

Research Article

Endogenous and exogenous antioxidants status in seminal plasma of skeletal fluorotic patients

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Abstract: Fluoride contamination in water (>1.5ppm) is the global problem for health in general. Fluoride has been reported to be a causative factor for male infertility. However, limited scientific literature is available on this aspect. The objective of the present study was to examine the fluoride induced oxidative burden and its association with endogenous and exogenous antioxidant status in seminal plasma of different grade of fluorotic patients (mild, moderate and severe). The lipid peroxide levels and antioxidant status namely superoxide dismutase, catalase, glutathione vitamins (A, C and E) were investigated in seminal plasma. Result of the present study showed that the LPO was found to be gradually increased with severity of the disease. The endogenous and exogenous antioxidants were found to be decreased in severe fluorotic patients. On the basis of the results it may conclude that fluoride exposure promote oxidative stress and alteration in vitamins in seminal plasma. These alterations may induce pathophysiological activities due to lack of vitamin and antioxidant defense against ROS. However, further in depth studies is required for the understanding of pathophysiology of Fluorosis.

Keywords: Seminal plasma, Fluorosis, Antioxidant, Vitamins, NRCFPI.

INTRODUCTION:

Fluorosis poisoning from long-term exposure to high levels is a serious health problem in many parts of the world where drinking water contains more than 1.5 ppm of fluoride [1]. An estimated 66.6 million people (17 states in India) are at risk of acquiring fluorosis [2]. In Rajasthan, people of 22 districts (out of 32) are presently consuming fluoride [3-4] greater than permissible limit. There are various researches have been conducted to explore the relationship between fluoride ingestion and reproductive structure in animal models [5-6]. The reproductive toxic effects are abnormal spermatozoa, loss of spermatogenesis in rats, decreased sperm quality and quantity. The mechanisms by which fluoride produce such effects are still not clear. Animal studies demonstrated that fluoride generates reacting oxygen species (ROS). ROS have been implicated in a variety of conditions relevant to the function of spermatozoa. Several studies has been reported that spermatozoa itself release hydrogen peroxide and superoxide radical [7-8] results is the loss of motility and peroxidation of spermatozoal lipids [9-10]. ROS exert both toxic actions and participate in physiological processes such as capacitation and several erectile functions. The seminal plasma is rich in superoxide dismutase (SOD) activity [11-12]. SOD is the first antioxidant enzyme which reflects to reduce superoxide radicals. Catalase is an also important enzyme to deplete hydrogen peroxide. Therefore, a

greater understanding, at biochemical levels, of the fluoride toxicity is very important in clinical samples. Glutathione is a tripeptide endogenous antioxidant which plays a key role in the defense against oxidative damage. It is found within the sperm and in the seminal plasma. GSH is able to protect sperm plasma membrane by detoxifying cytotoxic aldehydes which produced during lipid peroxidation [13]. Moreover, It has also been reported that GSH when given exogenously to infertile men, was found to cause significant increase in sperm motility [14-15].

Vitamins are important for the protection of oxidative stress as an exogenous antioxidant [16]. It also helps to modulate sperm quality. Vitamin E (α -tocopherol) is implicated in neurological and immune functions and protects the cells from potential deleterious effects of free radicals. Vitamin E is also involved in the control of enzyme activity to stabilise biological membrane cells [17]. It is reported that vitamin C and E improved male fertility by increasing sperm concentration and total motile sperm and decreasing abnormal and dead sperm in rabbits [18] [19].

Keeping in view the paucity of information in relation to high fluoride exposure in population residing in endemic areas and its impact on reproductive system, the present study was undertaken. The significance of

this study is to investigate the fluoride induced oxidative stress in seminal plasma of fluorotic patients.

MATERIALS AND METHODS:

Chemicals. Nitroblue tetrazolium (NBT), thiobarbituric acid (TBA), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide (NADH), 5,5'-dithio bis 2-nitrobenzoic acid (DTNB), 4,5 methyl thiazol-2-yl 2,5 diphenyltetrazolium bromide (MTT), nicotinamide adenine dinucleotide phosphate (NADPH) trichloroacetic acid (TCA) and reduced glutathione (GSH) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Subject selection: A total of 60 men, reproductive age group (between 25 and 40 years) were selected from eastern region of Rajasthan, India where fluoride content in drinking water (Figure-1) was more than 2.0 ppm. The study group comprised subjects who were further allocated into three subgroups of 20 each on the basis of the severity of the disease. After the scoring of Chin Chan test (WHO) and serum fluoride levels subjects were divided into three categories.

The controls and subjects were recruited using personnel interview as a tool for data collection, detail information of the subjects were recorded on the predesigned proforma that includes age, educational level, socio-economic status, working schedule, duration of exposure, male contraceptive users, smoking, and other addiction history, marital status, and number of children, history of disease of the individual subjects, and his family (Table-1). All qualified subjects completed a face-to-face interview questionnaire, which included information about demographic parameters, living habits, and diseases of reproductive organs (previous or current genital diseases such as cryptorchidism, inguinal hernia, varicocele, epididymitis, gonorrhoea, chlamydia, and surgery for torsion of the testis).

Preparation of Seminal Plasma: Semen samples were collected from the subjects and controls in a clean, dry, sterilized, wide mouth, well stopper glass vial by masturbation after 2–5 days of abstinence. After liquefaction, semen samples were centrifuged at $1200 \times g$ in cold ($4^\circ C$) for 20 min for the separation of seminal plasma. The supernatant (seminal plasma) was centrifuged again at $10\,000 \times g$ in cold ($4^\circ C$) for 30 min to eliminate all possible contaminating cells and stored at $-20^\circ C$ until analyzed. All biochemical estimations were carried out on seminal plasma.

Lipid peroxidation: The thiobarbituric acid reacting substances (TBARS) of the sample were estimated spectrophotometrically at 532 nm and expressed as nmole of MDA /g tissue [12].

Endogenous antioxidants: The SOD activity was expressed as U/mg protein (1 unit is the amount of

enzyme that inhibits the reduction of NBT by one half in above reaction mixture). Catalase (CAT, EC 1.11.1.6) activity was assayed as per the method of Aebi *et al* (1974) [21] using hydrogen peroxide as substrate; the decomposition of H_2O_2 was followed at 240nm on spectrophotometer. The CAT activity was expressed as U/mg protein. Reduced glutathione was measured in deproteinized supernatant of the cerebellum. Tissue homogenate was deproteinated with tetrachloroacetic acid, centrifuged and supernatant was used for the estimation of reduced glutathione (GSH) with the help of Ellman reagent (5, 5' dithiobis (2-nitro benzoic acid). The optical density of the pale colour was measured on the spectrophotometer on 412 nm. An appropriate standard (pure GSH) was run simultaneously. The level of GSH was expressed as $\mu g / g$ tissue [34].

Exogenous antioxidant: Vitamins A and E were measured by highperformance liquid chromatography (HPLC) as per the modified method of Omu *et al.* [22]. α -tocopherol acetate and retinol acetate were pipetted into an Eppendorf tube. To this, seminal plasma was added and vortex mixed. Hexane extract of vitamins A and E was aspirated out in a glass tube, dried under nitrogen stream and dissolved into methanol. Finally, this preparation was injected into HPLC apparatus fitted with a reverse phase C-18 stainless steel column. The vitamins were eluted with methanol at a flow rate of 1.5ml/min for 15min. The peak height and the curve area of vitamins A and E and their acetate were measured on ultraviolet detector with 292nm filters to calculate the amount of these vitamins in seminal plasma.

RESULTS AND DISCUSSION:

Two decades before researchers were focused on reports of decreasing human sperm counts and increasing abnormalities in human testes due to environmental factors and occupational exposure of trace elements, environmental pollution, and pesticides [23-24]. Among environmental hazards, fluoride ion is able to exert powerful effects on various enzymes and endocrine gland functions that affect or control the status of oxidant/antioxidant systems in living organisms. F exposure is defined by the biochemical and physiological stress in the body by generating imbalance between ROS and antioxidants thereby inducing oxidative stress and inhibiting several groups of enzymes of the defense mechanism [25-26]. It is reported that Fluoride exposure leads to oxidative stress as indicated by an increased level of lipid peroxide products in the testis, epididymis, and epididymal sperm with respect to control [27-28]. As evident in our study (Fig-2), we observed significantly ($p < 0.01$) increased amount of lipid peroxide levels were observed by 11%, 44% and 69% in mild, moderate and chronic group of fluorosis. The concentration of MDA were reflects the severity of the diseases. Inkielewicz and Krechniak [29] reported that F exposure causes

oxidative stress in animals. Spermatozoa membrane contains large amount poly unsaturated fatty acid and

since they vulnerable to lipid peroxidation [30].

Table-1 Demographic data of control and subject

	Control (N=25)	Subjects		
		Mild (N=20)	Moderate(N=20)	Chronic (N=20)
Age	32.5±6.8	31.8 ±7.2	32.6 ±5.8	33.4 ±5.2
Socio-economic status	Lower (100%)	Lower (100%)	Lower (100%)	Lowe (100%)
Married	100%	100%	100%	100%
Literacy (H.Sc.)	100 %	100 %	100 %	100 %
Smokers	63%	56%	67%	69%
Alcoholic (Occasionally)	4.3%	3.5%	4.1%	4.3%
Drug addicted	Nil	Nil	Nil	Nil
Male Contraceptive	Nil	Nil	Nil	Nil

Data are expressed as mean ± SD (age) and rest of the parameters in percentage for control and subjects

SOD is present within both sperm and seminal plasma [31-32]. It is reported that SOD protect them from oxidative attack in cells [33]. In the present study, antioxidant enzymes namely SOD and CAT were found to be reduced significantly ($p < 0.05$) in moderate and chronic stages of fluorosis. The SOD was reduced by 20% and 34% in moderate and chronic subject respectively, when compared with controls. Activity of catalase was also reduced by 15% and 30% in moderate and chronic subject respectively as compared to age matched controls. In addition, many research provided evidenced that deficient catalase activity in infertile seminal plasma [34-36]. The glutathione is an important antioxidant. It was found to be significantly ($p < 0.05$) reduced by 19% in chronic fluorotic patient when compared with the controls. Our results is concomitant with the finding of the previous study they have reported a positive correlation of GSH and sperm quality [37-38].

Fluoride in ground water

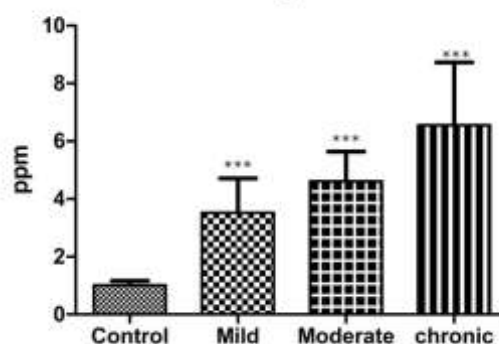


Figure1. The concentration of fluoride in ground water is expressed as mean±SD for control and subjects. Significance comparison was carried out using One way ANOVA followed by dunnett test.

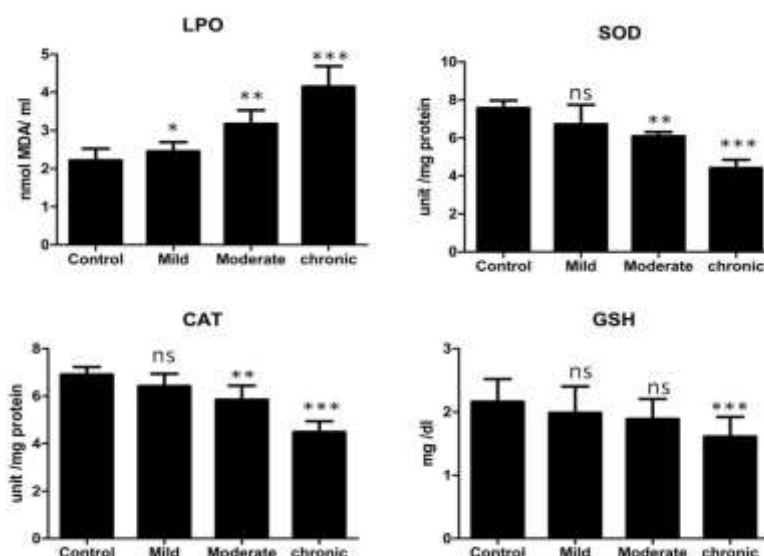


Figure 2. The lipid peroxide and antioxidant levels are expressed as mean ± SD for control and subjects. Significance comparison was carried out using One way ANOVA followed by dunnett test. Star of the columns (*) represents significance levels and ns represent not significant.

In the present study, three vitamins namely vitamins A, C and E were investigated that are naturally present in seminal plasma (Fig-3). These agents principally act by directly neutralizing free radicals. Several studies have been reported a significant reduction in non-enzymatic antioxidant activity in seminal plasma of infertile men. [39-43]. Earlier, It was reported that vitamin A deficiency causes degeneration of seminiferous tubular elements of testes and resulted in male infertility [44-45]. Vitamin A is also essential for testes and sperm. Moreover it is observed that lipid of testis was change with vitamin A deficiency [46].

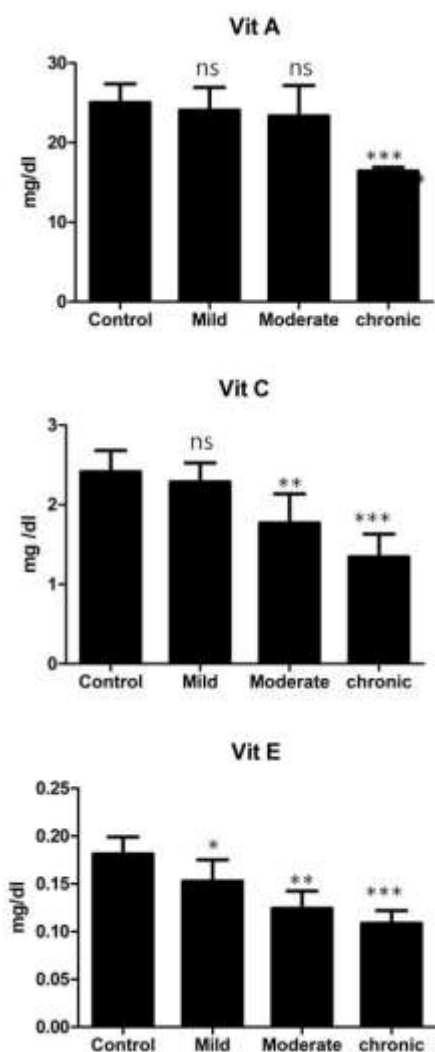


Figure 3. The concentrations of vitamins (A, C & E) are expressed as mean \pm SD for control and subjects. Significance comparison was carried out using One way ANOVA followed by dunnett test. Star of the columns (*) represents significance levels and ns represent not significant.

Our study indicated that reflection of reduced by 32% Vitamin A concentration in chronic fluoride intoxicated subject when compared with the controls. It is suggested that, reduced vitamin A concentration may reflects the degeneration of seminal lipids. On the other

hand the concentration of vit-C was found to be markedly ($p < 0.05$) reduced by 27% and 41% in moderate and chronic fluorotic patients respectively, as compared with the controls. While, the concentration of vitamin E were found to be significantly ($p < 0.05$) reduced by 16%, 31% and 29% in mild, moderate and severe fluorotic patients as compared to controls. It is suggested that fluoride gradually reduces the concentration of vitamin E in the seminal plasma as per the severity of the fluorosis. It is believed that Vitamin E (vit E) protects molecular and morphology of the cell, primarily through destruction of cell damaging ROS. Chinoy and Sharma [47] has also been reported that vitamin E is beneficial on fluoride induced reproductive functions and fertility. Human seminal plasma have several exogenous and endogenous antioxidants that play a vital role in the normal function of sperm. Many studies suggested that decreased levels of antioxidants in seminal plasma might be a potential cause of infertility.

CONCLUSION:

On the basis of results it may conclude that fluoride reduces the antioxidant status and increases lipid peroxide levels in seminal plasma which may reflect to male infertility. Intoxication of fluoride is more prone to increased burden of oxidative stress. Severity of the deaseses (fluorosis) is one of the factor for the reduced seminal quality. The present study suggested that incresed supplimentation is required to increse semen quality. However, further in depth studies is required for the understanding of pathophysiology of fluorosis and its association with reduced sperm quality.

References:

1. WHO, Environmental Health Criteria for Fluorosis and Fluoride; World Health Organization, Geneva, 1984: 1-136.
2. ICMR, RMRCT, Update, A Biannual newsletter of regional medical research centre for tribals, Jabalpur, 2004; 1 : 2.
3. Samal, U.N. and B.N. Naik; Dental Fluorosis in school children in the vicinity of an Aluminium factory in India. Fluoride, 1988; 21:142-148.
4. Yadav AK, Jain PK and Lal S; Geochemical study of fluoride in groundwater of Behror tehsil of Alwar district (Rajasthan). Res. J. Chem. Environ, 2003; 7: 43-47.
5. Jiang CX, Fan QT, Cheng XM, Cui LX; Relationship between spermatogenic cell apoptosis and serum estradiol level in rats exposed to fluoride. Wei Sheng Yan Jiu, 2005; 34X: 1:32-34.
6. Xu R, Shang W, Liu J, Duan L, Ba Y, Zhang H, Cheng X, Cui L; Influence of fluorine on expression of androgen-binding protein and inhibin B mRNA in rat testis sertoli cells. Wei Sheng Yan Jiu, 2010; 39:5:615-617.
7. Grivevau JF, Renard P. and le Lannou D; Superoxide anion production by human spermatozoa as a part of the

- ionophore-induced acrosome reaction process. *Int. J. Androl.*, 1995;18: 67–74.
8. Zhang H. and Zheng RL.; Promotion of human sperm capacitation by superoxide anion. *Free Rad. Res.*, 1996; 24: 261–268.
 9. MacLeod, J.; The role of oxygen in the metabolism and motility of human spermatozoa. *Am. J. Physiol.* 1995;138:512–518.
 10. Storey, B.T.; Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol. Hum. Reprod.*, 1987;3: 203–213.
 11. Kobayashi T, Miyazaki T, Natori M. and Nozawa S; Protective role of superoxide dismutase in human sperm motility. Superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. *Hum. Reprod.* 1991; 6: 987–991.
 12. Miesel R, Jędrzejczak P, Sanočka D, Kurpisz MK; Severe antioxidant deficiency in human semen samples with pathological spermogram parameters. *Andrologia*, 1997; 29:77–83.
 13. Eskiocak S, Gozen AS, Yapar SB, Tavas F, Kilic AS, Eskiocak M; Glutathione and free sulphhydryl content of seminal plasma in healthy medical students during and after exam stress. *Hum Reprod.* 2005; 20(9): 2595-2600.
 14. Agarwal A and Prabakaran SA; Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. *Ind J Exp Biol.*, 2005; 43:11: 963-974.
 15. Fatma A., Monia R, Habib B A, Kerkeni A, Ali S, Mounir A; Altered Antioxidant Status and Increased Lipid Per-Oxidation in Seminal Plasma of Tunisian Infertile Men. *Int. J. Biol. Sci.*, 2012; 8(1):139-149.
 16. Pincemail J, Deby C, Camus G; Tocopherol mobilization during intensive exercise. *Eur J Appl Physiol*, 1988; 57:189–91.
 17. Feki M, Souissi M, Mebazaa A; Vitamin E deficiency: risk factor in human disease? *Ann. Med. Interne*, 2001; 152: 398-406.
 18. Yousef MI, Abdallah GA, Kamel KI; Effect of ascorbic acid and Vitamin E supplementation on semen quality and biochemical parameters of male rabbits. *Anim. Reprod. Sci.*, 2003; 76: 99-111.
 19. Najjar A, Rouatbi M, Ben Mrad M; Le rythme de recolte affecte t-il le spermogramme du lapin et la fertilité des lapines. In *Proc.: 16èmes Journées Scientifiques sur les Résultats de la Recherche Agricole*, 2009;16: 336-343.
 20. Ohkawa H, Ohishi N, Yagi K; Assay for lipid peroxides in animal tissue by thio-barbituric acid reaction. *Anal Biochem.*, 1979; 95: 351-358.
 21. Aebi H. Catalase In: Bergmeyer HU; *Methods of Enzymatic Analysis*. 2: New York: Academic Press Inc., 1974; 673–684.
 22. Omu AE, Fatinikun T, Mannazhath N, Abraham S; Significance of simultaneous determination of serum and seminal plasma α -tocopherol and retinal in infertile men by high-performance liquid chromatography. *Andrologia.*, 1999;31:347–354.
 23. Carlsen E, Giwercman A, Keiding N; Evidence for decreasing quality of semen during past 50 years.,1992; 255:609.
 24. Giwercman A, Carlsen E, Keiding N; Evidence for increasing incidence of abnormalities of the human testis: a review. *Environ Health Perspect*, 1993; 101(2):65–71.
 25. Basha PM, Sujitha NS; Combined impact of exercise and temperature in learning and memory performance of fluoride toxicated rats. *Biol Trace Elem Res.*,2012;150(1-3):306-13.
 26. Yamaguti PM, Simoes A, Ganzerla E, Souza DN, Nogueira FN, and Nicolau J; Effects of Single Exposure of Sodium Fluoride on Lipid Peroxidation and Antioxidant Enzymes in Salivary Glands of Rats, *Oxidative Medicine and Cellular Longevity*, 2013; doi: 10.1155/2013/674593.
 27. Susheela AK, Kumar A; Ultrastructural studies on the Leydig cells of rabbits exposed to chronic fluoride toxicity. *Environ Sci.*, 1997; 5:79–94.
 28. Ghosh D, Das S, Sarkar S, Maiti R, Jana D, Das UB; Testicular toxicity in sodium fluoride treated rats: Association with oxidative stress. *Reprod Toxicol.*, 2002;16:385–90.
 29. Inkielewicz I, Krechniak J; Fluoride effects on glutathione peroxidase and lipid peroxidation in rats. *Fluoride*, 2004; 37(1):7-12.
 30. Alvarez JG, Storey BT; Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol Reprod Dev.*, 1995;42(3):334-46.
 31. Mennella M, Jones R; Properties of spermatozoal superoxide dismutase and lack of involvement of superoxides in metal-ion-catalysed lipid-peroxidation and reactions in semen. *Biochem J*, 1980; 191:289-297.
 32. Zini A, de Lamirande E, Gagnon C; Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. *Int J Androl*, 1993; 16:183-188.
 33. Kobayashi T, Miyazaki T, Natori M, & Nozawa S; Protective role of superoxide dismutase in human sperm motility: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. *Human Reproduction*, 1991; 6 (7):987–991
 34. Miesel R, Jędrzejczak P, Sanočka D, Kurpisz MK.; Severe antioxidant deficiency in human semen samples with pathological spermogram parameters. *Andrologia*, 1997; 29:77-83.
 35. Sanočka D, Miesel R, Jędrzejczak P, Chelmonska-Soyta AC, Kurpisz M; Effect of reactive oxygen species and the activity of antioxidant systems on human semen; association with male infertility. *Int J Androl*, 1997; 20:255-264.
 36. Zini A, Garrels K, Phang D; Antioxidant activity in the semen of fertile and infertile men. *Urology*, 2000; 55:922-926.
 37. Bhardwaj A, Verma A, Majumdar S and Khanduja KL; Status of Vitamin E and reduced glutathione in semen

- of Oligozoospermic & azoospermic patients. Asian J Androl. 2000; 2 (3): 225-228.
38. Chaudhari A.R, Piyali Das, Ramji Singh; Study of oxidative stress and reduced glutathione levels in seminal plasma of human subjects with different fertility potential. Biomed Res. 2008; 19 (3): 207-210.
39. Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM, Ames BN; Smoking and low antioxidant levels increase oxidative damage to sperm DNA. Mutat Res, 1996; 351:199-203.
40. Smith R, Vantman D, Ponce J, Escobar J, Lissi E; Total antioxidant capacity of human seminal plasma. Hum Reprod, 1996; 11:1655-1660.
41. Koca Y, Ozdal OL, Celik M, Unal S, Balaban N; Antioxidant activity of seminal plasma in fertile and infertile men. Arch Androl, 2003; 49:355-359.
42. Mostafa T, Tawadrous G, Roaia MM, Amer MK, Kader RA, Aziz A; Effect of smoking on seminal plasma ascorbic acid in infertile and fertile males. Andrologia, 2006; 38:221-224.
43. Song GJ, Norkus EP, Lewis V; Relationship between seminal ascorbic acid and sperm DNA integrity in infertile men. Int J Androl, 2006;29:569-575.
44. Thompson, J.N., Howell, J.M., & Pitt, G.A.G. Proc. Roy. Soc. London B. 1964;159, 510-534.
45. Suzuki A, Sato M, Handa S, Muto Y, Yamakawa T; Decrease of seminolipid content in the testes of rats with vitamin A deficiency determined by high performance liquid chromatography. J Biochem. 1977; 82(2):461-7.
46. Butler, W. R., A. D. Johnson and W. R. Gomes. Effect of short-term vitamin A deficiency on testicular lipids in the rat. J. Reprod. Fertil. 1968; 15:157.
47. Chinoy NJ, Sharma A; Amelioration of fluoride toxicity by vitamin E and D in reproductive functions of male. Fluoride, 1998; 31:203-216.