Effect of Vitamin E on Sperm Parameters, Chromatin Quality and Testosterone Hormone in Mice

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Abstract: The present study is carried out to investigate the protective effect of vitamin E (vit.E) on chromatin quality and sperm parameters (count, motility, viability and morphology) in mice**.** Antioxidants are the main defense factors against oxidative stress induced by free radicals. Vitamin E is believed to be the primary component of the antioxidant system of the spermatozoa and is one of the major membrane protectants against ROS and LPO attack. There is a relationship between activity of these antioxidant and function of sperm. Vitamins E and C which are belong to non-enzymatic antioxidant are used as a supplemented drug to improve sperm quality in male infertility. **Material and methods:** 16 adult male mice were divided equally into two groups each containing 8 mice .mice of group 1 served as control fed on basal diet, group 2 received basal diet and vitamin E (100 mg/kg, intraperitoneal) for 35 days. Blood was taken for the determination of serum testosterone. Finally, right tail of epididymis was cut in Ham's F10. Released sperm were used to analyze number, motility, morphology (Pap-staining) and viability (Eosin-Y staining) of the sperm and DNA integrity and chromatin condensation assessments were ready by standard cytochemical techniques including. (AOT): Acridine orange is a metachromatic fluorescence explore for the dimension of sperm nuclear DNA susceptibility to in situ acid-induced denaturation by distinctive between double-stranded DNA and single-stranded DNA. (AB): Aniline blue selectively stains lysine-rich histones and has been used for the purpose of those sperm chromatin condensation anomalies that are related to residual histones. (TB): Toluidine blue is a metachromatic dye which determines both the quality and the quantity of sperm nuclear chromatin. **Result:** In vitamin E mice, a significant increase was found in sperm number, sperm motility, sperm viability and sperm morphology compared to control group. A significant increase was also found in sperm AB+ (Mature sperm), TB- (normal) and Ao+ (double-stranded DNA) in vitamin E group compared to control group .the results suggest an increase in concentration testosterone in vitamin E mice compared to control mice, **Conclusion:** It was concluded, Vit.E not only is able to improvement the sperm parameters but also increases sperm chromatin quality in mice. Also vitamin E increases the level of testosterone hormone in mice.
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Introduction:

The World Health Organization (WHO) defines infertility as the inability of a couple to achieve conception or bring a pregnancy to term after 1 year or more of regular, unprotected sexual intercourse. Conception is normally achieved within 12 months in 80%–85% of couples using no contraceptive measures. Although certain cases of male infertility are due to anatomical abnormalities, such as varicocele, ductal obstructions, or ejaculatory disorders, an estimated 40%–90% of cases are due to deficient sperm production of unidentifiable origin. Infertility is a major clinical concern, affecting 15% of all reproductive age couples. Male factors, including decreased semen quality, are responsible for 25% of these cases. Currently, the etiology of suboptimal semen quality is poorly understood, and many physiological, environmental, and genetic factors, including oxidative stress, have been implicated (Mohammad K 2011).

Vitamin E is one of the most important antioxidative molecules, residing mainly in the cell membranes. It is thought to interrupt reactions with lipid peroxidation and is a free radical scavenger generated during the univalent reduction of molecular oxygen and also normal activity of oxidative enzymes (Palamanda JR 1993). These radicals will lead to peroxidation of phospholipids in the mitochondria of the sperm and thus to their ultimate immotility (De LamirandeE 1992). It is possible that vitamin E enhances the production of the scavenger antioxidant enzymes (Suleiman SA 1996).

Recently, the generation of oxidants, also described as reactive oxygen species (ROS), in the male reproductive tract has become a real concern because of their potential toxic effects, at high levels, on sperm quality and function (Sharma RK 1996). In the context of human reproduction, a balance normally exists between ROS production and antioxidant scavenging activities in the male reproductive tract. As

a result of such balance, only minimal amounts of ROS remain, and they are needed for the regulation of normal sperm functions, such as sperm capacitation, the acrosome reaction, and sperm–oocyte fusion (Aitken RJ 1999). The production of excessive amounts of ROS in semen can overwhelm the antioxidant defense mechanisms of spermatozoa and seminal plasma and causes oxidative stress (Sikka SC 2001). Production of high levels of ROS in the reproductive tract is detrimental not only to the fluidity of the sperm plasma membrane but also to the integrity of DNA in the sperm nucleus (Aitken RJ 1999). Strong evidence suggests that DNA fragmentation commonly observed in the spermatozoa of infertile men is mediated by high levels of ROS (Kodama H 1997).

It has been now documented that Vit.E is a potent scavenger of free radicals and is able to prevent the membrane damage mediated by free radicals (Gurel A 2005). In addition, the antioxidant's role of this vitamin has been reported in reducing testicular oxidative stress (Kutlubay R 2007).

A new approach to the microscopic assessment of sperm for investigation of male fertility is the evaluation of sperm nuclear chromatin (Talebi AR 2011 B). Hence male gamete supplies 50 % of the embryonic genome, any anomalies in sperm chromatin can affect embryonic development. It is generally accepted that there is a clear relation between sperm chromatin/DNA damage and reproductive outcomes (Talebi AR 2011 A). Furthermore sperm chromatin condensation has a key role in male fertility, early embryonic growth and pregnancy results (Talebi AR 2006). In the process of spermatogenesis, the extent of sperm chromatin compaction changes deeply when histones are replaced in a stride mode by testis-specific nuclear proteins, transitional proteins and finally by protamines. Each anomalies during expression of sperm-specific nucleoproteins change sperm chromatin structure and may cause male infertility (Talebi 2011 A). The inter and intra-molecular disulphide bonds of protamine molecules are crucial for sperm nuclear compaction and stabilisation. It is believed that this kind of nuclear compaction protects sperm genome from external damages include oxidative stress, temperature height and acid-induced DNA denaturation (Carrell DT 2007).

Materials and methods:

Animals and treatments:

During the course of this experiment, we followed the recommendations set forth by our Institutional Animal Care and Use Committee for the handling, maintenance, treatment, and killing of the animals. Detailed information about animals and treatments has been reported previously (Manjanatha 2006). Totaly 16 Adult male mice (10 weeks old, 35g) that they divided to 2 group each containing 8 mice. mice of group 1 served as control fed on basal diet ,group 2 received basal diet and vitamin E (100 mg/kg , intraperitoneal)(Gavazza M 2001) , They were held in cages and were housed in a controlled environment with a temperature range of 25±3℃ and mean relative humidity of 50±5%. The experimental proposal was agreed by our university ethics committee. On the 21st day after the last treatment, samples of blood were taken from the mice so that testosterone may be analysed, afterward the mice were killed.

Epididymal sperm preparation:

After 35 days (one duration of spermatogenesis in mice is about 32 days), a small part of the cauda epididymis of each mouse was dissected and located in 1 mL of pre-warmed Hams F10 medium (37℃, 5% CO2). Gentle tearing of the tissue was done to make spermatozoa swim out into the culture medium. The dishes were placed in the incubator for 15 min.

Sperm analysis:

Sperm count:

The dissected epididymis of each animal was transferred into 10 ml Ham's F10 medium and cut to small slices, in order to swim out the sperm in to the medium. After 10 min of diffusion, 1 ml of the solution was diluted with 9 ml formaldehyde fixative. The diluted solution was transferred into each chamber of Neubauer hemocytometer and sperm heads was manually counted under a microscope. Sperm count was performed according to WHO guidelines (WHO 1999) and data were expressed as the number of sperm per ml. (Momeni H R 2009)

Sperm motility:

Assessment of sperm motility was done according to WHO protocol (WHO 1999). In brief, 10 µl of the sperm suspension was placed on a microscopic slide and cover sliped. A minimum of five microscopic fields were assessed to evaluate sperm motility on at least 200 sperm for each animal. The percentage of sperm motility was analyzed for following motion parameters: Motility was expressed as percentage of progressive (fast and slow) and non-progressive spermatozoa (Momeni H R 2009).

Sperm viability test:

Eosin-nigrosin staining was used to asses' sperm viability according to WHO protocol (WHO 1999). Briefly,eosin (1%, Merck, Germany) and nigrosin (10%,Merck, Germany) was prepared in distilled water. One volume of sperm suspension was mixed with two volume of 1% eosin. After 30 second, an equal volume of nigrosin was added to this mixture. Thin smears were then prepared and observed under

alight microscope at ×100 magnification. Viable sperm remained colorless while nonviable sperm stained red. (Momeni H R 2009).

Sperm morphology:

For studying the sperm morphology, a drop of sperm suspension was smeared on to a clean glass slide. The smear was then air dried and fixed in amixture of equal parts ethanol and ether. The slides were then stained with Papanicolaou stain. Dried stained slides were scanned under oil immersion (100 objectives) for morphological abnormalities. A total of 100 sperms per sample were classified according to their morphology; such as normal, coiled mid piece, hair pin (a kink at the annulus, usually 180°), bent tail (a kink at the annulus, usually90°), coiled tail, double head, amorphous head, triangular head, pin head and cytoplasmic droplet. Sperm abnormality was expressed as percent. (Zohre Zare 2010)

Sperm chromatin/DNA evaluation:

DNA integrity and chromatin condensation assessments were ready by standard cytochemical techniques including acridine orange test (AO), aniline blue (AB), toluidine blue (TB) and chromomycin A3 (CMA3) .All dyes and chemicals were purchased from Sigma Aldrich Company (St Louis,MO, USA).(Talebi AR 2011 C).

Aniline blue (AB) staining:

Aniline blue selectively stains lysine-rich histones and has been used for the purpose of those sperm chromatin condensation anomalies that are related to residual histones. To do this staining, air-dried smears were set from washed semen samples and then fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature. Each smear was stained with 5% aqueous AB stain in 4% acetic acid (pH=3.5) for 7 min. In light microscopic evaluation, 200 spermatozoa were counted in different areas of each slide using×100 eyepiece magnification (Talebi AR 2011 A).

Toluidine blue (TB) staining:

Toluidine blue is a metachromatic dye which determines both the quality and the quantity of sperm nuclear chromatin condensation/DNA fragmentation via binding to phosphate groups of DNA strands (Joao Ramalho 2009). Briefly, air-dried sperm smears were permanent in fresh 96% ethanol–acetone (1: 1) at 4 ℃ for 30 min and then hydrolysed in 0.1 N HCl at 4 ℃ for 5 min. after that, the slides were rinsed 3 times in distilled water for 2 min and in the end stained with 0.05% TB in 50% citrate phosphate for 10 min at room temperature. In each sample, at least 200 spermatozoa were counted under light microscopy using × 100 eyepiece magnifications (Talebi AR 2008).

Acridine orange test (AO):

Acridine orange is a metachromatic fluorescence probe for demonstration of degree of sperm nuclear DNA susceptibility to in-situ acid-induced denaturation by distinction between native double-stranded DNA (green fluorescent) and denatured single-stranded DNA (red fluorescent). Briefly, the air-dried smears were fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) at 4℃ for at least 2 hrs. Each sample was stained by freshly prepared AO (0.19 mg/ml in McIlvain phosphate–citrate buffer (pH=4) for 10 min. Smears were assessed on the same day using fluorescent microscope (Zeiss Co., Jena, Germany) with a 460-nm filter (Talebi AR 2011 A).

Statistical analysis:

Statistical analysis was performed by spss 18 for Windows (SPSS Inc., Chicago, IL, USA). Student's t-test was applied to evaluate the data and the term 'statistically significant' was used to signify a two-sided *P value*< 0.05 for sperm parameters and cytochemical tests.

Results:

Table 1 shows the means and statistical analysis of the various sperm parameters in two groups. This table reveals that sperm count, rapid and total motilities, morphology and viability were significantly different (P<0.05) between Groups A and B. Table 2 shows the results of analysis of sperm chromatin and DNA integrity. Regarding to AO and AB tests, we saw significant differences (P<0.05) between two groups, but TB staining didn't have any differences between control and diabetic mice.

It should be noted that in AB staining, the percentages of unstained or pale blue stained (normal spermatozoa) and dark blue stained (abnormal spermatozoa) were reported.

In TB staining, the chromatin quality of sperm was assessed according to metachromatic staining of sperm heads in following scores: 0, light blue (good chromatin); 1, dark blue (mild abnormal chromatin); 2, violet; and 3, purple (severe chromatin abnormality). So, the sum of spermatozoa with score 1, score 2 and score 3 was considered as TB+ or sperm cells with abnormal chromatin, whereas score 0 spermatozoa were considered as TB- or spermatozoa with normal chromatin. For AO, the percentages of green (normal double-stranded DNA) and orange/red (abnormally denatured DNA) fluorescence spermatozoa per sample were calculated.

Table 3 shows the Results of the Effect of vitamin E on the Serum Testosterone, Evaluation of the blood serum Testosterone analysis revealed that the concentration of Testosterone in vitamin group was increased in comparison to the control group,

Table 1: The results of semen analysis in control (Group A) and vitamin E mice (group B).

Variables	Control mice (group A)	Vitamin E mice (group B)	p-value
	Mean±SD	Mean±SD	
Count($\times 10^6$)	110±17.493	132.5±19.116	0.038*
Rapid motility(%)(Grade a)	20.75±3.011	27.75±3.327	0.038*
Slow motility(%)(Grade b)	23.25±4.862	27.625±4.627	0.13
Non progressive motility(%)(Grade c)	32±4.375	26±7.946	0.105
Immotile sperm(%)(Grade d)	24±4.276	17.625±5.998	0.038*
Total motility(%)(Grade a,b,c)	76±4.276	82.375±5.998	0.038*
Normal morphology	75.875±6.728	83.75±7.382	0.028*
Viability (%)	78.125±5.083	74.5±5.723	0.015*

*Statistically significant (T-Test), P value <0.05

Table 2: The results of sperm chromatin/ DNA evaluation in control (group A) and vitamin E mice (group B)

Variables	Control mice (group A)	Vitamin E mice (group B)	p-value
	Mean±SD	Mean±SD	
AO	8.375±3.02	5±1.603	0.021*
TB	20.5±2.39	17.875±3.181	0.105
AB	22.5±4.72	16±4.84	0.01*

*Statistically significant, P value <0.05

Table 3: the Results of the Effect of Vitamin E on the Serum Testosterone in control (Group A) and vitamin E mice (group B)

Variables	Control mice (group A)	Vitamin E mice (group B)	p-value
	Mean±SD	Mean±SD	
Testosterone	3.25±1.582	6.087±2.494	0.01*

*Statistically significant, P value <0.05

Discussion:

Tocopherol (vitamin E) is the primary lipidsoluble small molecule antioxidant in biologic systems. As such, tocopherol was present, as expected, in only small amounts in seminal plasma (0.3 to 0.5 µmol/l). Ascorbate and tocopherol cooperate to protect lipid structures against peroxidation. Ascorbate recycles tocopherol by repairing its tocopheroxyl radical, thereby permitting it to function again as a free radical chain-breaking antioxidant (Buettner GR 1993).

VE could also improve the sperm motility in human. Treatment of asthenospermic patients with oral VE significantly decreased the lipid peroxidation in sperm and improved sperm motility (Suleiman 1996). There is evidence to show that infertile men possess substantially more sperm DNA damage than do fertile men and that this DNA damage may adversely affect reproductive outcomes(Zini A 2008) .More recent studies of infertile men with high levels of sperm DNA damage have shown that antioxidant therapy is effective in improving sperm DNA integrity or pregnancy rates. In men with unselected infertility, the effect of dietary antioxidants on sperm DNA integrity is equivocal with one of two controlled trials showing a benefit of antioxidants on sperm DNA integrity.

The levels of sperm-derived ROS (measured in sperm preparation shaving minimal leukocyte contamination) have been associated with sperm DNA damage, although no ROS thres hold level above which sperm DNA damage is detected has been established (Irvine DS 2000) (Barroso G 2000) (Saleh

RA 2003). Moreover, the levels of sperm DNA oxidation are higher in infertile compared to fertile men (Shen HM 1999) (Kodama H 1997) .Studies Caoshowed that increased oxidative stress, enzymatic and non-enzymatic antioxidant is reduced levels in leydig cells and an important factor for impaired spermatogenesis and consequently a significant reduction in epididymal sperm count(Cao L 2004) Our results also raise the sperm count and normal sperm in the vitamin E group than the control group and This is due to the effect of vitamin E on antioxidant enzymes oxidant effects could improve. Also, movement and viability in the vitamin E group compared to the control group showed significant increases the study is consistent with Armit and colleagues studied the vitamin E reduced the LPO caused by FeAA, and improved sperm motility and viability in vitro under induced oxidative stress. (Amrit KB 2008).

The other parameters which were compared between diabetic mice and control ones were sperm chromatin quality. As it was mentioned before, the incidence of any anomaly in testicular expression and incorporation of every category of sperm-specific nucleoprotein may change sperm chromatin structure and will cause male infertility (Talebi 2011 A)

Spermatozoa have only two defence mechanisms against oxidative attack of their DNA; the packaging arrangement of the DNA, and the seminal plasma. During spermatogenesis, the chromatin becomes highly condensed within a protamine matrix (Sidney 1986). The DNA is organized into loops, attached at their bases to the nuclear matrix, anchored to the base of the sperm tail by the nuclear annulus and stabilized by disulphide bonds (Ward, 1993; Barone 1994). This tight packing of the DNA reduces exposure to free radical attack. The second line of defense is the antioxidant capacity of its seminal plasma (Lewis 1995; 1997).

Previous studies have shown that antioxidant treatment improves sperm nuclear DNA integrity in men with elevated sperm DNA damage (Kodama 1997, Geva1998, Comhaire2000, Keskes-Ammar 2003). However, reports concerning the clinical usefulness of antioxidants in the treatment of male infertility are controversial (reviewed in Agarwal 2004). This is the first study in which the possible effect of this treatment by Aniline blue (AB) staining, Toluidine blue (TB) staining and Acridine orange test (AOT)

In TB- staining, although we saw the difference in percentage of TB- reacted spermatozoa between two groups, but it was not statistically significant. This showed that Vitamin E a small amount can makein both the quality and the quantity of nuclear chromatin condensation and reduction the sperm DNA fragmentation. Hammadeh showed that DNA damage is induced by oxidative assaults but the physiological antioxidant situation in the seminal plasma does not seem to be strongly associated with either a reduction in the DNA damage or an increase in the decondensation (Hammadeh 2006).

In AO test, we saw notable difference between two groups. As the AO test has the potential to differentiate the single-stranded DNA from double-strand ones, it can be concluded that the vitamin E has decreased the denaturation of sperm DNA strands. It should be considered that this finding was obtained by other researcher but using different assessment. According to our results, Ciara M.Hughes and colleagues showed DNA damage was induced by 30 Gy X-irradiation. DNA strand breakage was measured using the comet assay. Sperm DNA was protected from DNA damage by ascorbic acid (600 mM), alpha tocopherol (30 and 60 mM) and urate (400 mM). These antioxidants provided protection from subsequent DNA damage by X-ray irradiation. In contrast, acetyl cysteine or ascorbate and alpha tocopherol together induced further DNA damage. Supplementation in vitro with the antioxidants ascorbate, urate and alpha tocopherol separately has beneficial effects for sperm DNA integrity. (Ciara M.Hughes 1998).

In AB staining that shows the sperm cells with excessive histones, we did found a significant difference between groups. We can say that the vitamin E does have improvment effects on histone-protamines replacement during the testicular phase of sperm chromatin packaging. To compare our data with others, we didn't see any similar study by this test in literature.

It is indicated that the level of oxidative stress is high in hyperglycemia state (Steger RW 1997, Mahesh T 2004), due to excess production of reactive oxygen species (ROS) and decreased efficiency of anti-oxidant enzyme defences (Steger RW 1997). Oxidative stress is harmful to sperm function and a significant factor in the etiology of male infertility (KartikeyaMakker 2009). In addition, oxidative stress impairs male fertility by changing the cell function like sperm motility (KartikeyaMakker 2009 , Agarwal A 2005), increase in DNA damage by induction of gene mutations, DNA denaturation, base pair oxidation and DNA fragmentation (Agarwal A 2005,).

In conclusion, our study showed that in the cases of vitamin E, almost Majority of the sperm parameters had a statistically significant Increase in comparison with controls and also we demonstrated that spermatozoa of vitamin E mice had more chromatin condensation and Increase DNA integrity than spermatozoa of control group.

References:

1. Palamanda JR, Kehrer JP. Involvement of vitamin E and protein thiols in the inhibition of microsomal lipid peroxidation by glutathione.*Lipids*. 1993;28(5):427–431.
2. De Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. *J Androl*. 1992; 13(5):368–378.
3. Suleiman SA, Ali ME, Zaki ZM, el-Malik EM, Nasr MA. Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl*. 1996; 17(5):530–537.
4. Mohammad K, Moslemi, SamanehTavanbakhsh. Selenium–vitamin E supplementation in infertile men: effects on semen parameters and pregnancy rate. International Journal of General Medicine 2011;4 :99–104.
5. Aitken RJ. The Amoroso lecture: the human spermatozoon—a cell in crisis? J ReprodFertil 1999; 115:1- 7.
6. Sikka SC. Relative impact of oxidative stress on male reproductive function.Curr Med Chem 2001; 8:851- 62.
7. Kodama H, Yamaguchi R, Fukuda J, et al. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. FertilSteril 1997; 65:519-24.
8. Gurel A, Coskun O, Armutcu F, Kanter M, Ozen OA. Vitamin E against oxidative damage caused by formaldehyde in frontal cortex and hippocampus: biochemical and histological studies. *J ChemNeuroanat* 2005; 29: 173-178.
9. Kutlubay R, Oguz EO, Can B, Guven MC, Sinik Z, Tuncay OL. Vitamin E protection from testicular damage caused by intraperitonealaluminium.*Int J Toxicol* 2007; 26: 297-306.
10. Manjanatha MG, Aidoo A, Shelton SD, Bishop ME, McDaniel LP, Lyn-Cook LE, and Doerge DR. Genotoxicity of acrylamide and its metabolite glycidamide administered in drinking water to male and female Big Blue mice. Environ Mol Mutagen 2006;47:6–17.
11. Gavazza M, CataláA.The effect of alpha-tocopherol on the lipid peroxidation of mitochondria and microsomes obtained from rat liver and testis.Mol Cell Biochem. 2001; 225(1):121-8.
12. Carrell DT, Emery BR, Hammoud S .Altered protamine expression and diminished spermatogenesis: What is link?HumReprod. 2007; 13:313–327.
13. Joao Ramalho – Santos, sandraAmaral, pauloj, oliveira. Diabetes and the impairment of reproductive function: possible role of mitochondria and reactive species. Bentham science.2009; 4(1): 1573-3993
14. World Health Organization (WHO) laboratory manual forthe examination of human semen and sperm-cervicalmucus interaction.1999, 4th ed. Cambridge universitypress.
15. Momeni H R, soleimaniMehranjani M, Abnosi M H, Mahmoodi M. Effects of vitamin E on sperm parameters andreproductive hormones in developing rats treated withpara-nonylphenol.Iranian Journal of Reproductive Medicine. 2009;7(3):111-116
16. ZohreZare, HosseinEimani, Moslem Mohammadi, MahmoodMofid, HosseinDashtnavard. The Effect of Orally Administered L-carnitine on Testis Tissue, Sperm Parameters and Daily Sperm Production in Adult Mice .Yakhteh medical journal 2010; 11(4): 382-389
17. Talebi AR, Vahidi S, AflatoonianA,et al. Cytochemical evaluation of sperm chromatin and DNA integrity in couples with unexplained recurrent spontaneous abortions. J Androl.2011;1-9**A**
18. Talebi AR. Sperm nuclear maturation.1th Ed.Nova science publishers,Inc.New York ;2011:45-55. **B**
19. TalebiAR, Mohammad Ali Khalili, HosseinNahangi, AbulghasemAbbasi, Morteza Anvari. Evaluation of epididymalnecrospermia following experimental chronic spinal cord injury in rat.Iranian Journal of Reproductive Medicine.2006; 5(4): 171-176
20. Talebi AR. Abbasi A. Khalili,etal.Effect of ethanol consumption on chromatin condensation and DNA integrity of epididymal spermatozoa in rat. Androl 2011;45:403-409 **C**
21. Talebi AR, Moein MR, Tabibnejad N, Ghasemzadeh J. Effect of varicocele on chromatin condensation and DNA integrity of ejaculated spermatozoa using cytochemicaltests.Andrologia 2008; 40: 245–251
22. Lolis D, Georgiou I, Syrrou M, Zikopoulos K, Konstantelli M. Chromomycin A3-staining as an indicator of protamine deficiency and fertilization. Int J Androl 1996; 19:23–27.
23. Buettner GR. The pecking order of fl'ee radical's andantioxidants, lipid peroxidation, alpha-tocopherol and ascorbate. *Arch Biochem Biophys*1993; 300: 535-543.
24. Suleiman, S.A., Ali, M.E., Zaki, Z.M.S., El-Malik, E.M.A., Nasr, M.A. Lipid peroxidation and human sperm motility: protective role of vitamin E. J. Androl 1996; 17: 530– 537.
25. Armand Zini&Maria San Gabriel &AbdulazizBaazeem, Antioxidants and sperm DNA damage: a clinical perspective, J Assist Reprod Genet 2009; 26:427–432.
26. Zini A, Boman JM, Belzile E, Ciampi A. Sperm DNA damage isassociated with an increased risk of pregnancy loss after IVF andICSI: systematic review and meta-analysis. Hum Reprod 2008; 23:2663–8.
27. Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA, Aitken RJ.DNA integrity in human spermatozoa: relationships with semenquality. J Androl 2000; 21:33–44.
28. Barroso G, Morshedi M, Oehninger S. Analysis of DNAfragmentation, plasma membrane translocation of phosphatidylserine and oxidative

stress in human spermatozoa. Hum Reprod 2000; 15:1338–44.
29. Saleh RA, Agarwal A, Sharma RK, Said TM, Sikka SC, ThomasAJ Jr. Evaluation of nuclear DNA damage in spermatozoa frominfertile men with varicocele. FertilSteril 2003; 80:1431–6.
30. Shen HM, Chia SE, Ong CN. Evaluation of oxidative DNAdamage in human sperm and its association with male infertility. JAndrol 1999; 20:718–23.
31. Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T.Increased oxidative deoxyribonucleic acid damage in the spermatozoaof infertile male patients. FertilSteril 1997; 68:519–24.
32. Cao L, Leers-Sucheta S, Azhar S. Aging alters the functional expression of enzymatic and non-enzymatic anti-oxidant defense systems in testicular rat Leydig cells. J Steroid BiochemMol Biol 2004; 88(1):61-7.
33. AmritKaurBansal, Gurmail Singh Bilaspuri. Antioxidant effect of vitamin E on motility,viability and lipid peroxidation of cattle spermatozoa under oxidative stress .Animal Science Papers and Reports 2008; 27 (1): 5-14.
34. Sidney, R. and Grimes, J.R . Nuclear proteins in spermatogenesis. Comp. Biochem. Physiol 1986 ;83**:** 495–500.
35. Ward, W.S. DNA loop domain tertiary structure in mammalian spermatozoa. Biol. Reprod 1993;48**:** 1193–1201.
36. Barone, J.G., De Lara, J., Cummings, K.B. and Ward, W.S., DNA organisation in human spermatozoa. J. Androl 1994; 15**:**139–144.
37. Lewis, S.E.M., Sterling, S. Young, I. and Thompson, W., Comparison of individual antioxidants and total antioxidant capacity of sperm and seminal plasma in fertile and infertile men.Fertil.Steril 1997;67:142–147.
38. Kodama H, Yamaguchi R, Fukuda J, Kasai H and Tanaka T., Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertilemale patients. FertilSteril 1997;68:519–524.
39. Geva E, Lessing JB, Lerner-Geva L and Amit A . Free radicals, antioxidants and human spermatozoa: clinical implications. Hum Reprod 1998;13: 1422–1424.
40. Comhaire FH, Christophe AB, Zalata AA, Dhooge WS, Mahmoud AM andDepuydt CE . The effects of combined conventional treatment, oralantioxidants and essential fatty acids on sperm biology in subfertile men.Prostaglandins LeukotEssent Fatty Acids 2000;63:159–165.
41. Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghozzi H,Hammami S, Zghal K, Fki H, Damak J and Bahloul A . Sperm oxidativestress and the effect of an oral vitamin E and selenium supplementon semen quality in infertile men. Arch Androl 2003;49: 83–94.
42. Agarwal A, Nallella KP, Allamaneni SSR and Said TM . Role of antioxidantsin treatment of male infertility: an overview of the literature.Reprod Biomed Online 2004; 8:616–627.
43. Hammadeh DE, RadwanM, Al-Hasani S,. Comparision of reactive oxygen species consentration in seminal plasma and semen parameters in partners of pregnant and non-pregnant patients after IVF/ICSI, Reproductive Bio Medicine online 2006; 13:696-706
44. Steger RW, Rabe MB: The effect of diabetes mellitus on endocrine and reproductive function. ProcSocExpBiol Med 1997; 214: 1–11.
45. Mahesh T, Menon VP. Quercetin alleviates oxidative stress in streptozotocin-induced diabetic rats. J. of Phytother.Res. 2004; 18:123 – 7.
46. KartikeyaMakker, Ashok Agarwal and Rakesh Sharma.Oxidative stress and male infertility .Indian J. Med. Res 2009; 129: 357-367.
47. Agarwal A, Said TM. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. Br J UrolInt 2005; 95: 503–7.
48. Ciara M.Hughes, Sheena E.M.Lewis, Valerie J.McKelvey-Martinand W.Thompson. The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. Human Reproduction 1998;13(5) 1240–1247.

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