Experimental therapeutic intervention with ascorbic acid in ethanol induced testicular injuries in rats

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Ascorbic acid treatment significantly increased the activities of testicular 3β, 3β-HSD and 17β-HSD. Moreover, the treatment was also associated with significant decrease in oxidative stress in the testis. Ethanol induced oxidative stress and decreased steroidogenesis can be reversed by treatment with ascorbic acid.

Keywords: Ethanol, Ascorbic acid, Oxidative stress, Steroids, Testes.

IPC Code: Int. Cl. A61

Alcohol abuse is well known to impair reproductive performance in experimental animals and human. Alcoholics are often found having fertility abnormalities with low sperm count and impaired sperm motility. Chronic alcohol intake in men can cause impaired testosterone production and shrinkage of the testes (i.e., testicular atrophy). Ethanol significantly augmented lipid peroxidation in the testis and inhibited the conversion of both dehydroepiandrosterone and androstenedione to testosterone 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, superoxide dismutase, glutathione peroxidase levels in the testes of alcohol treated rats.

Ascorbic acid is a terminal water-soluble small antioxidant that protects lipids against peroxidation. In vitro studies have shown that low-density lipoprotein oxidation can be prevented by naturally occurring antioxidants such as ascorbic acid, α-tocopherol, carotenoids, etc.

The administration of antioxidants in patients with ‘male factor’ infertility has begun to attract considerable interest. Moreover, there is no effective treatment for alcohol induced infertility and testicular damage. 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 ascorbic acid in experimental chronic 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.

Animals — Male Wistar rats (10-12 weeks of age) weighing 100-120 g were used. The animals were housed in plastic cages of size 14"x9"x8" (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 into 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 + ascorbic acid treated rats (1.6 g ethanol + 1 g ascorbic acid/kg body weight/day for 4 weeks, orally).

Group IV: Ethanol followed by ascorbic acid treated rats (1.6 g ethanol/kg body weight/day for 4 weeks, followed by 1 g ascorbic acid/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 1.6 g/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 1.6 g/kg body weight/day for 4 weeks was chosen for this study. Ethanol and ascorbic acid were freshly 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. Tissues were removed, cleaned of 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. All subsequent processing were carried out at 0-4 °C.

Lipid peroxidation studies — Extent of lipid peroxidation was estimated by the method of Sinnhuber et al.\(^{11}\). Tissue homogenates 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) 0.25M] 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 1000 g 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.\(^{12}\).

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 N H\(_2\)SO\(_4\)) were added and incubated at 37°C for 3hr. The tube was taken, chilled for 10 min in an ice bath and cold H\(_2\)SO\(_4\) was added, placed the test tubes in ice bath, and kept in refrigerator for 30 min. Tubes were then centrifuged and absorbance was recorded at 540 nm against the blank.\(^{13}\)

Determination of reduced glutathione content — The reduced glutathione content of the tissue was measured by the method of Ellman\(^{14}\). 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-HC 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)\(^{15}\), superoxide dismutase (EC 1.15.1.1)\(^{16}\), glutathione peroxidase (EC 1.11.1.9)\(^{17}\), glutathione reductase (EC 1.6.4.1)\(^{18}\), glutathione-S-transferase (EC 2.5.1.18)\(^{19}\), testicular \(\Delta^5\), \(\Delta^3\)-hydroxy steroid dehydrogenase (\(\Delta^5\), \(\Delta^3\)-HSD)\(^{20}\) and testicular 17\(\beta\)-hydroxy steroid dehydrogenase (17\(\beta\)-HSD)\(^{21}\) were determined as per standard procedures.

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

The present study was undertaken to evaluate the effect of exogenous ascorbic acid on ethanol induced testicular oxidative stress and decreased steroidogenesis.

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, 24.44% increase in body weight was observed when rats were administered with ascorbic acid, while abstinence 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 than controls. Ascorbic acid treated rats had
significantly 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 ascorbic acid had significant protective effect on tissue lipid peroxidation in comparison to ethanol treated and abstaining rats (Table 2).

Non enzymatic antioxidant defense system—Ascorbic acid treated rats had significantly high reduced glutathione (GSH) and ascorbic acid than ethanol treated and abstaining rats (Table 2).

Enzymatic antioxidant defense system—Ascorbic acid treatment had significantly increased testicular catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase activities in the testis in comparison to ethanol treated and abstaining rats. However, the activity of glutathione S-transferase was significantly decreased in drug treated groups (Table 3).

Steroidogenic enzyme activities—Only group III, which was treated with ascorbic acid along with ethanol, had significantly high steroidogenic enzyme

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**Table 1—Effect of exogenous ascorbic acid on body weight and weight of the testis**

<table>
<thead>
<tr>
<th></th>
<th>Body weights</th>
<th>Weight of testis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Group I</td>
<td>108.3 ± 7.5</td>
<td>151.6 ± 10.5 (42%)</td>
</tr>
<tr>
<td>Group II</td>
<td>109.1 ± 6.6</td>
<td>127.8 ± 7.7 (17%)</td>
</tr>
<tr>
<td>Group III</td>
<td>108.3 ± 6.8</td>
<td>132.1 ± 8.54 (24.4%)</td>
</tr>
<tr>
<td>Group IV</td>
<td>108.3 ± 6.8</td>
<td>132.1 ± 8.53 (24.4%)</td>
</tr>
<tr>
<td>Group V</td>
<td>109.1 ± 5.8</td>
<td>136.5 ± 7.4 (26%)</td>
</tr>
</tbody>
</table>

*P* values <0.05 compared with *control group*, *ethanol treated group*, and *abstained group.*

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**Table 2—Effect of exogenous ascorbic acid on tissue levels of protein, iron, ascorbic acid, thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH)**

<table>
<thead>
<tr>
<th></th>
<th>Protein (mg/100mg tissue)</th>
<th>Ascorbic acid (mg/g tissue)</th>
<th>TBARS (nmol H₂O₂ consumed/min/mg protein)</th>
<th>GSH (µg/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>21.89 ± 0.83</td>
<td>1.86 ± 0.15</td>
<td>15.27 ± 0.30</td>
<td>2.14 ± 0.04</td>
</tr>
<tr>
<td>Group II</td>
<td>19.27 ± 0.30</td>
<td>1.69 ± 0.058</td>
<td>20.82 ± 0.13</td>
<td>1.55 ± 0.04</td>
</tr>
<tr>
<td>Group III</td>
<td>20.60 ± 0.31</td>
<td>1.84 ± 0.108</td>
<td>19.05 ± 0.14</td>
<td>1.94 ± 0.09</td>
</tr>
<tr>
<td>Group IV</td>
<td>20.58 ± 1.9</td>
<td>1.83 ± 0.102</td>
<td>19.32 ± 0.29</td>
<td>1.86 ± 0.04</td>
</tr>
<tr>
<td>Group V</td>
<td>19.31 ± 0.11</td>
<td>1.70 ± 0.037</td>
<td>20.27 ± 0.56</td>
<td>1.56 ± 0.06</td>
</tr>
</tbody>
</table>

*P* values <0.05 compared with *control group*, *ethanol treated group*, and *abstained group.*

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**Table 3—Effect of exogenous ascorbic acid on tissue activities of superoxide dismutase (SOD), catalase, glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione S-transferase (GST)**

<table>
<thead>
<tr>
<th></th>
<th>SOD (µmol pyrogallol auto-oxidized/min/mg protein)</th>
<th>Catalase (nmol H₂O₂ decomposed/min/mg protein)</th>
<th>GR (nmol NADPH oxidized/min/mg protein)</th>
<th>GPx (µmol CDNB conjugate formed/min/mg protein)</th>
<th>GST (µmol CDNB conjugate formed/min/mg protein)</th>
<th>17 β-HSD (absorbance/min/mg protein)</th>
<th>3β-HSD (absorbance/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>21.18 ± 0.93</td>
<td>2.03 ± 0.10</td>
<td>1.61 ± 0.043</td>
<td>0.171 ± 0.023</td>
<td>11.53 ± 0.64</td>
<td>0.0166 ± 0.001</td>
<td>0.0198 ± 0.002</td>
</tr>
<tr>
<td>Group II</td>
<td>15.39 ± 0.65</td>
<td>1.60 ± 0.564</td>
<td>1.34 ± 0.039</td>
<td>0.125 ± 0.010</td>
<td>13.69 ± 0.79</td>
<td>0.0131 ± 0.000</td>
<td>0.0155 ± 0.000</td>
</tr>
<tr>
<td>Group III</td>
<td>16.91 ± 0.873</td>
<td>1.81 ± 0.076</td>
<td>1.51 ± 0.017</td>
<td>0.160 ± 0.009</td>
<td>11.91 ± 0.286</td>
<td>0.0122 ± 0.0001</td>
<td>0.0170 ± 0.0011</td>
</tr>
<tr>
<td>Group IV</td>
<td>16.70 ± 0.573</td>
<td>1.72 ± 0.086</td>
<td>1.49 ± 0.046</td>
<td>0.155 ± 0.014</td>
<td>12.16 ± 0.326</td>
<td>0.0139 ± 0.0015</td>
<td>0.0167 ± 0.0015</td>
</tr>
<tr>
<td>Group V</td>
<td>15.73 ± 0.16</td>
<td>1.64 ± 0.031</td>
<td>1.32 ± 0.022</td>
<td>0.139 ± 0.006</td>
<td>12.89 ± 0.33</td>
<td>0.0130 ± 0.0016</td>
<td>0.0156 ± 0.0016</td>
</tr>
</tbody>
</table>

*P* values <0.05 compared with *control group*, *ethanol treated group*, and *abstained group.*
activities than ethanol treated and abstained rats (Table 4).

Discussion

The study demonstrates the adverse effect of ethanol on testicular androgenic activities and its protection by ascorbic acid administration. Attempts were also made to study the ethanol-induced testicular oxidative stress and its correction by ascorbic acid.

Lower level of ascorbic acid leads to infertility and increased damage to sperm genetic material. This dietary antioxidant acts by reducing free radical production, trapping free radicals, interrupting the peroxidation process or reinforcing the natural antioxidant defense. Ascorbic acid prevents cellular degenerative diseases associated with lipid peroxidation, reduce reactive oxygen species (ROS) generation and stimulate steriodogenic activity in cyclophosphamide treated rats.

The decrease in Δ5, 3β-HSD and 17β-HSD activities in ethanol-treated rat may be the result of an elevation in testicular conjugated dienes and MDA. The elevation in testicular free radicals in ethanol-treated rats was further supported by the diminution in scavenger enzymes against free radicals. Diminution in the testicular weights in ethanol-treated rats also supports the inhibition in testicular steriodogenesis. 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.

Ascorbic acid administration in ethanol-treated rats resulted in a significant elevation in the activities of testicular Δ5, 3β-HSD and 17β-HSD, which may be due to the direct stimulatory effect of this vitamin on the enzymes. Restoration of testicular steriodogenesis after ascorbic acid co-administration in ethanol-treated rat may protect the androgenic and gametogenic activity. Restoration of testicular steriodogenesis may also be due to the antioxidant effect of ascorbic acid against oxidative stress induced by ethanol. The latter possibility is supported by the fact that ascorbic acid reversed the testicular MDA and conjugated diene levels and restored scavenger system.

From the results it may be concluded that ascorbic acid co-administration has a protective effect on ethanol-induced testicular steriodogenic dysfunction. Moreover, ascorbic acid also ameliorates the ethanol-induced oxidative stress. Ascorbic acid 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. The protective effect of ascorbic acid may have some clinical implication in patients with a history of alcoholism.

References

13. Roe J H & Kather C A, The determination of ascorbic acid in whole blood and urine through the 2, 4-dinitrophenyl hydrazine derivative of dehydroascorbic acid, J Biol Chem, 147 (1947) 399.