

## Effects of Tocotrienols on Sperm Parameters, Testes Weight and Ultrastructure in Sprague Dawley Rats

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**Abstract:** Vitamin E is divided equally into 2 families, tocopherols and tocotrienols. Being an antioxidant, vitamin E has been reported to improve disorders related to oxidative damage in many organ systems. Sources of vitamin E can be found in many foods including palm oil which is rich in tocotrienols. Studies on the effects of vitamin E on male fertility have shown encouraging results. This study was conducted to observe the effects of feeding tocotrienols from palm oil to male Sprague-Dawley rats on sperm parameters, testes weight and sperm ultrastructure. Blood nitric oxide (NO) levels were also measured. Thirty-five rats were separated equally into 5 groups: initial group (sacrificed before experiment for base line values), control group (fed commercial pellets only), vehicle group (palm oil), low dose and high dose of tocotrienol treatment groups. The four groups were sacrificed at the end of the six weeks experiment and sperm parameters (motility, viability and count) were measured while sperm ultrastructure was observed via transmission electron microscopy (TEM). Cardiac blood was taken for NO analysis. Testes were also weighed. At high dose, it is found that sperm parameters increased significantly ( $p < 0.05$ ) but there was no significant change in NO readings. Testes weight does not show any changes while TEM showed lowered mitochondrial cristae distortion in both treatment groups. High dose of tocotrienol increase sperm parameters suggesting that the mechanism for better male fertility is related to better cristae membrane integrity in sperm.

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### I. Introduction

Most studies focus on the consequences of a lack of vitamin E on the biological body. Vitamin E is fat soluble and found in many foods, including milk, eggs, meats and leafy vegetables (Ganong, 2005). Vitamin E itself is actually made up of a collection of 8 different but related analogues, 4 of them being tocopherols and the other four tocotrienols (Herrera and Barbas, 2001). In most natural foods, tocopherols make up the bulk of vitamin E, which is also the reason when research is done on vitamin E, tocopherols are given instead of tocotrienols (Butterfield et al., 2002; Taber and Stevens, 2011; Boccardi et al., 2016).

Vitamin E has long since been linked to fertility as studies by Evans and Bishop in 1922 showed that vitamin E is a dietary factor vital for rat fertility (Mustacich *et al.*, 2007). Al-Attar (2011) found that male mice on seven weeks treatment of lead, mercury, cadmium and calcium in their drinking water showed normal seminiferous tubule structures upon vitamin E supplementation through intraperitoneal injection during the same period. Research on sperm parameters and functions improvement with vitamin E on its own or in combination with other vitamins in most male species including human were reported in the last two decades. A review by Archaniolo et al., (2014) on clinical trials on supplementation of vitamin E, vitamin A, vitamin C, arginine, cCarnitine, N-Acetyl-Carnitine, glutathione, Coenzyme Q10, selenium and zinc on males with oligoasthenoteratozoospermia (OAT) showed improve semen parameters (sperm count, sperm motility and morphology) and pregnancy rates. A recent report by Alizadeh, et al., (2016) also showed better sperm kinematics with dietary vitamin E as compared to supplementation with omega 3 and omega 6 in rats. The reason for its function in fertility was due to their properties as antioxidants protecting sperm cells from free radical damage (Review by Ko et al., 2014).

Free radicals are any molecular species which has an unpaired electron in an atomic orbital rendering it unstable and highly reactive as they can either donate or accept an electron from other molecules (Cheeseman and Slater, 1993). Free radicals attack important macromolecules leading to cell damage and homeostatic disruption (Rao et al., 2006). Antioxidants are stable molecules with free radical scavenging properties capable of donating an electron to a free radical thus neutralizing it and reducing its ability to cause cellular damage (Halliwell, 1995; Hancock et al., 2001). Glutathione, ubiquinol, and uric acid are antioxidants produced during normal metabolism in the body but other principle micronutrients like vitamins E, C and  $\beta$  carotene must be supplied in the diet (Wolf, 2005). As a balance is needed to control oxidative damage in the body, a reduction in micronutrients intake will favour elevation of free radicals level and cause oxidative stress (Mc Cord 2000, Rao et al., 2006; Lobo et al., 2010). Reactive Oxygen Species (ROS) are reactive molecules and free radicals derived from molecular oxygen produced as byproducts during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalyzed oxidation (Review by Novo and Parola, 2008).

ROS have been implicated in many diseases, among them neurodegenerative diseases (Butterfield et al., 2002). In the reproductive system, elevated levels of ROS have been associated with infertility cases in males (Tremellen, 2008; Makker, et al., 2009). Rajesh Kumar et al., (2002) and Kothari et al., 2010 reported that oxidative stress (OS) in the male reproductive system may cause sperm damage causing impaired motility, increase lipid peroxidation, DNA damage or fragmentation (sperm apoptosis) and decreased oocyte-sperm fusion. Koopers et al., (2008) and Kothari et al., (2010) reports that the main source of sperm produced ROS is the mitochondria via the formation of superoxide. Mitochondrial cristae are folds of the mitochondrial inner membrane providing an increase in surface area to enable the electron transport chain and chemiosmosis produce ATP in the final steps of cellular respiration (Zink et al., 2009). The mitochondrial ultrastructure is therefore important as any defects have been associated with decrease sperm motility in humans (Pelliccione et al., 2011).

Nitrite is a central component of the nitric oxide (NO) cycle as it measures NO metabolism and its changes in health and disease (Piknova and Schechter, 2011). It has been used as a biomarker of some health conditions as it is both an NO precursor and a product of NO oxidation. Therefore a simple measurement of nitrite levels may provide an insight into the NO pathway activity and efficiency. Even though research before was dedicated to tocopherol, only recently has tocotrienol research become more widespread due to the realization that it has its own unique functions (Sen et al., 2007; Agarwal et al., 2010 ; Peh et al., 2016). Tocotrienols rich in palm oil (palm oil tocotrienol-rich fraction) reduce the spermatotoxic effects occurring in spermatozoa of rats exposed to organophosphate insecticides fenitrothion by significantly increasing sperm counts, motility, and viability and decreased abnormal sperm morphology (Taib et al., 2014). In a similar study by Jegede et al., (2015), red palm oil rich in tocotrienol attenuates lead acetate induced testicular damage in adult male Sprague-Dawley rats.

We report here a study to observe the effects of feeding tocotrienols from palm oil for 6 weeks to male Sprague-Dawley rats on sperm parameters, ultrastructure, testicles weight and nitrite levels.

## **II. Materials And Methods**

### **Experimental design**

Thirty five Sprague-Dawley rats of 360-400g in weight were divided equally into five groups: initial group (sacrificed before experiment for baseline values), control (fed only commercial pellets), vehicle (fed palm oil), low dose (500 mg/kg tocotrienol) and high dose (1500 mg/kg tocotrienol). Treatment last for 42 days (6 weeks) and the rats were sacrificed. The cardiac blood was taken for Griess assay, testes excised and weighed and the cauda epididymis excised for cauda epididymal sperm collection. The right and left cauda epididymal sperm were used for two different purposes i.e., the right cauda for transmission electron microscopy (TEM) while the left cauda for measuring sperm parameters.

### **Cauda epididymal sperm collection**

Cauda epididymal sperm concentration was carried out according to Kempinas and Lamarno-Cavarlho, (1988) with some modifications. Briefly the right cauda was pooled in 10 ml of 0.1M Phosphate Buffer Saline at pH 7.4 and 37<sup>0</sup>C with 1% Bovine Serum Albumin, minced and debris removed via sedimentation. The supernatant was collected and the sperm pelleted via centrifugation at 5000 rpm for 5 minutes for transmission electron microscope (TEM) observation. The left cauda was mashed in 4ml of 0.1M Phosphate Buffer Saline at pH 7.4 and 37<sup>0</sup>C with 1% Bovine Serum Albumin and measured for sperm count and motility using the Makler Counting Chamber (Sefi Medical Instruments Ltd., Daimler St. Santa Ana, CA).

### **Measurement of sperm parameters**

Sperm count and motility was conducted according to Makler (1980) and WHO (2010). Examination was conducted under a normal light microscope using the 20X objective.

Briefly for counting sperm motility, approximately 10 $\mu$ l of sperm suspension was placed on the stage of the microscope chamber. Sperm motility was defined as sperm moving forward. The percentage motility would be the number of motile sperm divided by the total number of sperm (motile and non-motile) according to the formula below:

$$\text{Percentage of motile sperm (\%)} = \frac{\text{Number of motile spermatozoa}}{\text{Total number of spermatozoa (motile + non-motile)}} \times 100\%$$

For sperm count analysis, the concentration of sperm were counted in 3 x 10 squares either horizontally or vertically and only the sperm heads within the squares of the grid were counted. The total count of sperm in the area would be according to the formula below:

$$\text{Sperm count} = \frac{\text{Total sperm count from 3 x 10 squares}}{3} \times 10^6/\text{ml}$$

For sperm viability analysis, smears were prepared by placing approximately 10 $\mu$ l of the sperm suspension and Eosin/Nigrosin stain at the edge of a clean glass slide and smearing across the slide. The slide was air dry and viewed under light microscope (X 40). Live spermatozoa would appear as clear whereas dead spermatozoa would show a deep pink / purple colour. A total of 100 cells were counted in a battlement fashion. The percentage of sperm viability would be according to the formula below:

$$\text{Sperm viability (\% Live)} = \frac{\text{Number of live spermatozoa}}{\text{Total number of spermatozoa}} \times 100\%$$

#### **Nitric oxide determination using Griess Assay**

Griess assay was carried out to measure nitric oxide (NO) levels in cardiac blood according to Guevera et al., (1998), Miranda et al., (2001), Moorcroft et al., (2001) and Giustarini et al., (2008). Briefly, semen was dispensed into Eppendorf tubes and centrifuged at 3000 g for 10 minutes to collect the seminal plasma (supernatant). The NO level in the seminal plasma was then determined by using Griess reaction for colorimetric assay of nitrites, with a detection limit of approximately 100 nM. The purple azo derivative was monitored at an absorbance of 548 nm using a spectrophotometer. The readings obtained were estimated from a nitrite standard curve, prepared earlier to quantify the level of NO present in the seminal plasma.

#### **Transmission Electron Microscopy (TEM)**

Standard TEM procedures (fixation, dehydration, infiltration, embedding, ultrathin sectioning, staining and viewing) were carried out. Briefly, sperm pellet of approximately 1.0mm thick was sliced and immediately transferred to a small bottle containing 1-2 ml Karnovsky's fixative (4°C). The tissue was fixed for 2-4 hours at 4°C and later the fixative discard and replaced with buffer solution. The tissue was later fixed in osmium tetroxide for 15-30 minutes and later the block was rinse with distilled water for a few minutes. Tissue was then transferred to a bottle containing 4% aqueous uranyl acetate solution for 10 minutes and then rinse with distilled water several times. The pellet slices were then dehydrated into a series of ascending alcohol solutions (70%, 90%, and 95% for 15 minutes each and in 100% alcohol for 2 changes of 30 minutes each). The sample was then infiltrated with 100% propylene oxide for 2 changes of 30 minutes each. Later the infiltration solution was change to propylene oxide and resin mixture with ratio of 1: 1 for an hour followed by 1:3 for 2 hours and later in 100% resin overnight. Finally the specimen was infiltrated in 100% resin for 2 hours. Subsequently the specimen was embedded into beam capsules and filled with pure fresh resin, labelled and de-gassed in a vacuum embedding oven or vacuum chamber. Polymerization of the specimen process occurs in the oven at 45°C for 2-3 hours. Then the oven was set up to a higher temperature (65°C) overnight. Sample blocks were then removed and allowed to cool at room temperature. The capsules were removed using a Beam Presser and sectioning was performed with an ultramicrotome (50nm – 90 nm thick) and collected onto 3mm copper grids. The sections were stained with uranyl acetate for 10 minutes and wash with 50% filtered alcohol followed by lead stain for 5 minutes and then rinse with double distilled water. Stained sections were viewed under JOEL 1200EX electron microscope.

#### **Analysis of results**

The results were then analysed with One-Way ANOVA and Duncan Multiple Range Test for each parameter for significant difference between groups and Pearson's Correlation to identify the relationship between NO readings from Griess assay and sperm parameters. The degree of significance (p) of each parameter tested was obtained in the form of means  $\pm$  SEM (standard error mean) and interpreted. A p value <0.05 was considered statistically significant.

### **III. Results**

The results showed a significant increase (p<0.05) in sperm motility, viability and count in groups administered high doses of tocotrienol. Testes weights were not significantly affected by treatment of any sort and there was no correlation between parameter changes and NO concentration (Table 1)

**Table 1:** Sperm parameters, testes weight and nitrite levels

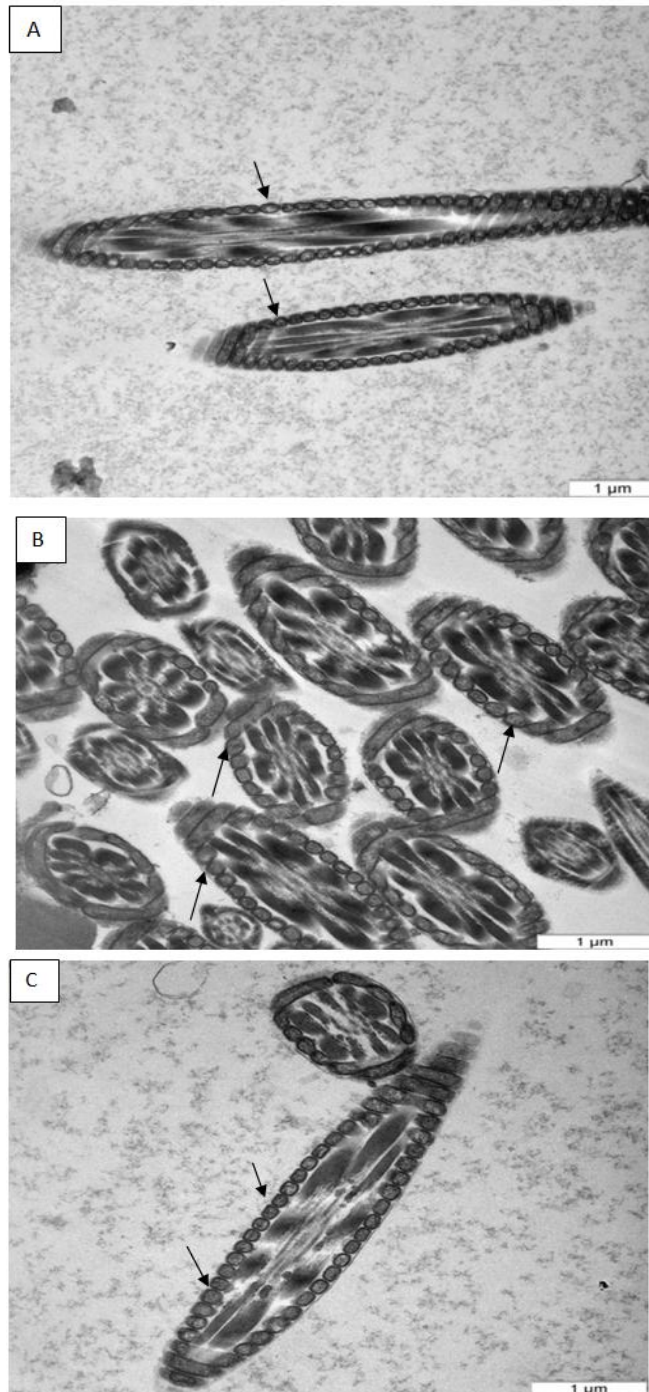
Parameters	Groups				
	Initial	Control	Vehicle	Low	High
Motility (%)	59.78±7.91	57.42±7.10	57.07±6.85	62.24±6.39*	68.40±6.42*
Count (10 <sup>6</sup> /ml)	24.67±9.39	23.24±3.42	27.29±6.45	26.92±4.96*	33.71±7.18*
Viability (%)	71.86±4.66	70.79±4.48	70.88±4.27	72.25±3.49*	78.14±3.23*
Weight of Testes (g)	1.74±0.19	1.78±0.16	1.67±0.12	1.67±0.17	1.75±0.13
Nitrite: Conc (µM)	17.00	26.53	15.40	13.30	13.03

± = standard deviation

n = 7

\* significant at p<0.05

TEM results showed lowered mitochondrial cristae distortion in both treatment groups but showed no significant changes in tail structure.



**Figure 1:** Figures (top to bottom) show initial reading group (A), low dose (B) and high dose (C) groups. Mitochondrial cristae show no changes in shape (arrows) when treatment with tocotrienol was given.

#### **IV. Discussions**

Findings were consistent with other studies using vitamin E, which had been extensively performed and have shown results. Studies have repeatedly showed that sperm parameters were improved by vitamin E but different studies show difference in improvement. The reason for this improvement was not known but scientists studying the effects of antioxidants found that vitamin E protects sperm membrane from lipid peroxidation and at the same time increase motility (Agarwal et al., 2014). Antioxidants have been shown to increase motility and probably viability and its role in combating lipid peroxidation which causes membrane damage leading to cell death. In addition, lipid peroxidation may also cause damage to the primitive germ cells in the testis and cause reduced sperm formation, which explains the increased count in high dose group rats. Another possible mode of action is the reduction of DNA fragmentation which can be decreased with administration of antioxidants (Greco et al., 2005), and possibly explain the increased count, motility and viability as DNA fragmentation is associated with cell death (apoptosis) and its ability to fertilize the ovum.

Testicle weight, in our experiment, showed no significant difference in any group. This is not expected as a study by Momenti et al., (2009) found that vitamin E maintains the weight of damaged testicles. Perhaps the protective effect of vitamin E is tocopherol specific, thus no effect on testicular weight is observed or its effect is reparative instead of improving, thus when the testicles are not damaged by a foreign substance (as with p-NP in the Momenti study), the effect is not seen. The effect may also be histological and perhaps in the future histological observation can be done. Another possible reason is that the sample number for each group is too small and does not represent the general population, thus some groups will have too many rats with starting testicle weights that are not of normal reading. This can be avoided by increasing the number of subjects in each group.

Griess assay also showed that blood nitrite level reduces proportionately with the dosage of tocotrienol administration but this difference is found to be insignificant. Tocotrienol treatment reduces nitric oxide level in the blood which can be directly related to the levels in the seminal plasma. Balercia et al. (2004) found that normozoospermic men will have a lower semen NO concentration compared to asthenozoospermic men. The fact that our analysis showed that it is not significant and subsequently showing not significant correlation with sperm parameters, may be due to the possibility that NO levels in the blood need not fall too low to cause a difference in sperm performance. The effect of nitric oxide levels on sperm parameters was reviewed by Doshi et al., (2012). Based on the review, studies have shown that physiological levels of nitric oxide are essential for conducting various sperm functions (capacitation, acrosomal reaction, zona pellucida binding) as well as sperm parameters (motility, morphology, viability). In contrast, some studies suggest higher levels have been shown to be detrimental to the sperm parameters mentioned. Perhaps the proposed non-significant reduction in NO level was sufficient to promote sperm performance. This is hypothesized and further study to correlate blood NO levels with semen NO levels are needed.

Mitochondrial functionality, as reported by Amaral et al., (2013), is crucial for sperm as it play an important role in ensuring sperm functionality and fertilizing ability. The mitochondria control production of mROS which is required for motility, capacitation and fertilization and its apoptotic pathway prevent DNA damaged sperm from fertilizing the ovum (Amaral et al., 2013). In our study, NO levels were reduced in tocotrienol treated groups and vehicle groups but were higher in control groups, however the results were not significant. As such we could not correlate the mitochondrial cristae shapes that were maintained in all treated and vehicle groups but were slightly distorted in control group (ultrastructure not shown) with the reduced or increased levels of nitric oxide. In the future, a study to measure nitric oxide levels in blood and semen with mitochondrial cristae integrity ultrastructure should be carried out to observe their possible correlation.

#### **V. Conclusion**

At high dose treatment of tocotrienol, all sperm parameters measured are increased significantly but is not due to a significant decrease in blood NO. Testes weight also do not show visible significant change pattern. We suggest that the increase in sperm parameters may be due to the maintenance of mitochondrial cristae integrity by reduced NO levels as less distortion is seen in treatment groups.

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#### **References**

- [1]. Agarwal, A., virk, G., Ong, C and du Plessis, S.S. (2014). Effect of oxidative stresson male reproduction. World Journal of Mens Health. 32(10): 1-17
- [2]. Agarwal, B.B., Sundaram, C., Prasad, S. and Kannappan, R. (2010). Tocotrienols, the vitamin E of the 21st century: Its potential against cancer and other chronic diseases. Biochemical Pharmacology 60(11): 1613-31



- [3]. Alizadeh, A., Taleb, Z., Ebrahimi, B., Esmaeili, V., Shaverdi, A., Nasr, J., Kheimeh, A. and Yazdi, R.S. (2016). Dietary Vitamin E Is More Effective than Omega-3 and Omega-6 Fatty Acid for Improving The Kinematic Characteristics of Rat Sperm. *Cell Journal* 18(2): 262–270.
- [4]. Amaral, A., Lourenco, B., Marques, M. and Ramalho-Santos, J. (2013). Mitochondria functionality and sperm quality. *Reproduction* 146:163-174
- [5]. Arcaniolo, D., Favilla, V., Tiscione, D., Pisano, F., Bozzini, G., Creta, M., Gentile, G., Fabris, F.M., Pavan, N., Veneziano, I.A., and Cai, T. (2014). Is there a place for nutritional supplements in the treatment of idiopathic male infertility? *Archivio Italiano di Urologia e Andrologia* 164 : 86- 3
- [6]. Balercia G., Moretti S., Vignini A., Magagnini M., Mantero F., Boscaro M, Ricciardo-Lamonica G. and Mazzanti L. (2004). Role of nitric oxide concentration on human sperm motility, *Journal of Andrology* 25(2): 245-249.
- [7]. Boccardi, V., Baroni, M., Mangialasche, F., and Mecocci, P. (2016). Vitamin E family: Role in the pathogenesis and treatment of Alzheimer's disease. *Alzheimer's & Dementia: Translational Research & Clinical Interventions*. 2(3): 182-91
- [8]. Butterfield, D.A., Castegna, A., Drake, J., Scapagnini, G. and Calabrese, V. (2002). Vitamin E and Neurodegenerative Disorders Associated with Oxidative Stress. *Nutritional Neuroscience*. 5(4): 229-239
- [9]. Cheeseman, K. H. and Slater T.F. (1993). An introduction to free radicals chemistry. *British Medical Bulletin*. 49:481–93
- [10]. Doshi, S.B., Khullar, K., Sharma, R.S. and Agarwal, A. (2012). Role of reactive nitrogen species in male infertility. *Reproduction Biology Endocrinology*. 10: 109
- [11]. Ganong, W.F. (2005). Energy Balance, Metabolism, and Nutrition. In *Review of Medical Physiology*, ed Ganong W.F, pp 315. New York: The McGraw Hill Companies.
- [12]. Giustarini, D., Rossi, R. Milzani, A., and Diale-Donne, I. (2008). Nitrite and nitrate measurement by Griess reagent in human plasma: evaluation of interferences and standardization. *Methods Enzymology*. 440: 361-80
- [13]. Greco E., Iacobelli M., Rienzi L., Ubaldi F., Ferrero S. and Tesarik J. (2005). Reduction of the Incidence of Sperm DNA Fragmentation by Oral Antioxidant Treatment, *Journal of Andrology* 26(3): 349-353
- [14]. Guevara, I., Iwanejko, J., Dembińska-Kieć A., Pankiewicz, J., Wanat, A., Anna, P., Gołabek, I., Bartuś, S., Malczewska-Malec, M and Szczudlik, A. (1998). Determination of nitrite/nitrate in human biological material by the simple Griess reaction. *Clinica Chimica Acta* 274(2): 177–188
- [15]. Halliwell, B. (1995). How to characterize an antioxidant - An update. *Biochemical Society Symposium* 61:73–101
- [16]. Hancock, J.T., Desikan, R., and Neill, S.J. Neill. (2001) Role of Reactive Oxygen Species in Cell Signaling Pathways. *Biochemical and Biomedical Aspects of Oxidative Modification*. 29(2):345-350
- [17]. Herrera E. and Barbas C. (2001). Vitamin E: action, metabolism and perspectives. *Journal of Physiology and Biochemistry*, 57(2):43-56.
- [18]. Jegede, A. I., Offor, U., Azu, O.O., and Akinloye, O. (2015). Red Palm Oil Attenuates Lead Acetate Induced Testicular Damage in Adult Male Sprague-Dawley Rats. *Evidence-Based Complementary and Alternative Medicine*. , Article ID 130261, 7 pages
- [19]. Kempinans, W.G. and Lamano-Carvalho, T.L. (1988). A method for estimating the concentration of spermatozoa in the cauda epididymis. *Laboratory Animals*. 22: 154-156
- [20]. Koppers, A.J., De Iulius, G.N., Finnie, J.M., McLaughlin, E.A. and Aitken, R.J. (2008). Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *Journal of Clinical Endocrinology and Metabolism* 93: 3199–3207.
- [21]. Kothari, S., Thompson, A., Agarwal, A. and du Plessis, S.S. (2010). Free radicals: their beneficial and detrimental effects on sperm function. *Indian Journal of Experimental Biology* 48(5): 425-35
- [22]. Lobo, V., Patil, A., Phatak, A. and Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacology Review*. 4(8): 118-126
- [23]. Makker, K., Agarwal, A., and Sharma, R. (2009). Oxidative stress and male infertility. *Indian Journal of Medical Research*, 129:357-66
- [24]. Makler, A. (1980). The improved ten-micrometer chamber for rapid sperm count and motility evaluation. *Fertility and Sterility*. 33(3): 337-338
- [25]. Mc Cord, J.M. (2000). The evolution of free radicals and oxidative stress. *American Journal of Medicine*. 108:652–9
- [26]. Miranda, K.M., Espey, M.G., and Wink, D.A. (2001). A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. *Nitric Oxide*. 5(1): 62–71
- [27]. Momenti H.R., Mehranjani M.S., Abnosi M.H. and Mahmoodi M. (2009). Effects of vitamin E on sperm parameters and reproductive hormones in developing rats treated with para-nonylphenol, *Iranian Journal of Reproductive Medicine* 17(3): 111-116.
- [28]. Moorcroft, M.J., Davis, J. and Compton. R.G. (2001). Detection and determination of nitrate and nitrite: a review. *Talanta*. 54(5): 785-803
- [29]. Mustacich D.J. and Bruno R.S., Traber M.G. (2007). Vitamin E, In *Vitamin and Hormones (76): Vitamin E*, ed Mustacich D.J. and Bruno R.S., Traber M.G. pp 1-21, New York: Academic Press
- [30]. Novo, E. and Parola, M. (2008). Redox mechanisms in hepatic chronic wound healing and fibrogenesis. *Fibrogenesis and Tissue Repair*. 1:5
- [31]. Peh, H.Y., Tan, W.S., Liao, W. and Wong, W.S. (2016). Vitamin E therapy beyond cancer: Tocopherol versus tocotrienol. *Pharmacology Therapeutics*. 162:152-69.
- [32]. Pelliccione, F., Micillo, A., Cordeschi, G., D'Angeli, A., Necozone, S., Gandini, L., Lenzi, A., Francavilla, F. & Francavilla, S. (2011). Altered ultrastructure of mitochondrial membranes is strongly associated with unexplained asthenozoospermia. *Fertility and Sterility* 95: 641–646.
- [33]. Piknova, B. and Schechter, A. (2011). Measurement of Nitrite in Blood Samples Using the Ferricyanide-Based Hemoglobin Oxidation Assay. *Methods Molecular Biology*. 704: 39-56
- [34]. Rajesh Kumar T, Doreswamy K, and Shrilatha B. Muralidhara. (2002). Oxidative stress associated DNA damage in testis of mice: induction of abnormal sperms and effects on fertility. *Mutation Research*. 513:103–11
- [35]. Rao, A.L., Bharani, M., and Pallavi, V. (2006). Role of antioxidants and free radicals in health and disease. *Advance Pharmacology Toxicology*. 7:29–38.
- [36]. Sen C.K., Khanna S. and Roy S. (2007). Tocotrienols in health and disease: the other half of the natural vitamin E family. *Molecular Aspects of Medicine*, 28(5-6): 692-728.
- [37]. Suleiman S.A., Elamin Ali M., Zaki Z.M.S., El-Malik E.M.A and Nasr M.A. (1996), Lipid peroxidation and human sperm motility: Protective role of vitamin E, *Journal of Andrology* 17(5): 530-537
- [38]. Taib, I.S., Budin, S.B., Ghazali, A.R., Jayusman, P.A. and Mohamed, J. (2014). Fenitrothion Alters Sperm Characteristics in Rats: Ameliorating Effects of Palm Oil Tocotrienol-Rich Fraction. *Experimental Animal*. 63(4): 383-93

- [39]. Traber, M. G. and Stevens, J.F. (2011). Vitamins C and E: Beneficial effects from a mechanistic perspective. *Free Radical Biology and Medicine*. 51(5): 1000-13
- [40]. Tremellen, K. (2008). Oxidative stress and male fertility: a clinical perspective. *Human Reproduction Update*. 14:245-58
- [41]. World Health Organization (2010). WHO Laboratory manual for the examination and processing of human sperm. Fifth edition.
- [42]. Wolf, G. (2005). The discovery of the antioxidant function of vitamin E: The contribution of Henry A. Mattill. *Journal of Nutrition*. 135:363–6.
- [43]. Zink, M., Rabi, R. and Reichert, A.S. (2009). Assembly of the Mitochondrial Respiratory Chain : Cristae formation—linking ultrastructure and function of mitochondria. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 1793: 5–19