 80 µM [Figure 4].

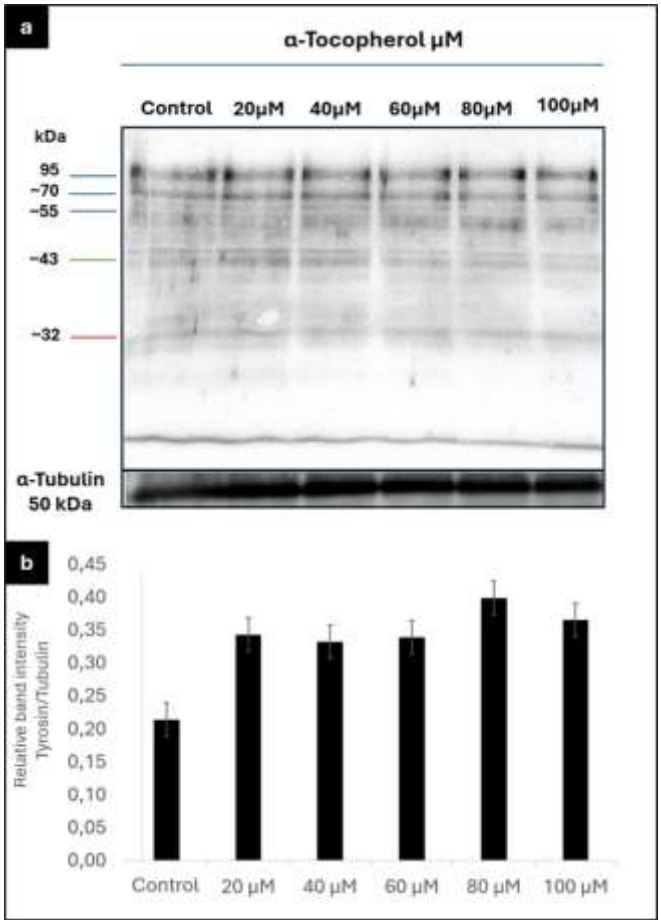

Figure 4: Western immunoblotting was performed using an antibody recognizing general tyrosine phosphorylation as an indicator of sperm capacitation after alpha-tocopherol treatment. (a) The Western blot results showed several clearly detectable bands at ~32 kDa, ~43 kDa, ~55 kDa, ~70 kDa, and ~95 kDa. The membrane was stripped and re-probed with a tubulin antibody as a loading control. (b) Analysis of tyrosine phosphorylation levels using ImageJ revealed the highest band intensity at an α-tocopherol concentration of 80 µM. The experiment was repeated at least three times with consistent results.

This finding is consistent with established theories suggesting that enhanced sperm motility is closely associated with the capacitation process. Capacitation involves signal transduction initiated by elevated cAMP levels, which subsequently stimulate the activation of protein kinase A (PKA). Activated PKA facilitates the activation of SRC kinase, which phosphorylates tyrosine residues, thereby promoting increased tyrosine phosphorylation and enabling the capacitation of sperm cells.(Aitken, 2017b; Aitken & Nixon, 2013; Dcunha et al., n.d.)

Phosphorylation, particularly tyrosine phosphorylation, is crucial for sperm motility, capacitation, and hyperactivation. This study identified five distinct protein bands (~32 kDa, ~43 kDa, ~55 kDa, ~70 kDa, and ~95 kDa) with tyrosine phosphorylation, consistent with previous findings in human and boar sperm.(Sepideh et al., 2009; Tardif et al., 2001) ImageJ analysis showed higher band intensities in α-tocopherol-treated groups, with the highest at 80 µM, indicating enhanced tyrosine phosphorylation. This suggests that α-tocopherol promotes capacitation and hyperactivation by reducing ROS-induced oxidative stress, preventing lipid peroxidation, and maintaining sperm membrane integrity and fluidity.

AKT phosphorylation

Detection of Akt protein phosphorylation was performed to observe the effect of α-tocopherol addition on sperm cell survival using western blot. Akt phosphorylation is indicated by a band at ~60 kDa. This study show an increase in Akt phosphorylation expression with increasing α-tocopherol concentrations. The highest band intensity was observed at an α-tocopherol concentration of 100 µM [Figure 5].

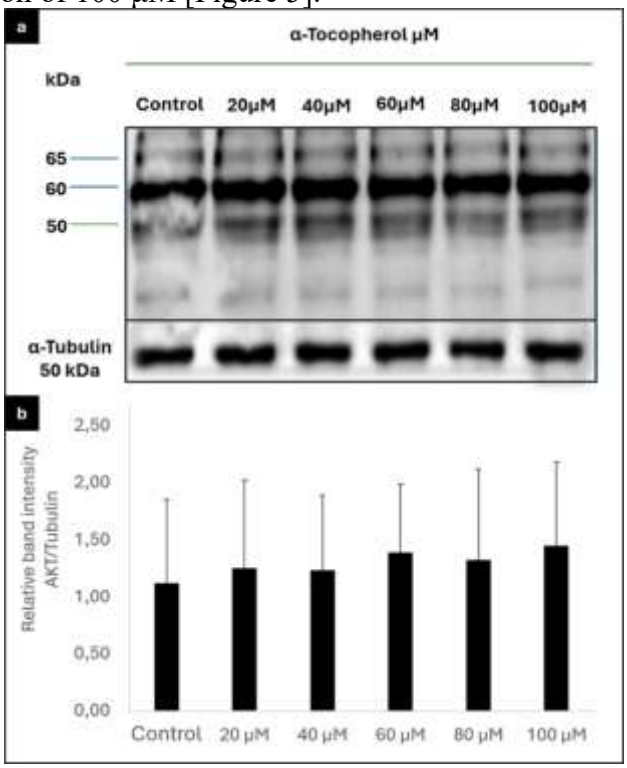

Figure 5: Western blot analysis of sperm Akt phosphorylation after treatment with a various concentration of α-tocopherol. (a) The results show phosphorylated Akt protein at 60 kDa. (b) Analysis of Akt phosphorylation levels at the 60 kDa band using ImageJ reveals the highest band intensity at concentration of 100 µM.

Akt regulates cell survival and apoptosis and is critical for sperm motility, viability, and mitochondrial membrane stability. This study identified Akt phosphorylation at ~60 kDa, with increased band intensity in α-tocopherol-treated groups, peaking at 100 µM. The results align with Zingg et al., who found that α-tocopherol stimulates PI3K, leading to Akt activation, which inhibits apoptosis by phosphorylating Bad and activating anti-apoptotic proteins.(Zingg et al., 2014) By reducing oxidative stress and lipid peroxidation, α-tocopherol supports PI3K-Akt signaling, enhancing sperm survival and function.

Sperm caspase 3 levels

Caspase 3 protein detection was performed to observe the effect of α-tocopherol on sperm cell apoptosis activity. We measured the activation of caspase 3 protein in the human sperm after being treated with α-tocopherol using western immunoblotting. The data show incubation of α-tocopherol at concentrations of 20 µM to 100 µM for 120 minutes at 37°C showed decrease in caspase 3 protein expression indicated bands at ~32 kDa. The band at 50 kDa represents a non-specific band. Decrease in caspase 3 expression was along with increasing α-tocopherol concentrations. The lowest band intensity was observed at an α-tocopherol concentration of 80 µM [Figure 6].

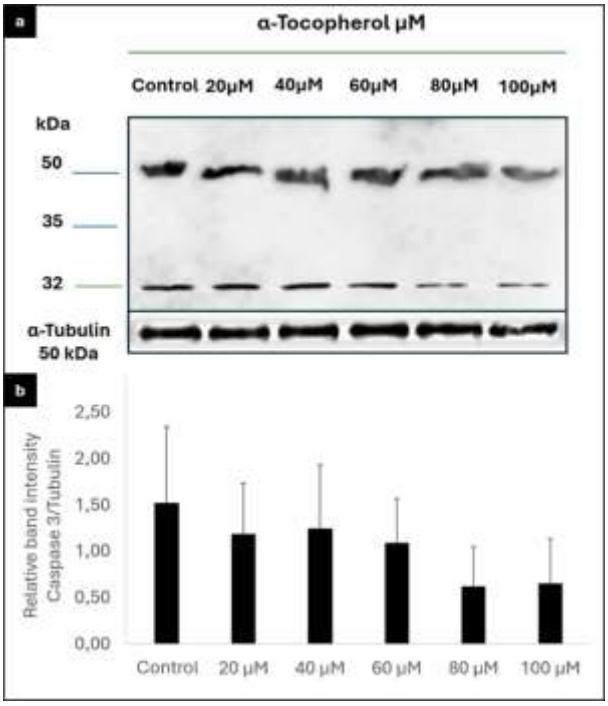

Figure 6: Western blot analysis of caspase 3 expression after treatment with α-tocopherol. (a) Decrease in caspase 3 expression was detected at ~50 kDa and ~32 kDa. (b) Analysis of caspase 3 levels at the ~32 kDa band using ImageJ shows the lowest band intensity at an α-tocopherol concentration of 80 μM.

Caspase 3 protein serves as a marker for the final stage of apoptosis and is commonly used to detect apoptosis in cells. Caspase 3 is activated by caspase 8 and 9, with two bands observed at approximately 32 kDa and 50 kDa. The 50 kDa band is likely non-specific due to antibody binding to other epitopes. Caspase 3 is expressed as a proenzyme with a molecular weight of 32 kDa and is cleaved into 17 kDa and 12 kDa subunits upon activation.(Nestal de Moraes et al., 2011) The reduction in caspase 3 expression after α-tocopherol treatment suggests a decrease in oxidative stress, thereby suppressing apoptosis and promoting sperm cell survival.

CONCLUSION

While α-tocopherol supplementation did not significantly lower MDA levels, it had positive impacts on motility, membrane integrity, capacitation, and survival pathways of human sperm. Thus α-tocopherol may be a promising antioxidant for improving sperm quality. Further study using sperm from infertile patients caused by asthenozoospermia is suggested to observe the improving effect of α-tocopherol.

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