Protective effect of ascorbic acid against ethanol-induced reproductive toxicity in male guinea pigs

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Abstract

The present study was undertaken to elucidate the effect of ascorbic acid on alcohol-induced reproductive toxicity and also to compare it with that of abstention. A total of thirty-six male guinea pigs were divided into two groups and were maintained for 90 d as control and ethanol-treated groups (4 g/kg body weight (b.wt.)). After 90 d, ethanol administration was stopped and animals in the control group were divided into two groups and then maintained for 30 d as the control and control + ascorbic acid groups and those in the ethanol-treated group as ethanol abstention and ethanol + ascorbic acid (25 mg/100 g b. wt.) groups. Animals treated with ethanol showed a significant decline in sperm quality ($P < 0.001$), decreased activity of steroidogenic enzymes ($P < 0.05$) and reduced serum testosterone ($P < 0.05$), luteinising hormone and follicle-stimulating hormone levels, decrease in the activity of testicular succinate dehydrogenase, adenosine triphosphatase, sorbitol dehydrogenase and reduction in fructose content ($P < 0.05$). It also caused an increase in testicular malondialdehyde levels ($P < 0.05$) and decrease in the levels of glutathione content ($P < 0.001$) of testes. Ascorbic acid levels in testes and plasma were also reduced ($P < 0.001$) in ethanol-fed animals. Ascorbic acid supplementation altered all these parameters and produced a better and faster recovery from alcohol-induced reproductive toxicity than abstention. The mechanism of action of ascorbic acid may be by reducing the oxidative stress and improving antioxidant status, which eventually changed the microenvironment of testes and enhanced the energy needed for motility of sperms, improved the sperm morphology and elevated the testosterone and gonadotropin levels.

Key words: Alcoholism: Reproductive toxicity: Ascorbic acid: Abstention

Chronic alcoholism exerts toxic effects on the male reproductive system[1]. Chronic and persistent alcohol use is known to induce sexual dysfunction[2]. Prolonged alcohol abuse in men can cause testosterone deficiency and testicular atrophy, which can lead to impotence, sterility and feminisation[3]. Alcohol is well documented as a direct testicular toxin[4] and can cause significant deterioration in sperm concentration, motility and morphology in alcoholic men[5,6]. Numerous experimental data indicate that free radical mechanisms contribute to ethanol-induced testicular alterations[7]. Animal studies have indicated that depletion in antioxidant status occurs as a result of oxidative stress due to excess alcohol intake[8,9].

Ascorbic acid or L-ascorbate is a major water-soluble chain-breaking antioxidant that is able to neutralise free radicals. It is essential for the maintenance of the structural and functional integrity of testes[10]. Ascorbic acid plays a significant role in semen integrity and fertility, both in human subjects[11,12]

and experimental animals[13], and contributes to about 65% of the total antioxidant capacity of seminal plasma found intracellularly and extracellularly[14]. Guinea pigs maintained on a high-ascorbic acid diet showed reduction in ethanol-induced toxicity as compared with low ascorbic acid-supplemented animals. High dietary levels of ascorbic acid were found to be protective against hepatic steatosis, necrosis and elevated levels of serum glutamate oxaloacetate transaminase and serum glutamate pyruvate transaminase in guinea pigs caused by chronic ethanol consumption[15].

Although significant progress has been made in understanding the pathogenesis of alcoholic testicular damage, treatment options are limited. At present, abstention from taking alcohol seems to be the only effective therapy adopted to bring down the toxic effects of alcohol. Hence, an effective and economic way to overcome the alterations induced by chronic alcohol abuse has become important. Earlier studies conducted in

our laboratory have shown that ascorbic acid mitigates alcohol-induced hepatotoxicity[16]. Hence, we extended our studies to analyse the impact of ascorbic acid on ethanol-induced reproductive toxicity. Animal models have been developed to study chronic alcohol abuse by the oral administration of alcohol. Like human beings, guinea pigs are unable to synthesise ascorbic acid due to the lack of a key biosynthetic enzyme, L-gulonolactone oxidase[17]. The present study investigated the effect of ascorbic acid supplementation during abstention in chronic alcoholic guinea pigs on reproductive toxicity and compared it with those animals in abstention.

Materials and methods

Experimental animals

Sexually matured male guinea pigs (*Cavia porcellus*) of Hartley strain, weighing between 400 and 450 g, procured from Small animals breeding station, Mannuthy, Thrissur, Kerala, India, were used. The animals were maintained under standard animal husbandry conditions. The animals were housed in polypropylene cages, kept under controlled conditions of temperature of $28 \pm 2^\circ$C, 45–60 % relative humidity and exposed to a 12 h dark−12 h light cycle. Male guinea pigs were fed with guinea pig feed (Sai Feeds) and water was given *ad libitum*. The study protocol was approved by the institutional animal ethics committee (IAEC-KU-6/09-10-BC-MI (21)). Animals were handled using the laboratory animal welfare guidelines[18]. Absolute alcohol was purchased from M/s Merck Limited. Ethanol diluted with distilled water (1:1, v/v) was given by oral administration. Ascorbic acid was purchased from M/s SRL Limited. Ascorbic acid was freshly dissolved in distilled water during treatment and given orally by gastric tube.

Experimental design

Phase I: Dose-dependent studies. Dose-dependent studies were carried out to find the optimum dose of ascorbic acid to be given for regression of alcohol-induced testicular toxicity.

Groups. A total of forty-two guinea pigs were first grouped into two and maintained for 90 d as follows: group 1: control (C1) (*n* 12) and group 2: ethanol treated (4 g/kg body weight (b.wt.)/d) (E) (*n* 30). The dose of alcohol was selected from previous studies[19]. Animals in the control group were given glucose solution equivalent to the energy supplied by ethanol (isoenergetic). Ethanol administration was stopped after 90 d, and six animals in the ethanol-treated group and the control group were killed after overnight fasting and the testis and the blood were collected for biochemical analysis. The animals in the ethanol-treated group were further divided into four groups of six guinea pigs each to which ascorbic acid of different doses was supplemented: E + AA (1 mg/100 g b.wt.) (*n* 6), E + AA (10 mg/100 g b.wt.) (*n* 6), E + AA (25 mg/100 g b.wt.) (*n* 6) and E + AA (50 mg/100 g b.wt.) (*n* 6). The remaining animals in the control group (C2) (*n* 6) were fed with a normal diet. The period of regression was 30 d. Previous studies[16] in our laboratory have shown that administration of ethanol for 90 d, at a dose of 4 g/kg b.wt.,

is ideal to create a chronic alcohol model, and reversal for 30 d using ascorbic acid has been effective in protection against alcohol-induced hepatotoxicity. Hence, the duration of the study was 90 d for toxicity induction and 30 d for reversal. The experimental design is schematically represented in Fig. 1.

At the end of the experimental period, animals were fasted overnight, anaesthetised by ketamine hydrochloride and then killed. The testes were dissected out and cleaned with ice-cold PBS, blotted dry and immediately transferred to ice-cold containers for biochemical and sperm characteristic analysis. Blood was collected for various biochemical analyses.

Phase II. A total of thirty-six guinea pigs were first grouped into two: group 1: control (C) (*n* 18) and group 2: ethanol (4 g/kg b.wt./d) treated (E) (*n* 18). Alcohol at a dose of 4 g/kg b.wt. was given for a period of 90 d. The control group was given isoenergetic glucose solution. Blood was collected weekly from the ear of guinea pigs, and the weekly progression and regression of ethanol toxicity was assessed by assaying the activity of the toxicity marker, γ-glutamyl transpeptidase (GGT), in serum. After 90 d, ethanol administration was stopped and six animals each from the control and the ethanol-treated groups were killed after overnight fasting. The control group was used to evaluate whether the toxicity was induced after 90 d of alcohol administration in the ethanol-treated group by the assay of liver function markers alanine aminotransferase, aspartate aminotransferase and GGT in serum. All other parameters were analysed only in the ethanol-treated group. The blood was collected and the testis and accessory reproductive organs, epididymis and seminal vesicle, were dissected from the ethanol-treated group for various biochemical analysis. The remaining animals in the control group were maintained as control (C) (*n* 6) and ascorbic acid-treated groups (C + AA) (25 mg/100 g b.wt.) (*n* 6) and the remaining animals in the ethanol-treated group were further divided into two groups as the ascorbic acid-treated groups (E + AA) (25 mg/100 g b.wt) (*n* 6) and the ethanol abstention group (EAG) (*n* 6). The experimental design is schematically represented in Fig. 2.

The dose of ascorbic acid was selected from phase I studies. The period of regression was 30 d. At the end of the experimental period (90 + 30 d), animals were fasted overnight, anaesthetised by ketamine hydrochloride and then killed. The testis, epididymis and seminal vesicle were removed and their absolute and relative b.wt. (relative b.wt. = organ weight/final b.wt. × 100) were determined. The testis was dissected out and cleaned with ice-cold PBS, blotted dry and immediately transferred to ice-cold containers for various biochemical evaluations. Blood was collected in clean, dry test-tubes, and allowed to clot at room temperature. The clear serum was removed after centrifugation and used immediately for the assay of alanine aminotransferase, aspartate aminotransferase and GGT and stored at −20°C until the assay of hormones. For plasma, blood was collected in heparinised tubes, centrifuged at 3000 *g* for 10 min and stored at −20°C for HPLC analysis of ascorbic acid.

Fig. 1. Schematic representation of experimental design of phase I study. C, control group; E, ethanol-treated group; b.wt., body weight; E + AA, ascorbic acid-supplemented group.
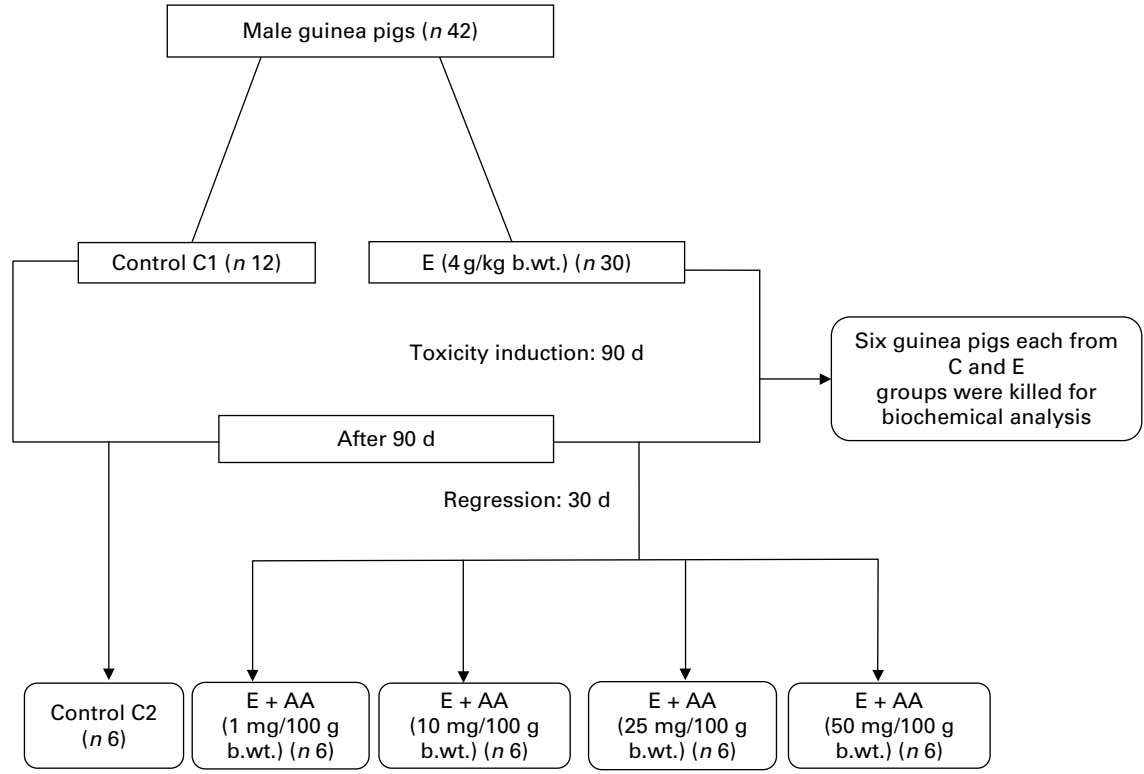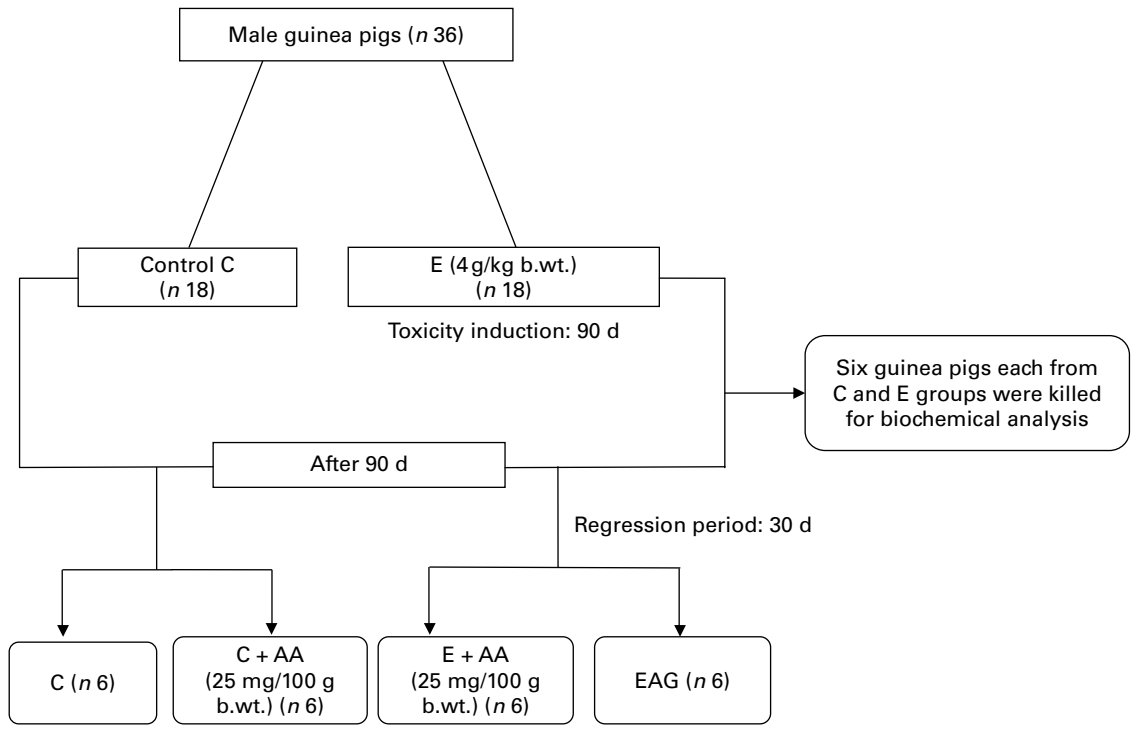
Fig. 2. Schematic representation of experimental design of phase II study. C, control group; E, ethanol-treated group; b.wt., body weight; C + AA, control + ascorbic acid group; E + AA, ascorbic acid-supplemented group; EAG, ethanol abstention group.

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Methods

Semen characteristics. Seminal content of epididymis was obtained by cutting the cauda epididymis using surgical blades and squeezed onto a sterile clean watch glass. This content was diluted ten times with 2·9 % sodium citrate dehydrate solution and thoroughly mixed to estimate the percentage progressive motility and sperm concentration[20]. One drop of the suspension was smeared on a glass slide and stained by eosin−nigrosin stain to determine the percentage of sperm cell viability and morphological abnormalities[20].

Biochemical parameters. Activity of alanine aminotransferase, aspartate aminotransferase[21] and GGT[22] in serum was assayed. Succinate dehydrogenase (SDH) activity[23] and adenosine triphosphatase (ATPase) activity in testes[24] were estimated. Activity of sorbitol dehydrogenase (SORD) in seminal vesicle[25] and the seminal fructose content[26] were determined. Glutathione (GSH) content[27] and ascorbic acid levels in testes[28] were measured. Malondialdehyde (MDA) levels[29] and total protein in testes[30] were estimated.

Assay of serum testosterone, luteinising hormone and follicle-stimulating hormone. Serum testosterone was assayed using a solid-phase RIA kit obtained from the Diagnostic Products Corporation. The sensitivity of the testosterone assay was 4 pg/l. Follicle-stimulating hormone (FSH) and luteinising hormone (LH) were measured using an electrochemiluminescence immunoassay using a Boehringer Manheim Elecsys 2010 Immuno analyser (Roche Diagnostics). The detection limits were 0·10 mIU/ml for FSH and LH.

Determination of ascorbic acid in plasma by HPLC. One part plasma with four parts of 6 % metaphosphoric acid were mixed in a polypropylene storage vial. The vial contents were vortexed and centrifuged at 10 000 g for 15 min at 4°C and the supernatant was used for analysis. HPLC analysis was done by the Shimadzu Prominence SCL-20AHT (Shimadzu) system and the separation of ascorbic acid was done by isocratic gradient elution using a Luna 5S NH2 100A column (Phenomenex). The length of the column was 250 mm × 4·6 mm and the particle size was 5 μm. The mobile phase was HPLC-grade water (eluent A) and methanol (eluent B) in a 1:1 ratio, the total flow rate was 1·0 ml per min and the time of analysis was 15 min. The detector's wavelengths were set at 268 nm. The injection volume was 20 μl and the temperature of the column was thermostated at 40°C.

Assay of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase. Assay of 3β-hydroxysteroid dehydrogenase (3β-HSD) was carried out according to the colorimetric assay of Shivanandappa & Venkatesh[31]. Tissue was homogenised in 0·1 M-Tris−HCl and centrifuged at 12 000 g at 40°C. To 0·05 ml homogenate, 1 ml NAD, 1 ml dehydroepiandrosterone (DHEA) and 0·9 ml water were added and incubated for 1 h at 37°C. The reaction was stopped using 2 ml phthalate buffer. The turbidity was removed by centrifugation at 1000 g for 20 min. Supernatant was read at 490 nm. Activity of 17β-hydroxysteroid dehydrogenase (17β-HSD) was measured according to the method of Jarbak *et al.*[32]. The testicular supernatant (1 ml) was mixed with 440 μM of sodium pyrophosphate buffer (pH 10·2), 25 mg crystalline bovine serum albumin and 0·3 μM of testosterone. The total volume was made up to 3 ml. Enzyme activity was measured after the addition of 1·1 μM-NADP to the incubation mixtures in a spectrophotometer at 340 nm. For a detailed description of the procedures, see Supplementary material (available online).

Statistical analysis

Data are presented as means with their standard errors. The degree of significance was set at $P < 0.05$. One-way ANOVA and *post hoc* Tukey-honestly significant difference test were used to determine the differences among the groups. All the analyses were carried out using the SPSS/PC (version 17.0; SPSS) software package program.

Results

Phase I

The dose-dependent studies showed (Table 1) that animals supplemented with ascorbic acid at a dose of 25 mg/100 g b.wt. and 50 mg/100 g b.wt. showed significant ($P < 0.001$)

Table 1. Effect of different doses of ascorbic acid supplementation after alcohol administration on sperm count, testicular malondialdehyde (MDA) and serum γ-glutamyl transpeptidase (GGT) (phase I study)

(Mean values with their standard errors of six guinea pigs in each group)

Groups	Sperm count (millions/ml)		Testicular MDA (m mol/100 g tissue)		Serum GGT (IU/ml)	
	Mean	SEM	Mean	SEM	Mean	SEM
C1	44·4[a]	4·3	1·58[a]	0·15	6·39[a]	0·61
E	24·4[b]	2·4	3·12[b]	0·30	19·12[b]	2·31
C2	43·7[a]	3·5	1·62[a]	0·16	6·78[a]	0·74
E+AA (1 mg/100 g b.wt.)	25·7[b]	2·5	2·95[d]	0·28	16·72[c]	1·60
E+AA (10 mg/100 g b.wt.)	29·0[b]	2·6	2·80[d]	0·28	14·14[c]	1·60
E+AA (25 mg/100 g b.wt.)	39·0[a]	3·1	2·18[c]	0·21	7·85[a]	0·74
E+AA (50 mg/100 g b.wt.)	38·2[a]	3·0	2·30[c]	0·19	8·71[a]	0·83

C1, control group maintained for first 90 d; E, ethanol-treated group; C2, control group maintained for last 30 d; E+AA (1 mg/100 g b.wt.), 1 mg/100 g body weight ascorbic acid-supplemented group; E+AA (10 mg/100 g b.wt.), 10 mg/100 g body weight ascorbic acid-supplemented group; E+AA (25 mg/100 g b.wt.), 25 mg/100 g body weight ascorbic acid-supplemented group; E+AA (50 mg/100 g b.wt.), 50 mg/100 g body weight ascorbic acid-supplemented group.

[a,b,c,d] Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

Table 2. Activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transpeptidase (GGT) in serum after 90 d of alcohol administration (phase II study)

(Mean values with their standard errors of six guinea pigs in each group)

Groups	ALT in serum (μmol of pyruvate liberated/min per mg protein)		AST in serum (μmol of oxaloacetate liberated/ min per mg protein)		Serum GGT (IU/ml)	
	Mean	SEM	Mean	SEM	Mean	SEM
C	174·46[a]	6·18	132·25[a]	13·20	5·36[a]	0·82
E	241·00[b]	19·28	268·57[b]	24·13	18·14[b]	1·45

C, control group; E, ethanol-treated group.
[a,b] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).

revival in sperm count and a decrease in elevated testicular MDA levels induced by ethanol administration, as compared with the control. The maximum effect was shown by the group supplemented with 25 mg/100 g b.wt. ascorbic acid. The low dose groups (1 and 10 mg/100 g b.wt. ascorbic acid) did not significantly restore the sperm count and testicular MDA levels. The activity of the toxicity marker, GGT, in serum, which was increased significantly ($P<0·001$) in the alcohol-ingested group, was reduced significantly ($P<0·001$) in groups supplemented with 25 and 50 mg/100 g b.wt. of ascorbic acid and maximum recovery was shown by the group supplemented with 25 mg/100 g b.wt. of ascorbic acid.

Phase II

The liver function markers alanine aminotransferase, aspartate aminotransferase and GGT (Table 2) in serum increased significantly ($P<0·001$) in the ethanol-treated group when compared with the control.

The b.wt. of guinea pigs (Table 3) decreased significantly ($P<0·05$) in the alcohol-administered group when compared with the control. The b.wt. of both the E + AA and EAG groups increased significantly ($P<0·01$). However, the change in b.wt. in the ascorbic acid-supplemented group ($P<0·001$) was significantly higher than in the EAG group.

The relative weight of testes and epididymis (Table 3) decreased significantly ($P<0·05$) in the ethanol-administered group, as compared with the control. The relative weight of these organs increased on ascorbic acid administration. The abstention group also showed an increase in relative testicular weight but was less significant when compared with the ascorbic acid-supplemented group. However, the abstention group showed an increase in relative epididymal weight similar to the ascorbic acid-supplemented group. No significant change was observed in relative organ weights of the seminal vesicle.

GGT in serum during the treatment period (Fig. 3) showed a gradual increase in activity upon administration of ethanol. Both ascorbic acid supplementation and abstention significantly ($P<0·05$) decreased the activity of GGT. However, a faster recovery was observed in the E + AA group than with abstention.

Sperm parameters, like percentage sperm motility, sperm count and viability (Table 4), decreased significantly ($P<0·001$) in the ethanol-treated guinea pigs. The percentage of abnormal sperms with marked morphological defects, like headless sperm, double-headed sperm, tailless sperm, looped tail and coiled tail sperms, increased significantly in the ethanol-treated group, as compared with the control. The sperm parameters were restored on ascorbic acid administration. Abstention was also able to reverse the sperm parameters, but was less significant when compared with the ascorbic acid-treated group.

The lipid peroxidation product, MDA (Table 5), increased significantly ($P<0·05$) in the alcohol-ingested group when compared with the control, which was reduced on ascorbic acid supplementation. The MDA concentration also showed a decline in the abstention group, but was less pronounced when compared with the ascorbic acid-supplemented group.

The concentrations of testicular GSH content and ascorbic acid in testes (Table 5) and plasma (Fig. 4) were reduced significantly ($P<0·001$) in the ethanol-loaded group, which were restored to near-normal levels on ascorbic acid administration. Abstention could also increase the levels of GSH and ascorbic acid, but to a lesser extent when compared with the ascorbic acid-supplemented group.

Table 3. Body and reproductive organ weights (phase II study)

(Mean values with their standard errors of six guinea pigs in each group)

Parameters	C		C + AA		E		EAG		E + AA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Initial body weight (g)	436[a]	4·0	430[a]	3·0	445[a]	4·0	448[a]	4·0	427[a]	3·0
Final body weight (g)	638[a]	5·0	653[a]	3·0	464[b]	4·0	506[c]	4·0	609[d]	5·0
Change in body weight (g)	204[a]	2·0	223[b]	2·0	19[c]	0·50	61[d]	1·0	174[e]	2·0
Testes (g)	2·69[a]	0·09	2·88[a]	0·10	1·26[b]	0·04	1·82[c]	0·06	2·49[a]	0·11
Relative weight of testes (g/100 g b.wt.)	0·42[a]	0·04	0·44[a]	0·04	0·27[b]	0·02	0·36[c]	0·03	0·41[d]	0·04
Epididymis (g)	0·74[a]	0·02	0·83[a]	0·03	0·44[b]	0·01	0·55[b]	0·02	0·69[a]	0·02
Relative weight of epididymis (g/100 g b.wt.)	0·12[a]	0·02	0·11[a]	0·02	0·09[b]	0·03	0·11[a]	0·02	0·11[a]	0·03
Seminal vesicle (g)	0·82[a]	0·03	0·91[b]	0·03	0·63[c]	0·02	0·69[c]	0·02	0·77[a]	0·02
Relative weight of seminal vesicle (g/100 g b.wt.)	0·13[a]	0·01	0·14[a]	0·02	0·14[a]	0·02	0·14[a]	0·01	0·13[a]	0·01

C, control group; C + AA, control + ascorbic acid group; E, ethanol-treated group; EAG, ethanol abstention group; E + AA, ascorbic acid-supplemented group; b.wt., body weight.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

Table 4. Sperm characteristic analysis (phase II study)

(Mean values with their standard errors of six guinea pigs in each group)

Groups	Sperm motility (%)		Sperm count (millions/ml)		Sperm viability (%)		Sperm abnormalities (%)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C	96.8[a]	3.6	46.8[a]	1.8	98.0[a]	3.5	1.2[a]	0.04
C+AA	98.4[a]	3.7	44.4[a]	1.7	94.6[a]	3.5	1.1[a]	0.03
E	58.3[b]	2.1	33.3[b]	1.2	53.4[b]	2.0	8.6[b]	0.3
EAG	75.0[c]	2.8	37.8[b]	1.4	67.7[c]	2.4	6.1[c]	0.2
E+AA	87.8[a]	3.3	41.6[a]	1.5	80.3[d]	3.0	3.9[d]	0.1

C, control group; C + AA, control + ascorbic acid group; E, ethanol-treated group; EAG, ethanol abstention group; E + AA, ascorbic acid-supplemented group.
[a,b,c,d] Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).
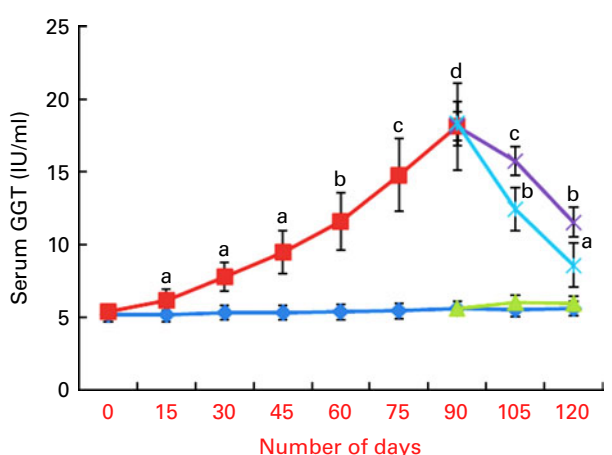

Fig. 3. Time-course measurement of activity of γ-glutamyl transpeptidase in serum. Values are means, with their standard errors of six guinea pigs in each group represented by vertical bars. [a,b,c,d] Mean values with unlike letters were significantly different ($P<0.05$). C (—), control group; C + AA (—), control + ascorbic acid group; E (—■—), ethanol-treated group; EAG (—), ethanol abstention group; E + AA (—), ascorbic acid-supplemented group. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

Serum testosterone, LH and FSH levels (Table 6) were significantly reduced ($P<0.05$) in the alcohol-administered group, but were elevated on ascorbic acid supplementation. Abstention did not significantly improve the testosterone concentration and gonadotropin levels, as compared with the ascorbic acid-treated group.

The activities of key steroidogenic enzymes, 3β-HSD and 17β-HSD (Table 7), were reduced significantly ($P<0.05$) in the ethanol-administered group, as compared with the control. The activities of these enzymes increased significantly ($P<0.05$) in the ascorbic acid-administered group and with abstention. The increase in the activities was more pronounced in the ascorbic acid-supplemented group when compared with abstention.

The concentration of fructose (Table 7) in seminal plasma and activity of SORD in seminal vesicle were reduced significantly ($P<0.05$) in the alcohol-administered group, as compared with the control. Ascorbic acid supplementation significantly ($P<0.05$) increased their concentration compared with the abstention group.

The SDH and ATPase activity (Table 7) in testes dropped significantly ($P<0.05$) in the alcohol-fed group when compared with the control. Their activities were brought back to near-normal level by the administration of ascorbic acid. Abstention also increased their activities, but was less significant compared with the ascorbic acid-fed group.

Discussion

Earlier reports[14] have implicated the role of reactive oxygen species-mediated oxidative stress in the pathophysiology of alcohol-mediated reproductive toxicity. Numerous studies are available regarding the effect of ascorbic acid on alcohol-induced hepatotoxicity. However, very less information is available regarding the effect of ascorbic acid in alcohol-mediated testicular dysfunctions in scorbutic animal models. Ascorbic acid is one of the important and essential vitamins for human health. It functions physiologically as a

Table 5. Effect of ascorbic acid supplementation and abstention after alcohol administration on concentration of malondialdehyde (MDA), glutathione (GSH) and ascorbic acid in testes (phase II study)

(Mean values with their standard errors of six guinea pigs in each group)

Parameters	C		C+AA		E		EAG		E+AA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Testicular MDA (mM/100 g tissue)	1.28[a]	0.02	1.41[a]	0.02	3.09[b]	0.09	2.35[c]	0.06	1.63[a]	0.02
Testicular GSH (mM/100 g tissue)	4.16[a]	0.23	4.23[a]	0.20	1.86[b]	0.14	2.57[c]	0.18	3.31[d]	0.20
Ascorbic acid (mg/100 g tissue)	4.95[a]	0.27	6.54[b]	0.35	2.95[c]	0.21	3.90[d]	0.25	5.00[a]	0.30

C, control group; C+AA, control + ascorbic acid group; E, ethanol-treated group; EAG, ethanol abstention group; E + AA, ascorbic acid-supplemented group.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P<0.05$).
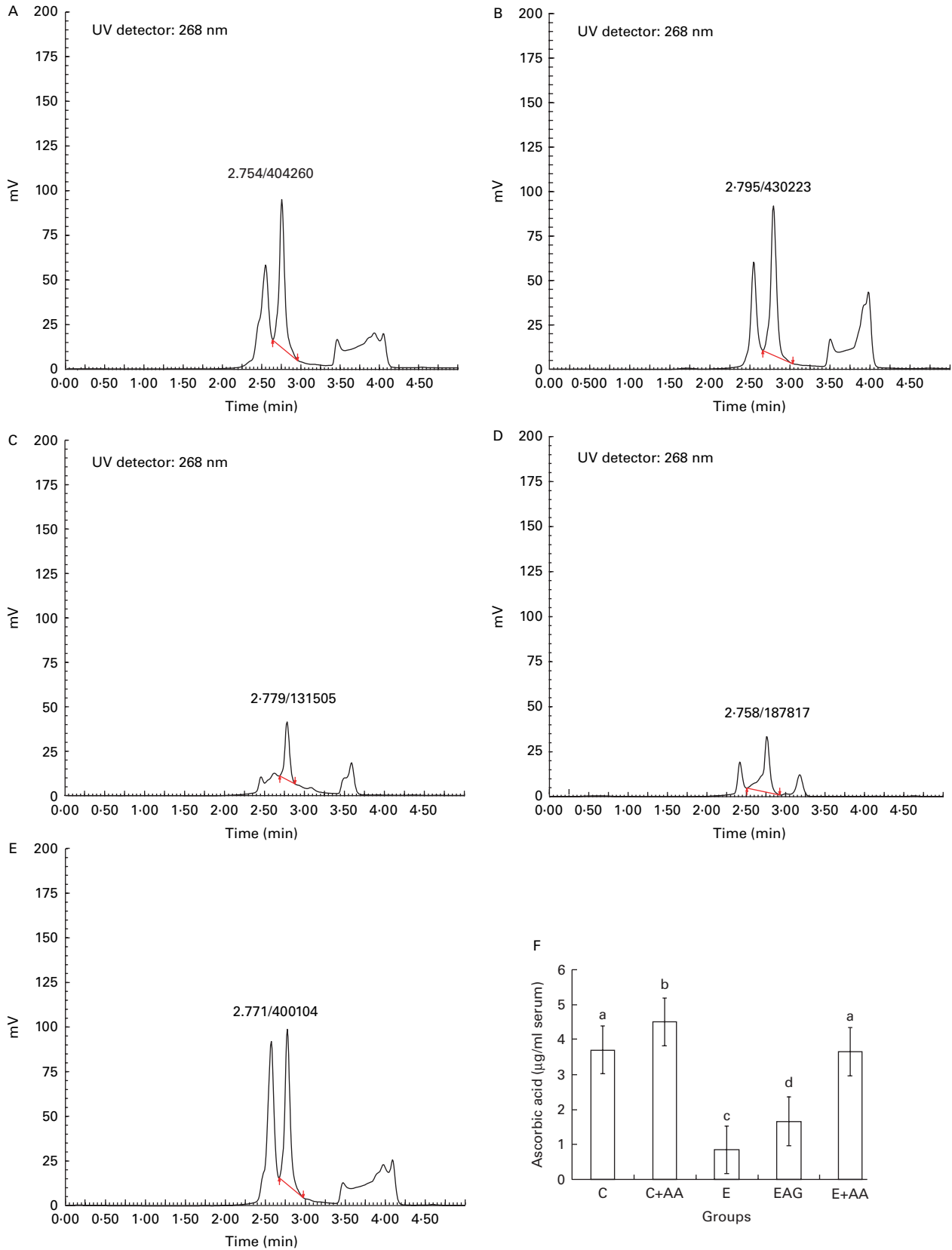
Fig. 4. HPLC chromatograms of ascorbic acid in serum. (A) C, control group; (B) C + AA, control + ascorbic acid group; (C) E, ethanol-treated group; (D) EAG, ethanol abstention group; (E) E + AA, ascorbic acid-supplemented group. The retention time was 2·7 min. (F) Graphical representation of ascorbic acid content in the serum. Values are means, with their standard errors of six guinea pigs in each group represented by vertical bars. [a,b,c,d] Mean values with unlike letters were significantly different ($P < 0.05$). (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

Table 6. Effect of ascorbic acid supplementation and abstention after alcohol administration on serum testosterone levels, luteinising hormone (LH) and follicle-stimulating hormone (FSH) (phase II study)

(Mean values with their standard errors of six guinea pigs in each group)

Groups	Testosterone (ng/ml)		LH (mIU/ml)		FSH (mIU/ml)	
	Mean	SEM	Mean	SEM	Mean	SEM
C	2·16[a]	0·08	1·63[a]	0·05	0·74[a]	0·08
C + AA	2·90[b]	0·11	1·80[b]	0·06	0·85[b]	0·11
E	1·60[c]	0·06	0·60[c]	0·05	0·40[c]	0·06
EAG	1·77[d]	0·07	0·77[d]	0·07	0·52[d]	0·07
E + AA	3·52[e]	0·11	1·52[a]	0·10	1·01[e]	0·11

C, control group; C+AA, control + ascorbic acid group; E, ethanol-treated group; EAG, ethanol abstention group; E+AA, ascorbic acid-supplemented group.
[a,b,c,d,e] Mean values within a column with unlike superscript letters were significantly different (*P*< 0·05).

water-soluble antioxidant by virtue of its high reducing power and acts as a cofactor for enzymes involved in the biosynthesis of collagen, carnitine and neurotransmitters, and quenches a variety of reactive oxygen species and reactive nitrogen species in the aqueous environment[33]. Several conditions, such as alcoholism, stress, smoking, infections and various pathological conditions, which increase oxidative stress, deplete the ascorbic acid reserves in the body and demand higher doses of ascorbic acid supplementation[34]. The new average daily intake level for human subjects that is sufficient to meet the nutritional requirement of ascorbic acid or RDA for adults (>19 years) are 90 mg/d for men and 75 mg/d for women[35]. We initially carried out a dose–response study to find the effective dose of ascorbic acid required to reduce alcohol-induced testicular alteration in guinea pigs. Further investigations were done using the selected dose to analyse the impact of ascorbic acid supplementation on ethanol-induced reproductive toxicity in male guinea pigs and also to compare it with abstention.

The dose–response study revealed that a dose of 25 mg/100 g b.wt. of ascorbic acid showed the maximum effect in regression of alcohol-induced testicular toxicity by bringing down MDA levels and restoring the sperm count to near-normal levels. The activity of the toxicity marker, GGT, in serum was also significantly reduced and was brought to a near-normal level by supplementing 25 mg/100 g b.wt of ascorbic acid. Hence, this dose was non-toxic and was selected for further studies.

The progression and regression of alcohol-induced toxicity were evaluated by the assay of GGT in the serum, a marker for ethanol-induced toxicity on a weekly basis. Both ascorbic acid supplementation and abstention could reduce the toxicity induced by alcohol, but a faster recovery was recorded in the case of ascorbic acid supplementation, indicating the role of ascorbic acid as a therapeutic agent to reduce alcohol-induced damage. This reduction in toxicity was also reflected in body weight gain and an increased relative organ weight of testes in the ascorbic acid-supplemented group, as compared with abstention.

The testicular MDA levels were elevated and levels of non-enzymatic antioxidants like GSH in testes and ascorbic acid levels in testes and plasma were diminished in alcohol- administered animals, suggesting a state of oxidative stress in testes. GSH and ascorbic acid are lines of defence against oxidative damage and facilitate protection against ethanol-induced reactive oxygen species production. Depletion in antioxidants enhances oxidative stress in testes. This is in agreement with the study of Grattagliano *et al.*[7]. Ascorbic acid supplementation was able to reduce the oxidative stress induced by chronic alcoholic intake by reducing the enhanced levels of MDA and by alleviating the depleted levels of GSH and ascorbic acid. Abstention could also lower oxidative stress, but to a smaller extent when compared with the ascorbic acid-administered group. The observation in the present study is in line with our[36] previous reports that supplementation of ascorbic acid reduced the depletion of GSH in the liver caused by the administration of ethanol. Hence, reduction in oxidative stress caused by alcohol may be one of the mechanisms of ascorbic acid to provide a protective effect against alcohol-induced toxicity.

Table 7. Effect of ascorbic acid supplementation and abstention after alcohol administration on testicular sorbitol dehydrogenase (SORD), fructose, succinate dehydrogenase (SDH), adenosine triphosphatase (ATPase), 17β-hydroxysteroid dehydrogenase (17β-HSD) and 3β-hydroxysteroid dehydrogenase (3β-HSD) (phase II study)

(Mean values with their standard errors of six guinea pigs in each group)

Parameters	C		C + AA		E		EAG		E+AA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
SORD (units*/mg protein)	2·71[a]	0·09	2·87[a]	0·10	1·25[b]	0·11	1·43[b]	0·09	2·08[d]	0·05
Fructose (mg/ml of seminal plasma)	2·08[a]	0·07	2·18[a]	0·08	0·78[b]	0·03	1·19[c]	0·04	1·51[d]	0·05
SDH (μg formazon formed/mg protein per 15 min)	8·32[a]	0·02	8·37[a]	0·01	5·20[b]	0·03	6·56[c]	0·02	7·94[a]	0·03
ATPase (μmol inorganic phosphate released/mg protein per 30 min)	5·47[a]	0·20	5·66[a]	0·21	3·65[b]	0·13	4·23[c]	0·16	4·96[d]	0·18
17β-HSD (units†/mg protein)	1·67[a]	0·07	1·78[a]	0·07	0·62[b]	0·05	0·79[c]	0·05	1·14[d]	0·06
3β-HSD (units†/mg protein)	7·40[a]	0·32	7·63[a]	0·32	4·21[b]	0·21	5·33[c]	0·22	6·04[d]	0·22

C, control; E, ethanol-treated group; C + AA, control + ascorbic acid-treated group; EAG, ethanol abstention group; E+AA, ascorbic acid-supplemented group.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different (*P*< 0·05).
* μg of fructose liberated/min at 37°C.
† Change in absorbance of 0·001/min at 340 nm.

Ethanol administration reduced circulating testosterone levels. This may be due to the decreased activities of the major steroidogenic enzymes, 3β-HSD and 17β-HSD, involved in testosterone biosynthesis in the ethanol-treated group. This is in accord with the studies of Maneesh et al.[37]. The reduction in testosterone levels can also be attributed to a suppressed hypothalamic–pituitary–gonadal axis, as there was a significant decrease in circulating LH and FSH levels in ethanol-administered animals. This is in agreement with the observation of Salonen & Huhtaniemi[38]. Ascorbic acid plays a key role in the synthesis of testosterone[39], and ascorbic acid deficiency can affect testosterone synthesis in testes. In the present study, ascorbic acid supplementation increased the activities of the steroidogenic enzymes, 3β-HSD and 17β-HSD, and also elevated serum testosterone, LH and FSH levels. However, abstention could not significantly restore the steroidogenic enzyme activities and circulating testosterone, LH and FSH levels, as compared with the ascorbic acid-supplemented group.

The quality of semen in ethanol-treated groups deteriorated as sperm parameters like percentage motility, viability and count were significantly reduced. There was a significant impairment in the morphology of sperm, as the percentage of abnormal sperms increased on excessive alcohol consumption. A low testosterone concentration may be responsible for the adverse effects of ethanol on sperm parameters, as a high level of testosterone in testes is critically required for normal spermatogenesis, development and maintenance of sperm morphology and normal physiology of seminiferous tubules[40,41]. The diminution of sperm parameters was restored by ascorbic acid supplementation. The findings are in agreement with studies of Ganaraja et al.[42]. Abstention from alcohol was also able to revive the sperm parameters. However, the reversal was more pronounced in the ascorbic acid-supplemented group than with abstention.

Fructose is a main source of energy for sperm motility. Fructose is produced from sorbitol by the coordinated function of SORD and serves as an energy source for spermatozoa[43,44]. The ethanol-treated animals showed a decrease in fructose content and SORD activity. This accounts for the reduced sperm motility in the ethanol-loaded groups. There were significant increases in fructose content and SORD activity on ascorbic acid supplementation, which further improved sperm quality. Abstention could not substantially increase the concentration of fructose and SORD activity, as compared with the ascorbic acid-supplemented group.

SDH is a key enzyme in the mitochondrial Kreb's cycle, which is mainly concerned with the aerobic oxidation of acetyl CoA and the generation of ATP. A decrease in SDH activity in the testes of ethanol-ingested guinea pigs indicates a reduction in aerobic oxidation, which could be the result of reduced oxygen transport to tissues[45]. Reduced aerobic oxidation and ATP generation in the testes could be responsible for the reduction in ATPase activity. Decreased SDH and ATPase activity with reduced fructose levels could explain the reduced sperm count and motility and the increased number of non-viable spermatozoa observed in the ethanol-administered animals. Ascorbic acid supplementation restored both SDH and ATPase activity, which accounts for the restoration of diminished sperm quality in the ascorbic acid-supplemented group. Abstention from alcohol was able to increase SDH and ATPase activity, but was less significant when compared with the ascorbic acid-supplemented group.

Conclusion

The present study demonstrates that ascorbic acid supplementation causes a reduction of alcohol-induced testicular toxicity to a greater extent than abstention. The mechanism may be by reducing oxidative stress and improving antioxidant status, which eventually change the microenvironment of testes and enhance the energy needed for motility of sperms, improve the sperm morphology and increase the testosterone levels. The increase in testosterone levels may be due to increased steroidogenic enzyme activities and elevated levels of LH and FSH of the hypothalamic–pituitary–gonadal axis. In short, therapeutic supplementation of ascorbic acid is effective in minimising the alterations produced by alcohol-induced testicular damage. Further studies are needed to complement and extrapolate the findings of the study to human subjects.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114512005739

Acknowledgements

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