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ROLE OF OXIDATIVE STRESS IN ETHANOL INDUCED GERM CELL APOPTOSIS - AN EXPERIMENTAL STUDY IN RATS

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ABSTRACT

The study was undertaken to evaluate the possible involvement of oxidative stress in the pathogenesis of ethanol induced testicular atrophy in rats. Adult male rats were orally administered ethanol at a dose of 1.6 g/kg body weight/ day for four weeks. Twenty-four hours after the last treatment the rats were sacrificed using anesthetic ether. Testes were removed and weighed. Apoptosis was studied by using the Feulgen reaction on 5 µ thin paraffin sections of testis. Testicular homogenate was prepared and centrifuged. The supernatant was used for the estimation of extent of lipid peroxidation and antioxidant defense status. There was significant reduction in body weight; and in testicular weight and diameter in ethanol treated rats. Extent of germ cell apoptosis was significantly high in ethanol treated rats. Ethanol treated rats showed significantly high tissue TBARS level and glutathione S-transferase activity; and low tissue ascorbic acid, reduced glutathione, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities. Chronic ethanol administration resulted in high oxidative stress in the testes either due to increased extent of lipid peroxidation or due to decreased antioxidant defenses, and thereby induces germ cell apoptosis leading to testicular atrophy.

KEY WORDS

Apoptosis, Ethanol, Oxidative stress, Rats, Testes.

INTRODUCTION

Alcohol abuse in men causes impaired testosterone production; shrinkage of the testes (1) reduced sperm counts, abnormal sperm shapes and altered sperm motility (2). The testis is sensitive to a variety of stressors and exposure to agents that induce germ cell apoptosis (3, 4). This organ has fairly high concentrations of antioxidant (5, 6). These facts indicate that the defense against oxidative stress plays critical roles in the maintenance of spermatogenesis and prevention of testicular atrophy. However the mechanism by which antioxidants protect germ cells from hazardous stress, which causes atrophy of the testis, remains obscure. Ethanol metabolism generates reactive oxygen species by inducing lipid peroxidation and decreases cellular levels of antioxidant (7). Therefore we hypothesized that ethanol might elicit oxidative stress in the testis, thereby inducing germ cell apoptosis. The present study describes the changes in oxidative stress in the testis and extent of germ cell apoptosis after administration of ethanol to the rat.

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MATERIALS AND METHODS

Chemicals were purchased from Sisco Research Laboratory, INDIA; Sigma Chemical Co., St. Louis, USA and Merck Ltd. India.

Wistar male rats (10-12 weeks of age) weighing 100-120 g were used. The animals were housed in plastic cages of size 14"×9"×8" (6 rats in each cage) in side a well-ventilated room. The room temperature was maintained at 22±2°C with a 12 h light/dark cycle. All rats had free access to a standard diet and tap water. Food and water were given *ad libitum.*

Animals were divided into two groups. Control and Ethanol treated. Ethanol treated rats were given ethanol orally at a dosage of 1.6 g ethanol/kg body weight/day for four weeks. Ethanol was diluted with double distilled water to get desired concentration. The control animals received a similar volume of the

vehicle alone for four weeks. After the experimental period rats were weighed and killed by cervical dislocation under light ether anesthesia. Testes were removed, cleared of the adhering tissues and weighed. Right testes were used for biochemical analysis and left testes were processed histopathological study. Tissues were immediately rinsed, perfused with ice cold normal saline, trimmed and stored in pre- cooled (-4°C) containers. Tissues were thawed on ice before analysis. Tissue levels of protein (8), thiobarbituric acid reactive substance (9), ascorbic acid (10), reduced glutathione (11), catalase (12), superoxide dismutase (13), glutathione peroxidase (14), glutathione reductase (15) and glutathione S-transferase (16) were assayed.

Tissue processing for detection of apoptosis

Fixation - Tissues were fixed in Bouin's fluid for a minimum of 48 hours.

Paraffin sectioning - Tissue blocks were processed for paraffin sections using routine histological procedures (17). 5 µ thick sections were taken and mounted on the gelatinized slides.

Detection of apoptosis - Apoptosis was detected in histological sections by using the Fuelgen reaction (18, 19). The sections were dewaxed with xylene and hydrated with descending grades of ethyl alcohol and placed in distilled water for 5 minutes. The sections were placed in Schiff's reagent for 45 minutes. It was then rinsed in bisulphite solution (10% potassium metabisulphite) followed by wash in distilled water. Counter staining of the sections were done with 1% light green. The slides were dehydrated with ascending grades of alcohol, cleared with xylene and finally mounted with DPX mounting media. The slides were observed under light microscope at 400X magnification for detection of apoptotic cells which stain brick red in colour.

Quantification of Apoptotic cells - Number of apoptotic cells were counted using a calibrated ocular grid at 400X magnification. Slides were coded in order to avoid experimenter's bias. From each testes a minimum of 5 sections were selected for quantification. The minimum distance between the two adjacent sections selected for quantification was 15 µ, in order to avoid the duplication of counting in the adjacent sections. In each section, 10 randomly selected seminiferous tubules were selected for quantification.

Apoptotic index (AI) was calculated in each section by dividing the number of Fuelgen stain positive germ cells per seminiferous tubule by the total number of total germ cells per seminiferous tubule (obtained from H and E slides) and the result was multiplied by 100. For this 100 rounded seminiferous tubule were randomly selected in each section. Mean AI of each case was calculated.

Statistical analysis

The data were presented as mean±SD. Statistical analysis was performed using Student's 't' test for unpaired data. Significance of difference was set at $P<0.05$.

RESULTS

Ethanol exposed rats showed significant decrease in testicular weight and diameter than controls (Table 1). The extent of lipid peroxidation in testes measured as thiobarbituric acid reactive substances were significantly high in ethanol treated rats (Table 2). There was significant reduction in ascorbic acid, reduced glutathione, superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in the testes of ethanol treated rats (Table 2 and 3). However, tissue activity of glutathione S-tranferase, an important antioxidant and a phase II drug-metabolizing enzyme, was significantly enhanced in ethanol treated rats (Table 3).

Extent of apoptosis was measured as apoptotic index (AI). Although in low numbers apoptotic germ cells were also observed in the control rats, the mean apoptotic index (AI) in ethanol treated rats was significantly higher than in controls. The spermatocytes (most frequent) and spermatogonia were the main germ cells undergoing apoptosis (Figure 2). Ethanol treated rats had more number of vacuoles varying sizes (Figure 4)

DISCUSSION

Apoptosis is a mechanism by which the body maintains cellular balance. In physiological processes this is delicately maintained and any disturbance in its control results in disease. It is an integral component of normal testicular function and has been hypothesized to limit the germ cell population and prevent maturation of aberrant germ cells (20). In the present study ethanol treated rats had showed significant reduction in testicular weight and diameter; and increased extent of apoptosis. During apoptosis, chromatin condensation takes place (21) which results in a free -OH group at the 3' end of the deoxyribose sugar of the condensed DNA. In Fuelgen reaction, Schiff's reagent will bind to the exposed aldehyde at the 3' end of the deoxyribose sugar staining the apoptotic cell brick red in colour.

The testis has been shown to be highly susceptible to ethanol as it crosses blood testis barrier and depresses spermatogenesis. The reduction in the testicular weight of ethanol treated rats may be due to reduced tubule size, spermatogenic arrest and inhibition of steroid biosynthesis of Leydig cells (22). Although the controlled generation of highly reactive oxygen species (ROS) serves as a second messenger

Table 1. **Mean weight (± SD) of the testis (in grams and grams per body weight) and mean diameter of testis and apoptotic index (± SD) in control and ethanol treated rats. n=6.* P<0.05 vs. the control group**

Parameters Experiment	Weight of testis		Diameter of testis (mm)	Apoptotic index
	In grams	In grams per body weight		
Control (n=6)	0.78 ± 0.073 (0.896-0.698)	0.511 ± 0.01 (0.518-0.509)	15.2 ± 1.73 (16.98-13.67)	8.5 ± 2.8 (11.2-6.02)
Ethanol treated (n=6)	0.623 ± 0.06* (0.698-0.543)	0.511 ± 0.01 (0.512-0.5)	12.5 ± 1.87* (14.26-10.82)	26.34 ± 3.3* (29.92-23.04)

Table 2. **Thiobarbituric acid reactive substances (TBARS), ascorbic acid and reduced glutathione (GSH) levels in control and ethanol treated rats. [Data is represented as mean ± SD from 6 observations. * P<0.05 vs. the control group]**

Parameters	Control (n=6)	Ethanol treated (n=6)
TBARS[1]	15.27±0.30 (15.33-15.09)	20.82±0.13* (21.98-19.67)
Ascorbic acid[2]	1.86±0.159 (2.0-1.67)	1.69±0.058* (1.76-1.62)
GSH[3]	2.14±0.04 (2.2-2.1)	1.55±0.04* (1.6-1.5)

[1] nmol/min/mg tissue [2] mg/g tissue
[3] µg/mg tissue

Table 3. **Specific activities of superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, glutathione-S-transferase in testes of control and ethanol treated rats** [Data is represented as mean ± SD from 6 observations. * P<0.05 vs. the control group]

Parameters	Control (n=6)	Ethanol treated (n=6)
Superoxide Dismutase[1]	21.18±0.93 (22.1-19.89)	15.39±0.65* (16.24-14.67)
Catalase[2]	2.03±0.10 (2.09-1.89)	1.60±0.54* (1.67-1.54)
Glutathione reductase[3]	1.61±0.043 (1.66-1.57)	1.34±0.039* (1.37-1.29)
Glutathione peroxidase[4]	0.171±0.023 (0.178-0.143)	0.125±0.010* (0.139-0.117)
Glutathione transferase[4]	11.53±0.64 (12.45-10.98)	13.69±0.79* (14.67-12.98)

1 µmol pyrogallol auto oxidized /min/mg protein
2 n mol H_2O_2 decomposed/min/ mg protein
3 nmol NADPH oxidized/min/mg protein
4 µmol CDNB conjugate formed/min/mg protein.

system in many different cell types, its uncontrolled production is considered as an important factor in the tissue damage (23). In the context of human reproduction, excessive ROS production that exceeds critical levels can overwhelm antioxidant defense strategies of spermatozoa and seminal plasma causes oxidative stress (24). Increased lipid peroxidation results in sperm immobilization, reduced acrosomal reaction and membrane fluidity (25) and DNA damages (26) and also causes high frequencies of single and double DNA strand breaks (27). High levels of ROS disrupt the inner and outer mitochondrial membranes resulting in the release of cytochrome-C protein that activates Caspases and induces apoptosis (28).

ROS are regularly formed during the process of normal respiration. However, the production is kept at physiologically low levels by intracellular free radical scavengers (29). It had demonstrated that the major sources of ROS in semen were derived from the spermatozoa and infiltrating leukocytes (30). Spermatozoa and seminal plasma have their own antioxidative mechanisms to protect ROS-induced cellular damage. GSH is a major nonprotein thiol in living organisms which plays a central role in coordinating the body's antioxidant defense processes. Conditions

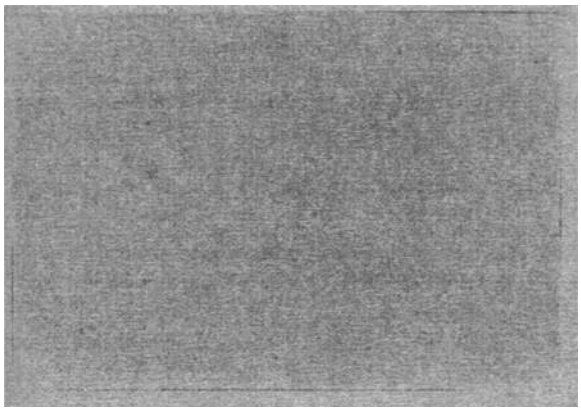

Fig. 1

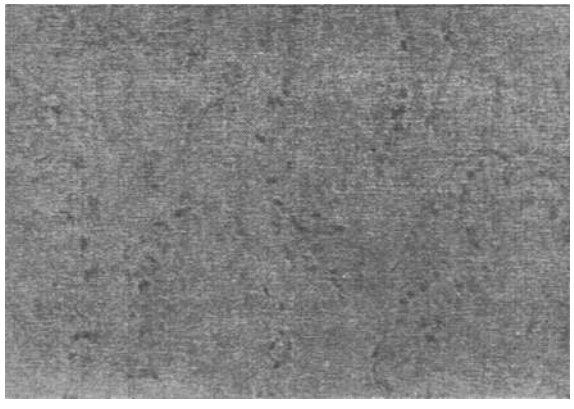

Fig. 2

Transverse section of testis of control (Figure: 1) and ethanol treated (Figure: 2) rats showing Fuelgen stain (40X) positive germ cells

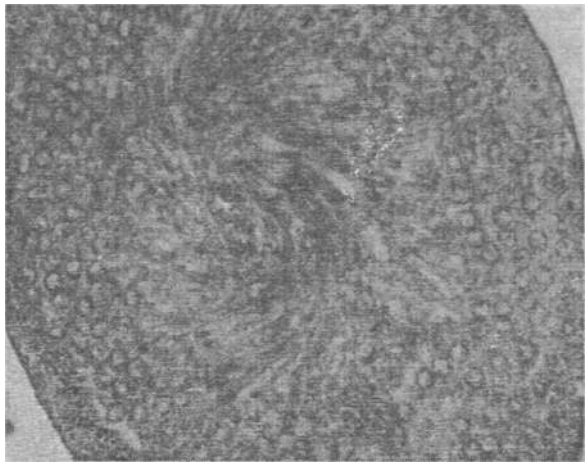

Fig. 3

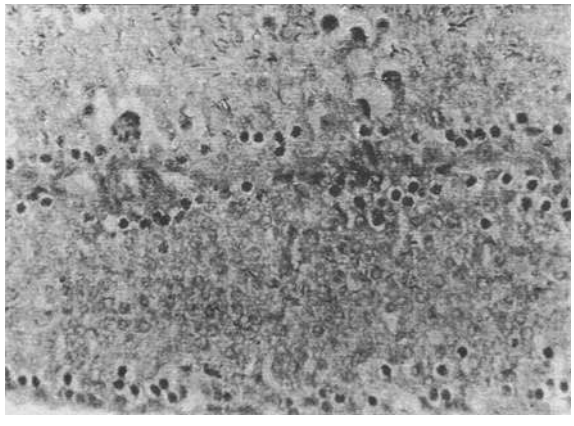

Fig. 4

Transverse section of testis of control (Figure: 3) and ethanol treated (Figure: 4) rats (H & E, 40X). Ethanol treated rats had showed vacuoles of varying sizes

that perturb intracellular levels of glutathione have been shown to result in significant alteration in cellular metabolism. The tissue glutathione concentration reflects its potential for detoxification and it is critical in preserving the proper cellular redox balance and for its role as a cellular protectant (31). GSH has a likely role in sperm nucleus decondensation and spindle microtubule formation (32). Ethanol induced depletion of glutathione supports the hypothesis that reactive oxygen intermediates generated during the metabolism of ethanol lead to glutathione oxidation and lipid peroxidation and are responsible for the toxic effects of ethanol.

SOD spontaneously dismutates (O_2) anion to form O_2 and H_2O_2 while catalase coverts H_2O_2 to O_2 and H_2O. SOD protects spermatozoa against spontaneous O_2 toxicity and LPO (33). Catalase is one of the most efficient enzymes which cannot be saturated by H_2O_2 at any concentration. SOD and catalase also remove (O_2.) generated by NADPH oxidase in neutrophils, and may play an important role in decreasing LPO and protecting spermatozoa (34). Superoxide dismutase protects dehydratases (dihydroxy acid d-aconitase, 6-phosphogluconate dehydratase and fumarases A and B) against free radical, superoxide (35).

Glutathione reductase is concerned with the maintenance of cellular level of GSH by effecting fast reduction of oxidized glutathione (GSSG) to reduced form (35). Glutathione peroxidase plays a significant role in the peroxyl scavenging mechanism and in maintaining functional integration of the cell membranes, spermatogenesis, and sperm morphology and motility (36). It is suggested that the metabolic pathway of testosterone biosynthesis requires protection against peroxidation and will be affected by a decrease in the activity of this enzyme (37). The increased tissue glutathione S-transferase activity in ethanol treated rats might be an adaptive defense

mechanism. Glutathione S-tranferase plays an essential role in eliminating toxic compounds by conjugation (38).

Oxidative stress in the testis is one of the major factors that induce germ cell apoptosis (39). The antioxidants protect germ cells against oxidative DNA damage (40), and play important roles in spermatogenesis (41, 42). ROS induced apoptosis in the testis was observed primarily with spermatocytes and spermatogonia. Differences in the susceptibility of cells to ethanol toxicity may be explained by the difference in their activities of antioxidants and related enzymes.

From the present study it can be concluded that testicular atrophy, decreased testicular weight and diameter in ethanol treated rats might be due to increased germ cell apoptosis, which might have been contributed by increased oxidative stress either due to increased free radical generation or due to decreased antioxidant defenses.

REFERENCES

1. Lieber, C.S. (1995) Medical disorders of alcoholism. N. Engl. J. Med. 333, 1058-1065.
2. Armstrong, I.S., Rajasekaran, M., Chamulitrat, W., Gatti, P., Hellstrom, W.J. and Sikka, S.C. (1999) Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. Free Radic. Biol. Med. 26, 869-880.
3. Lue, H., Hikim, A.P., Swerdolf, R.S., Im, P., Taing, K.S., Bui, T., Leung, A. and Wang. C. (1999) Single exposure to heat apoptosis in rats: role of intratesticular testosterone on stage specificity. Endocrinology 140, 1709-1717.
4. O'Bryan, M.K., Schlatt, S., Philips, D.J., de Krester, D.M. and Hedger, M.P. (2000) Bacterial lipopolysacharide-induced inflammation compromises testicular function at multiple levels *in vivo*. Endocrinology 141, 238-246.
5. Rao, A.V. and Shaha, C. (2000) Role of glutathione S-transferases in oxidative stress-induced male germ cell apoptosis. Free Radical Biol. Med. 29, 1015-1027.
6. Yang, Y., Cheng, J.Z., Singhal, S.S., Saini, M/, Pandya, U., Awasthi, S. and Awasthi, Y.C. (2001) Role of glutathione S-transferases in protection against lipid peroxidation. Over expression of hGSTA2-2 in K562 cells protects against hydrogen peroxide- induced apoptosis and inhibits JNK and caspase 3 activation. J. Biol. Chem. 276, 19220-19230.
7. Rosenblum, F.R., Gavaler, J.S. and Van Theil, D.H. (1987) Vitamin A at pharmacologic doses ameliorates the membrane lipid peroxidation injury and testicular atrophy that occurs with chronic alcohol feeding in rats. Alcohol Alcoholism 2, 241-249.
8. Lowry, O.H., Rosenbourgh, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265-275.
9. Sinnhuber, R.O., Yu, T.C. and Yu, T.C. (1958) Characterization of the red pigment formed in the thiobarbituric acid determination of oxidative capacity. Food Res. 23, 626-630.
10. Roe, J.H. and Kuther, C.A. (1943) The determination of ascorbic acid in whole blood and urine through the 2, 4-dinitrophenyl hydrazine derivative of dehydroascorbic acid. J. Biol. Chem. 147, 399-401.
11. Ellman, G.L. (1959) The sulphydryl groups. Arch. Biochem. Biophys. 32, 70-77.
12. Mahley, A.C. and Chance, B. (1954) In: D Glick (ed.). Methods in Biochemical Analysis. Wiley Inter Science, New York. 1, 357.
13. Marklund, S. and Marklund, G. (1974) Involvement of superoxide radical in the auto oxidation pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 7, 469-474.
14. Paliga, D.E. and Valentine, W.N. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, 1158-1159.
15. Goldberg and Spooner, J.R. (1983) In: Methods of Enzymatic Analysis (Ed. Bergmayer), Vol.III, 3rd edn. Academic press, Inc. Florida, 258-265.
16. Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974) Glutathione-S-tranferase, the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130-7139.
17. Culling, C.F.A., Allison, R.T. and Barr, W.T. (1985) Cellular pathology technique. 14th ed. London, Butterworths.
18. Bancroft, J.D. and Stevans, A. (1996) Theory and Practice of histological techniques. 3rd ed. London. Churchill Livingstone.
19. Wyllie A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284, 555-556.

20. Yin, Y., Stahl, B.C., Dewolf, W.C. and Morgentaler, A. (1998) p53-mediated germ cell quality control in spermatogenesis. Developmental Biology 204, 165-171.

21. Stites, D.P. (1976) Laboratory methods for detection of antigens and antibodies, In: Basic and Clinical and Immunology, Eds. Fudenberg, H.H., Stites, D.P., Cladwell, J.L. and Wells, J.V. Lange Medical Publications, California, 281-315.

22. Nandi, S., Banerjee, P.P. and Zirkin, B R. (1999) Germ cell apoptosis in the testes of Sprague Dawley rats following testosterone withdrawal by ethane 1, 2 dimethanesulfonate administration: Relationship of Fas? Biol. of Reprod. 61, 70-75.

23. Young, K.A., Zirkin, B.R. and Nelson, R.J. (1999) Short photoperiod induces testicular apoptosis in the white-footed mouse (peromyscus leucopus), Endocrinology, 140, 1331-1339.

24. Gil-Guzman, E., Ollero, M., Lopez, M.C., Sharma, R.K., Alvarez, J.G. and Thomas, A.J. Jr. (2001) Differential production of reactive oxygen species by subjects of human spermatozoa at different stages of maturation, Hum. Reprod. 16, 1922-1930.

25. Armstrong, J.S., Rajasekaran, M., Chamulitrat, W., Gatti, P., Hellstrom, W.J. and Sikka, S.C. (1999) Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. Free Radic. Biol. Med. 26, 869-880.

26. Duru, N.K., Morshedi, M. and Ochninger, S. (2000) Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. Fertil Steril. 74, 1200-1207.

27. Aitken, R.J. and Krausz, C. (2001) Oxidative stress, DNA damage and the Y chromosome. Reproduction, 122, 497-506.

28. Gandini, L., Lombardo, F., Paoli, D., Caponeechia, L., Familiari, G. and Verlengia, C. (2000) Study of apoptotic DNA fragmentation in human spermatozoa. Hum. Reprod. 15, 830-839.

29. Sikka, S.C. (2001) Relative impact of oxidative stress on male reproductive function. Curr. Med. Chem. 8, 851-862.

30. Sharma, R., Pasqualotto, F.F., Nelson, D.R., Thomas, A.J.Jr. and Agarwal, A. (2001) Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. J. Androl. 22, 575-583.

31. Mari, M., Wu, D., Nieto, N. and Cederbaum, A.I. (2001) CYP2E1-Dependent toxicity and up-regulation of antioxidant genes, J. Biomed. Sci. 8(1), 52-55

32. Irvine, D.S. (1996) Glutathione as a treatment for male infertility. Reviews of Reprod. 1, 6-12.

33. Alvarez, J.G., Touchstone, J.C., Blasco, L. and Storey, B.T. (1987) Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa.: superoxide dismutase dismutase as a major enzyme protectant against oxygen toxicity. J Androl. 8, 336-348.

34. Aitken, R.J. (1995) Free radicals, lipid peroxidation, sperm function. Reprod. Fertil. Dev. 7, 659-668.

35. Lenzi, A., Cualosso, F., Gandini, L., Lombardo, F. and Dondero, F. (1993) Placebo controlled, double-blind cross over trial of glutathione therapy, in male infertility. Hum. Reprod. 9, 2044-2050.

36. Calvin, H.I., Cooper, G.W. and Wallace, E. (1981) Evidence that selenium in rat sperm is associated with a cystine rich structural protein of the mitochondrial capsule. Gamete Res. 4, 139-149.

37. Chandra, R., Aneja, R., Rewal, C., Konduri, R., Das, K., and Agarwal, S. (2000) An opium alkaloid-Papaverine ameliorates ethanol induced hepatotoxicity: diminution of oxidative stress. Ind. J. Clin. Biochem. 15(2), 155-160.

38. Rommerts, F.F.G. (1990) Testosterone: an overview of biosynthesis transport metabolism and action. In: Testosterone action deficiency substitution Eds: Nieschlag and Hehre, H.M., (Springler-Verlag: Berlin) 1-22.

39. Emiko, Kasahara, Eisuke, F. Sato, Mami, Miyoshi, Rysei, Konaka, Keiichi, Hiramoto, Junzo, Sasaki, Masaaki, Tokuda, Yoshihisa, Nakanos and Masayasu, Inoue (2002) Role of oxidative stress in germ cell apoptosis induced by di (2-ethylhexyl) phthalate. Biochem. J. 365, 849-856.

40. Fraga, C.G., Motchnik, P.A., Shigenaga, M.K., Helbock, H.J., Jacob, R.A. and Ames, B.N. (1991) Ascorbic acid protects against endogenous oxidative damage in human sperm. Proc. Natl. Acad. Sci. U.S.A, 88, 11003-11006.

41. Chinoy, N.J., Mehta, R.R., Seethalakshmi, L., Sharma, J.D. and Chinoy, M.R. (1986) Effects of vitamin C deficiency ob physiology of male reproductive organs of guinea pigs. Int. J. Fertil. 31, 232-239.

42. Bensoussan, K., Morales, C.R. and Hermo. L. (1998) Vitamin E deficiency causes incomplete spermatogenesis and affects the structural differentiation of epithelial cells of the epididymis in the rat. J. Androl. 19. 266-288.