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In vivo effects of vitamin C on the cytotoxicity of post-ethanol serum

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Abstract—The consumption of alcohol is followed by the development in the serum of a non-dialysable cytotoxic activity against A9 cells. This cytotoxicity has been previously shown to reside mainly in unstable acetaldehyde–albumin complexes from which cytotoxic acetaldehyde molecules can be transferred to target cells. The cytotoxicity developing in serum albumin 8 hr after seven healthy volunteers drank 84 g ethanol over 45 min was abolished when the same volunteers were pre-treated with 1 g vitamin C daily for 3 days prior to alcohol consumption. The cytotoxicity was measured against A9 cells using two different indicators: (i) detachment of adherent cells and (ii) a decrease in the ability of cells to reduce tetrazolium. These data suggest that the administration of vitamin C may be useful in limiting those aspects of alcohol toxicity mediated by circulating acetaldehyde.

Key words: ascorbic acid; ethyl alcohol; acetaldehyde–albumin complexes; toxicity

When healthy volunteers drink 0.8–1.2 g ethanol per kg body weight over 20–45 min, either as ethanol (BP grade) or as wine, a non-dialysable cytotoxic activity appears in the serum with a peak 6–10 hr after the start of alcohol consumption [1, 2]. This cytotoxicity can be demonstrated in dialysed post-alcohol serum using three different indicators of cytotoxicity: (a) detachment of adherent A9 cells [1–3], (b) inhibition of [^{3}H]thymidine incorporation into the DNA of cell lines [4] and (c) impairment of PHA-stimulated lymphocyte transformation [5]. Three lines of evidence indicate that the post-ethanol cytotoxic activity resides in unstable Schiff bases formed during the first stage of the reaction between acetaldehyde and albumin [4]: (1) Sephacryl S300 gel filtration of post-alcohol serum reveals that the cytotoxic activity is in the albumin fraction, (2) complexes formed *in vitro* by the reaction of acetaldehyde with human serum albumin are cytotoxic and (3) the cytotoxicity of both post-alcohol serum and artificially-prepared acetaldehyde–albumin complexes falls sharply after treatment with the reducing agent sodium borohydride, a treatment known to stabilise unstable Schiff bases by addition of hydrogen across the double bond C = N [6, 7]. Other experiments have shown that the cytotoxicity results from the release of acetaldehyde from unstable acetaldehyde–albumin complexes and the preferential binding of the free acetaldehyde to the target cells [4].

Recently, we reported that when post-ethanol serum is exposed *in vitro* to 10–500 μg/mL ascorbic acid for 3 hr, its cytotoxic activity against A9 cells and PHA-stimulated normal human lymphocytes is reduced [8]. In the present study we have investigated whether the administration of vitamin C orally to healthy volunteers influences the development *in vivo* of cytotoxic activity in post-ethanol serum.

Materials and Methods

Seven healthy volunteers who had abstained from drinking any alcohol for at least 3 days drank 700 mL of white wine (84 g ethanol) over 45 min. Venous blood (20 mL) was obtained before the wine was consumed (pre-alcohol sample) and 8 hr after consumption began (post-alcohol sample). After an interval of 10–14 days, the same volunteers ingested 1 g of vitamin C per day every evening for 3 days during which period they again refrained from any alcohol consumption. Two hours after the third dose of vitamin C, they drank 700 mL of the same white wine over 45 min, after giving a pre-alcohol blood sample. The post-alcohol blood sample was taken 8 hr after the consumption of wine began, as before. All the blood samples were placed in plain glass tubes and allowed to clot. The sera were separated and stored at −70° until use.

Separation of albumin. Aliquots of pre- and post-alcohol sera were subjected to affinity chromatography on "Blue Sepharose CL-6B" and the albumin eluted as described earlier [4]. The albumin-containing fractions so obtained were pooled and the pool dialysed against RPMI 1640 at 4°. The cytotoxicity of the dialysed albumin preparations was then studied, after adjusting the albumin concentration to 0.5 mg/mL, by two different techniques as described below.

Cytotoxicity assay based on detachment of A9 cells. One millilitre aliquots of each of the dialysed albumin preparations obtained from pre- and post-alcohol sera were incubated at 37° for 16 hr with monolayers of adherent A9 cells within the wells of a multiwell tissue culture plate and the number of residual adherent cells determined as described previously [1, 3]. The cytotoxicity of an albumin preparation was considered to be inversely proportional to the number of residual adherent cells. Detachment of cells may indicate irreversible or reversible impairment of cell function or cell death or both.

Colorimetric MTT (tetrazolium) cytotoxicity assay [9]. Aliquots (200 μL) of each of the dialysed albumin preparations obtained from pre-and post-alcohol sera were interacted at 37° for 16 hr with monolayers of adherent A9 cells within the wells of a microtitre plate. The preparation of the A9 cell monolayers was as described previously [1], except that 200 μL volumes rather than 1 mL volumes of a suspension of A9 cells containing 3×10^5 cells/mL were dispensed into each well; the suspending fluid consisted of RPMI 1640 plus 10% (v/v) foetal calf serum. After the A9 cell monolayers were incubated with the albumin solutions, 20 μL of a solution containing 5 mg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; tetrazolium—Sigma Chemical Co., Poole, U.K.) per mL phosphate-buffered saline was added to each well and the plates incubated at 37° for a further 4 hr. The resulting dark blue crystals were dissolved, after removal of 100 μL of the supernatant, by adding to each well 200 μL of 0.04 N HCl in isopropanol as described by Mosmann [9]. The plates were read at a test wavelength of 540 nm, using a reference wavelength of 620 nm.

The MTT assay is based on the ability of various dehydrogenase enzymes in functioning mitochondria to cleave the tetrazolium ring and convert the yellow tetrazolium salt to dark blue crystals [9]. Reduced

Table 1. Effect of pre-treatment with 1 g vitamin C per day for 3 days on the difference in the number of adherent A9 cells with albumin preparations obtained from pre- and post-alcohol sera of seven healthy volunteers consuming 84 g ethanol over 45 min

State of volunteer	No of residual adherent A9 cells after incubation with albumin ($\times 10^5$/well)					Percentage difference between values with albumin from pre- and post-alcohol sera	
	Pre-alcohol serum		Post-alcohol serum				
	Mean	SD	Mean	SD	P*	Mean	SD
Not pre-treated with vitamin C	1.505	0.539	0.985	0.396	<0.02	−33.7	21.3
Pre-treated with vitamin C	1.352	0.535	1.325	0.504	>0.6	−0.4†	5.7

* Significance of difference from values given by albumin preparations from pre-alcohol sera (paired *t*-test).

† Significance of difference from corresponding data in the same volunteers studied without pre-treatment with vitamin C (paired *t*-test): $P < 0.01$.

Table 2. Effect of pre-treatment with 1 g vitamin C per day for 3 days on the difference in the absorbance at 540 nm with albumin preparations obtained from pre- and post-alcohol sera of seven healthy volunteers consuming 84 g ethanol over 45 min

State of volunteer	Absorbance at 540 nm					Percentage difference between values with albumin from pre- and post-alcohol sera	
	Pre-alcohol serum		Post-alcohol serum				
	Mean	SD	Mean	SD	P*	Mean	SD
Not pre-treated with vitamin C	0.203	0.043	0.138	0.064	<0.005	−25.1	16.7
Pre-treated with vitamin C	0.201	0.042	0.199	0.046	>0.9	−1.4†	6.8

* Significance of difference from values given by albumin preparations from pre-alcohol sera (paired *t*-test).

† Significance of difference from corresponding data in the same volunteers studied without pre-treatment with vitamin C (paired *t*-test): $P < 0.005$.

generation of blue crystals indicates impaired mitochondrial function (reversible or irreversible) or cell death or both.

Results

In each of the volunteers studied without pre-treatment with vitamin C, the number of residual adherent cells after incubating monolayers of A9 cells with albumin from post-alcohol serum was less than with albumin from pre-alcohol serum ($P < 0.02$), indicating that the former had become cytotoxic. On average, there was a 33.7% reduction with albumin from post-alcohol compared to that from pre-alcohol serum (Table 1). By contrast, when the same volunteers were studied after pre-treatment with vitamin C, the albumin from post-alcohol serum did not show cytotoxic activity (Table 1).

Table 2 shows the results of the colorimetric MTT (tetrazolium) assay performed on A9 cells reacted with albumin fractions obtained from pre- and post-alcohol serum, in the experiments with and without pre-treatment with vitamin C. In every subject, the absorbance in wells containing albumin from post-alcohol serum was lower than from pre-alcohol serum ($P < 0.005$), with an average reduction of 33.4%. By contrast, when the same subjects were re-investigated after pre-treatment with vitamin C, there was no significant difference between the absorbance in wells containing albumin from post-alcohol serum and pre-alcohol serum.

Figures 1 and 2 show the individual values for the cytotoxicity of albumin from post-alcohol serum measured by two different methods in the seven subjects studied. The figures emphasise that, whereas in every subject substantial cytotoxic activity developed in the serum albumin when alcohol was consumed without pre-treatment with vitamin C, little or no cytotoxicity developed when alcohol was consumed after pre-treatment with this vitamin.

Discussion

The results reported here indicate that the administration of 1 g vitamin C daily to healthy volunteers for 3 days prevents the development of cytotoxic activity in the

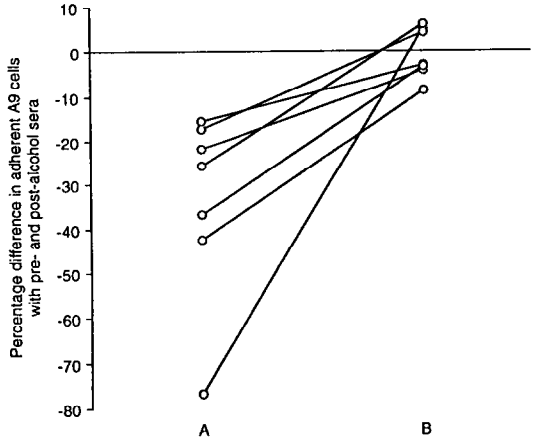

Fig. 1. Percentage difference in the number of adherent A9 cells after incubation of cells with albumin prepared from pre- and post-alcohol serum. (A) Subjects not pre-treated with vitamin C; (B) same subjects pre-treated with vitamin C. An increase in the percentage indicates decreased cytotoxicity.

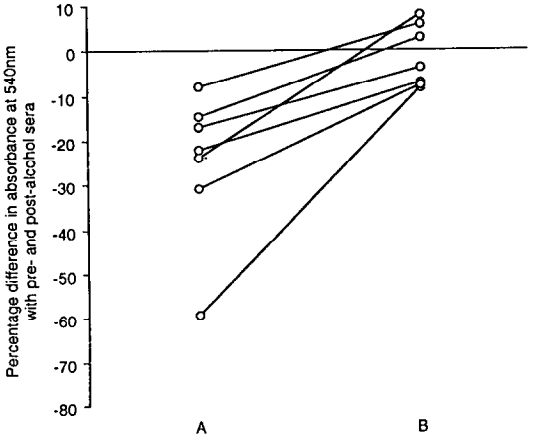

Fig. 2. Percentage difference in the absorbance at 540 nm after A9 cells were incubated with albumin prepared from pre- and post-alcohol serum and reacted with tetrazolium. (A) Subjects not pre-treated with vitamin C; (B) same subjects pre-treated with vitamin C. An increase in the percentage indicates decreased cytotoxicity.

albumin molecules of post-alcohol serum. This conclusion was reached using two different methods of assessing cytotoxicity, namely the detachment of adherent A9 cells and the ability of A9 cells (actually mitochondria) to cleave the tetrazolium ring (the colorimetric MTT assay). Thus, post-alcohol sera of vitamin C-treated subjects (i.e. sera taken 8 hr after the consumption of wine began) must be considered to lack appreciable quantities of unstable acetaldehyde–albumin complexes. These *in vivo* data are consistent with previous *in vitro* data indicating that the incubation of post-alcohol sera with vitamin C results in a reduction in cytotoxic activity [8]. The *in vivo* and *in vitro* effects of vitamin C may be related to the observation that this vitamin promotes the formation of stable adducts between acetaldehyde and the lysine moieties of bovine serum albumin by a mechanism that does not involve the reduction of Schiff bases [10]. At least in theory, the *in vivo* effects may also result from the prevention of the extracellular release of acetaldehyde by ethanol-metabolizing cells.

There are some data in experimental animals, and limited data in humans, that vitamin C may reduce the toxic effects of alcohol. Thus, high doses of ascorbic acid have been shown to increase the survival of and reduce blood ethanol levels in ethanol-intoxicated rats and mice [11], to reverse the impaired swimming behaviour of ethanol-intoxicated mice [12], to increase the rate of decline of blood ethanol in ethanol-infused guinea pigs [13] and to decrease serum aspartate and alanine aminotransferase activities, hepatic steatosis and hepatic necrosis in ethanol-treated guinea pigs [13, 14]. In human volunteers given ethanol orally, pretreatment with ascorbic acid caused significant enhancement of blood ethanol clearance, improved motor coordination and better colour discrimination in some of the subjects [15, 16]. It is also well established that chronic alcoholics have low plasma and white cell vitamin C levels [17] and it is common practice to administer vitamin C intravenously to such patients during conventional detoxification therapy.

It has been suggested that the enhancement of ethanol clearance caused by vitamin C may be mediated via an ascorbic acid-dependent ethanol oxidizing system that involves the peroxidic oxidation of ethanol by catalase and that the effects of this vitamin on ethanol-related behaviour changes may be caused by its involvement in neurochemical processes [16]. Recent data suggest that, by delivering acetaldehyde to various target cells, circulating unstable acetaldehyde–albumin complexes may contribute to alcohol-related tissue damage (especially to extrahepatic tissue damage) and that the persistence of such complexes in the circulation for several hours after blood alcohol levels have returned to normal serves to prolong the acetaldehyde-mediated toxicity of alcohol *in vivo* [1, 18]. If these ideas are correct, our data suggest that another mechanism by which vitamin C may have a protective effect against some aspects of alcohol toxicity could be by stabilizing potentially cytotoxic unstable acetaldehyde–albumin complexes or by preventing their formation.

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REFERENCES

1. Wickramasinghe SN, Gardner B and Barden G, Cytotoxic protein molecules generated as a consequence of ethanol metabolism *in vitro* and *in vivo*. *Lancet* **ii**: 823–826, 1986.
2. Wickramasinghe SN, Clague JR, Barrison IG, Walker JG, Barden G and Gardner B, Differences in the nature and kinetics of the ethanol-related circulating cytotoxic proteins in normal subjects and patients with alcohol-induced cirrhosis. *Alcoholism: Clin Exp Res* **13**: 723–729, 1989.
3. Wickramasinghe SN, Supernatants from ethanol-containing macrophage cultures have cytotoxic activity. *Alcohol Alcoholism* **21**: 263–268, 1986.
4. Wickramasinghe SN, Gardner B and Barden G,

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Circulating cytotoxic protein generated after ethanol consumption: identification and mechanism of reaction with cells. *Lancet* **ii**: 122–126, 1987.
5. Wickramasinghe SN and Barden G, Acetaldehyde-modified serum proteins inhibit mitogen-stimulated human lymphocyte transformation. *Br J Haematol* **73**: 23–28, 1989.
6. Donohue TM Jr, Tuma DJ and Sorrell MF, Acetaldehyde adducts with proteins: binding of [^{14}C]-acetaldehyde to serum albumin. *Arch Biochem Biophys* **220**: 239–246, 1983.
7. Tuma DJ and Sorrell F, Covalent binding of acetaldehyde to hepatic proteins: role in alcoholic liver injury. In: *Aldehyde Adducts in Alcoholism*. (Ed. Collins MA), pp. 3–17. Alan R Liss Inc., New York, 1985.
8. Wickramasinghe SN and Hasan R, *In vitro* effects of vitamin C, thioctic acid and dihydrolipoic acid on the cytotoxicity of post-ethanol serum. *Biochem Pharmacol* **43**: 407–411, 1992.
9. Mosmann T, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**: 55–63, 1983.
10. Tuma DJ, Newman MR, Donohue TM Jr and Sorrell MF, Covalent binding of acetaldehyde to proteins: participation of lysine residues. *Alcoholism: Clin Exp Res* **11**: 579–584, 1987.
11. Yunice AA and Lindeman RD, Effect of ascorbic acid and zinc sulfate on ethanol toxicity and metabolism. *Proc Soc Exp Biol Med* **154**: 146–150, 1977.
12. Busnel RG and Lehman AG, Antagonist effect of sodium ascorbate on ethanol-induced changes in swimming of mice. *Behav Brain Res* **1**: 351–356, 1980.
13. Yunice AA, Hsu JM, Fahmy A and Henry S, Ethanol–ascorbate interrelationship in acute and chronic alcoholism in the guinea pig. *Proc Soc Exp Biol Med* **177**: 262–271, 1984.
14. Susick RL Jr, Abrams GD, Zurawski CA and Zannoni VG, Ascorbic acid and chronic alcohol consumption in the guinea pig. *Toxicol Appl Pharmacol* **84**: 329–335, 1986.
15. Krasner N, Moore MR, Dow J and Goldberg A, Ascorbic acid and ethanol metabolism. *Lancet* **2**: 693–695, 1974.
16. Susick RL Jr and Zannoni VG, Effect of ascorbic acid on the consequences of acute alcohol consumption in humans. *Clin Pharmacol Ther* **41**: 502–509, 1987.
17. Majumdar SK, Patel S, Shaw GK, O'Gorman P and Thomson AD, Vitamin C utilization status in chronic alcoholic patients after short-term intravenous therapy. *Int J Vit Nutr Res* **51**: 274–278, 1981.
18. Wickramasinghe SN, Role of macrophages in the pathogenesis of alcohol-induced tissue damage. *Br Med J* **294**: 1137–1139, 1987.