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EXPERIMENTAL THERAPEUTIC INTERVENTION WITH ALPHA TOCOPHEROL IN ETHANOL INDUCED TESTICULAR INJURIES IN RATS

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ABSTRACT

Infertility is well-established harmful effect in chronic alcoholism and so far, there is no effective treatment for this condition. The study was conducted to determine the effects of alpha tocopherol on ethanol induced testicular injuries in male albino rats of Wistar strain. Five groups (n=6) of animals were used. Group I served as control. Group II received daily 1.6g ethanol/kg body weight/day for 4 weeks orally. Group III received 1.6g ethanol + 80mg alpha tocopherol/kg body weight/day for four weeks orally. Group IV received 1.6g ethanol /kg body weight for/day 4 weeks and followed by 80mg alpha tocopherol/kg body weight/day for four weeks orally. Group V received 1.6g ethanol/kg body weight/day orally for 4 weeks, followed by 4 weeks abstinence. Twenty-four hours after the last treatment the rats were sacrificed using anesthetic ether. Testes were removed and used for the estimation of extent of lipid peroxidation and tissue levels of antioxidants and steroidogenic enzymes. Alpha tocopherol treatment increased the activities of testicular Δ^5, 3β-HSD and 17β-HSD. Moreover, the treatment was also associated with significant decrease in testicular oxidative stress. Ethanol-induced oxidative stress and decreased steroidogenesis can be reversed by treatment with alpha tocopherol.

KEY WORDS

Ethanol, Alpha tocopherol, Oxidative stress, Steroids, Testes.

INTRODUCTION

Alcohol abuse is well known to impair reproductive performance in experimental animals and human (1). Alcoholics are often found having fertility abnormalities with low sperm count and impaired sperm motility (2). Chronic alcohol intake in men cause impaired testosterone production and shrinkage of the testes (i.e., testicular atrophy) (3). Ethanol significantly augmented lipid peroxidation in the testis (4) and inhibited the conversion of both dehydroepiandrosterone and androstenedione to testosterone (5) by decreasing the activities of 3β-hydroxy steroid dehydrogenase (3β-HSD) and 17β-hydroxy steroid dehydrogenase (17β-HSD). Mitochondrial enriched extracts obtained from the testes of alcohol treated rats showed significant increase in the malondialdehyde formation; moreover there was a significant decrease in glutathione (6), superoxide dismutase (7), glutathione peroxidase (8) levels in the testes of alcohol treated rats.

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As there is no effective treatment for alcohol induced infertility and testicular damage, the administration of antioxidants in patients with 'male factor' infertility has begun to attract considerable interest. Ascorbic acid administration has protective effect on ethanol induced testicular steroidogenic dysfunction and oxidative stress (9). Administration of β-tocopherol could be another modality to treat alcohol induced testicular injuries and there is little data exploring use of this compound in male infertility associated with excessive ROS production. With the understanding of the role of oxidative stress in alcohol-induced testicular injury, the present study has been undertaken to evaluate the role of oral alpha tocopherol in experimental alcohol-induced testicular injury.

MATERIALS AND METHODS

Chemicals : Fine chemicals were purchased from Sisco

Research Laboratory, India and Sigma Chemical Co., St. Louis, USA. All other chemicals used were of analytical grade and were purchased from Merck Ltd., India and Sisco Research Laboratories Ltd., India.

Drug : Vitamin E (Tocofer-400, Torrent Pharmaceuticals Ltd, India)-400mg/cap

Animals : Male Wistar rats (10-12 weeks of age) weighing 100-120g were used. The animals were housed in plastic cages of size 14"×9"×8" (6 rats in each cage) inside a well-ventilated room. The room temperature was maintained at 22 ± 2 °C with a 12-12 hr L:D cycle. All rats had free access to a standard diet and tap water. Food and water were given *ad libitum.* The experimental study protocol was approved by the Institutional Animal Ethics Committee, SMIMS, Gangtok and National Institutes of Health (NIH), Bethesda, MD, USA guidelines were followed for maintenance, handling, experimentation, sacrifice and disposal of animals.

Experimental design : The animals were divided in to following five groups of 6 each :

Group I (Control) : 1 g double distilled water/kg body weight/ day for 4 weeks, orally.

Group II : Ethanol treated rats (1.6 g ethanol/kg body weight/day for 4 weeks, orally).

Group III : Ethanol + alpha tocopherol treated rats (1.6 g ethanol + 80 mg alpha tocopherol /kg body weight/day for 4 weeks, orally).

Group IV : Ethanol followed by alpha tocopherol treated rats (1.6 g ethanol /kg body weight/ day for 4 weeks, followed by 80 mg alpha tocopherol /kg body weight/day for next 4 weeks, orally).

Group V : Ethanol treatment (1.6 g ethanol /kg body weight/day, orally) for 4 weeks and followed by 4 weeks abstination.

The dose of ethanol was determined from serial dose response studies in rats with doses of 0.8, 1.2, 1.6 and 2 g / kg body weight / day for 4 weeks. Ethanol orally at a dosage of 1.6g / kg body weight / day for four weeks produced features of liver injury comparable to those observed in clinical situations of moderate alcoholic liver disease. Therefore, the dose of 1.6g / kg body weight / day for 4 weeks was chosen for this study. Ethanol and alpha tocopherol were dissolved in double distilled water to get desired concentration. After the experimental period, rats were weighed and sacrificed by cervical dislocation under light ether anesthesia, Testes were removed, cleaned of the adhering tissues and weighed. 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.

Lipid peroxidation studies : Extent of lipid peroxidation was estimated by the method of Sinnhuber *et al*, (10). Testes were homogenized in ice-cold 0.25 M Tris buffer (pH 7.4). To this homogenate, TCA-TBA-HCl [Trichloroacetic acid (TCA) 15 % w/v, thiobarbituric acid (TBA) 0.375%, and hydrochloric acid (HCl) 0.25N] were added and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000g for 10 min. Levels of thiobarbituric acid reactive substances (TBARS) were measured spectrophotometrically at 532nm. Tissue protein was estimated by the method of Lowry *et al*, (11).

Determination of ascorbic acid in the tissue : Weighed sample was homogenized in ice cold 6% TCA in a pre-chilled mortar. The extract was shaken well in a test tube; activated animal charcoal was added, and allowed to stand for 15 min. To clear supernatant a drop of thiourea reagent (in 50% alcohol) and 2, 4-dinitrophenyl hydrazine (2% in 9 N H_2SO_4) were added and incubated at 37°C for 3hr.The tube was taken, chilled for 10 min in an ice bath and cold H_2SO_4 was added, placed the test tubes in ice bath, and kept in refrigerator for 30 min. Tubes were then centrifuged and absorbance were recorded at 540 nm against the blank (12).

Determination of reduced glutathione content : The reduced glutathione content of the tissue was measured by the method of Ellman (13). For calibration, a standard curve of reduced glutathione was prepared using varied concentrations of glutathione treated with DTNB.

Antioxidant enzyme assays : Tissue homogenates (25 w/v) were prepared in 50 mM Tris-HCl buffer (pH 7.4), using motor driven teflon homogenizer. Homogenates were centrifuged for 30 min (4°C, at 12,000 rpm) and the resultant supernatant fractions were used for various assays.

Activities of catalase (EC 1.11.1.6) (14), superoxide dismutase (EC 1.15.1.1) (15), glutathione peroxidase (EC 1.11.1.9) (16), glutathione reductase (EC 1.6.4.1) (17), glutathione-S-transferase (EC 2.5.1.18) (18), testicular Δ^5, 3β-hydroxy steroid dehydrogenase (Δ^5, 3β-HSD) (19) and 17β-hydroxy steroid dehydrogenase (17β-HSD) (20) were determined as per standard procedures.

Statistical analysis : The data were presented as mean ±

Table 1 : Effect of alpha tocopherol on body and testicular weights
[Values are mean ± SD from 6 animals in each group; Figures in parenthesis are % increase]

	Body weights		Weight of testis	
	Initial	Final	In gram	In g/body weight
Group 1	108.3 ± 7.5	151.6 ± 10.5 (42%)	0.781 ± 0.073	0.512 ± 0.003
Group II	109.1 ± 6.64	127.8 ± 7.7 (17%)	0.623 ± 0.063 [a]	0.508 ± 0.004
Group III	108.3 ± 6.8	133.1 ± 8.54 (25.9%)	0.768 ± 0.063 [a bc]	0.509 ± 0.006
Group IV	108.3 ± 6.8	132.1 ± 8.53 (24.4%)	0.764 ± 0.070 [a bc]	0.508 ± 0.006
Group V	109.1 ± 5.8	136.5 ± 7.4 (26%)	0.750 ± 0.068 [a]	0.509 ± 0.003

P values: <0.05 compared with [a] control group, [b] ethanol treated group, and [c] abstinated group.

SD. Statistical analysis was performed using Student's 't' test for unpaired data. Significance of difference was set at P<0.05.

RESULTS

Changes in body weights : Alcohol treated animals showed lower gain (17%) in body weight after 4 weeks of treatment than control group (42%). In the follow up treatments, 25.9% and 24.4% increase in body weight were observed in group III and IV respectively, while abstination showed 26% increase in body weight. However, these differences in body weights were not statistically significant (Table 1).

Changes in testicular weight : Ethanol exposed rats showed significant decrease in testicular weight compared to controls. Alpha tocopherol treated rats had significantly (P<0.05) higher testicular weight in comparison to ethanol treated and abstaining rats. But when adjusted for body weight the difference was not significant in any of the groups (Table 1).

Extent of lipid peroxidation : Extent of lipid peroxidation in the tissue was estimated by measuring level of thiobarbituric acid reactive substances (TBARS). Exogenous alpha tocopherol had significant (P<0.05) protective effect on tissue lipid peroxidation (Table 2).

Antioxidant defense system : Alpha tocopherol not only increased (p<0.05) testicular glutathione (GSH) content and activities of superoxide dismutase, catalase and glutathione reductase but reduced (P<0.05) testicular glutathione S-transferase activity. However its effect on tissue ascorbic acid and glutathione peroxidase was not significant (Table 3).

Steroidogenic enzyme activities : Alpha tocopherol treatment increased steroidogenic enzyme activities although the differences were statistically not significant (Table 3).

DISCUSSION

The study demonstrates the adverse effect of ethanol on testicular steroidogenic activities and its protection by alpha tocopherol administration. Attempts were also made to study the ethanol-induced testicular oxidative stress and its correction by alpha tocopherol. Alpha tocopherol is a well-known lipid soluble, dietary antioxidant (21) and it acts by reducing free radical production, trapping free radicals,

Table 2 : Effect of alpha tocopherol on tissue protein, ascorbic acid, thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) [Values are mean ± SD from 6 animals in each group]

	Protein (mg/100mg tissue)	Ascorbic acid (mg/g tissue)	TBARS (nmol H_2O_2 consumed/ min/mg protein)	GSH (µg/mg tissue)
Group I	21.89 ± 0.83	1.86±0.159	15.27±0.30	2.14±0.04
Group II	19.27±0.30[a]	1.69±0.058 [a]	20.82±0.13 [a]	1.55±0.04[a]
Group III	20.45±0.23[abc]	1.76 ±0.093	19.03±0.28 [ab]	1.99±0.10 [abc]
Group IV	20.47±0.29 [abc]	1.74 ±0.076	19.25±0.13 [ab]	1.84±0.08 [abc]
Group V	19.37±0.11[a]	1.70±0.037	20.27±0.56[a]	1.56±0.06[a]

P values: <0.05 compared with [a] control group, [b] ethanol treated group, and [c] abstinated group.

Table 3 : Effect of alpha tocopherol on tissue superoxide dismutase (SOD), catalase, glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), 17β-HSD and 3β-HSD [Values are mean ± SD from 6 animals in each group]

	SOD (µmol pyrogallol autooxidized /min/mg protein)	Catalase (nmol H_2O_2 decomposed/ min/mg protein)	GR (nmol NADPH oxidized/ min/ mg protein	GPx (µmol CDNB conjugate / formed min/mg protein)	GST (µmol CDNB conjugate formed/min/ mg protein)	17β-HSD (absorbance/ min/mg protein)	3β-HSD (absorbance /min/ mgprotein)
Group I	21.18 ± 0.93	2.03 ± 0.10	1.61 ± 0.043	0.171± 0.023	11.53 ± 0.64	0.0166 ± 0.001	0.0198 ± 0.002
Group II	15.39 ± 0.65[a]	1.60 ± 0.54[a]	1.34 ± 0.039[a]	0.125± 0.010[a]	13.69 ± 0.79[a]	0.0131 ± 0.00[a]	0.0155 ± 0.00[a]
Group III	17.00± 0.82[abc]	1.78± 0.08[abc]	1.44± 0.046[abc]	0.154± 0.014[a]	11.85± 0.50[bc]	0.0139 ± 0.001[a]	0.0168 ± 0.001[a]
Group IV	16.77± 0.45[abc]	1.74± 0.06[abc]	1.42± 0.040[abc]	0.152± 0.015[a]	12.15± 0.09[bc]	0.0136 ± 0.001[a]	0.0166 ± 0.001[a]
Group V	15.73 ± 0.16[a]	1.64 ± 0.03[a]	1.32 ± 0.022[a]	0.139± 0.006[a]	12.89 ± 0.33[a]	0.0130 ± 0.001[a]	0.0156 ± 0.001[a]

P values:<0.05 compared with [a] control group, [b] ethanol treated group, and [c] abstinated group.

interrupting the peroxidation process or reinforcing the natural antioxidant defense (22). Alpha tocopherol treatment significantly ameliorated aflatoxin-induced biochemical alterations (23) and lipid peroxidation (24) in the testis of mice. The protective effect of alpha tocopherol can be explained by the scavenging of free radicals before the damage cellular macromolecules.

The decrease in Δ^5, 3β-HSD and 17β- HSD activities in ethanol-treated rat may be the result of an elevation in testicular conjugated dienes and MDA (25). The elevation in testicular free radicals in ethanol-treated rats was further supported by the diminution in scavenger enzymes against free radicals (26). Diminution in the testicular weights in ethanol-treated rats also supports the inhibition in testicular steroidogenesis (27). As the body growth was also altered in ethanol-treated rats, the effect of ethanol on the testis may be due to its general toxicity other than its specific toxic effect on the target organ. Alpha tocopherol administration in ethanol-treated rats resulted in an elevation in the activities of testicular Δ^5, 3β-HSD and 17β-HSD, which may be due to the direct stimulatory effect of this vitamin on the enzymes (28, 29). Restoration of testicular steroidogenesis after alpha tocopherol administration in ethanol-treated rat may protect the androgenic and gametogenic activity (30). Restoration of testicular steroidogenesis may also be due to the antioxidant effect of alpha tocopherol (31) against oxidative stress induced by ethanol. The latter possibility is supported by the fact that alpha tocopherol reversed the testicular MDA and conjugated diene levels and restored scavenger system.

From the results it may be concluded that alpha tocopherol administration has a protective effect on ethanol-induced testicular steroidogenic dysfunction. Moreover, alpha tocopherol also ameliorates the ethanol-induced oxidative stress. Alpha tocopherol may execute its role by modulating testicular free radical production and/or stimulating testicular androgenesis. To answer which one is more important, further investigation is needed.

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