Human & Experimental Toxicology (2002) 21, 675–680
www.hetjournal.com

Effects of ascorbic acid and α-tocopherol on arsenic-induced oxidative stress

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Arsenic is an ubiquitous element in the environment causing oxidative burst in the exposed individuals leading to tissue damage. Antioxidants have long been known to reduce the free radical-mediated oxidative stress. Therefore, the present study was designed to determine whether supplementation of α-tocopherol (400 mg/kg body weight) and ascorbic acid (200 mg/kg body weight) to arsenic-intoxicated rats (100 ppm in drinking water) for 30 days affords protection against the oxidative stress caused by the metalloid. The arsenic-treated rats showed elevated levels of lipid peroxide, decreased levels of non-enzymatic antioxidants and activities of enzymatic antioxidants. Administration of α-tocopherol and ascorbic acid to arsenic-exposed rats showed a decrease in the level of lipid peroxidation (LPO) and enhanced levels of total sulfhydryls, reduced glutathione, ascorbic acid and α-tocopherol and so do the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase to near normal. These findings suggest that α-tocopherol and ascorbic acid prevent LPO and protect the antioxidant system in arsenic-intoxicated rats. *Human & Experimental Toxicology* (2002) **21**, 675–680.

Key words: antioxidants; arsenic; ascorbic acid; oxidative stress; tocopherol

Introduction

Arsenic is the first metalloid to be identified as a human carcinogen that causes cancer and many other toxic effects. The wide occurrence of arsenic in the environment makes human exposure to arsenic almost universal. The fraction of environmental arsenic that is biologically available from environmental sources, including drinking water and food, varies considerably. Populations exposed to drinking water contamination of arsenic include Taiwan, China, Eastern Europe, United States and India.[1] Chronic exposure to arsenic compounds is associated with several human diseases including blackfoot disease,[2] diabetes mellitus,[3] hypertension[4] and cancers of bladder, lung, skin and liver.[5]

Arsenic exerts its toxicity through reaction with various sulfhydryls that exist in the cell.[6] On the other hand, recent studies suggest that arsenic compounds during their metabolism in cells generate reactive oxygen species like superoxide, hydroxyl radical and hydrogen peroxide through which also they exert their toxicity.[7] Arsenic exposure has shown to depress the functions of antioxidant defence system leading to oxidative damage to cellular macromolecules including DNA, proteins and lipids.[8] Antioxidants protect the cellular machinery from peroxidative damage inflicted by reactive oxygen species.[9]

Ascorbic acid is the most widely cited form of water-soluble antioxidant and prevents oxidative damage to the cell membrane induced by radicals in the aqueous environment.[10] Hydrophilic ascorbic acid is not able to scavenge radicals within the interior of the membrane. α-Tocopherol is a primary scavenger of free radicals within the membrane.[11] Both these antioxidants act synergistically against reactive oxygen species. The aim of this study was to evaluate the effects of ascorbic acid and tocopherol on arsenic-induced changes in antioxidant system.

Materials and methods

Sodium arsenite, ascorbic acid and α-tocopherol were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were of reagent grade. Male albino rats of Wistar strain weighing approximately

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Received 11 February 2002; revised 20 September 2002; accepted 16 October 2002

10.1191/0960327102ht307oa

120–130 g were used. The animals were divided into five groups namely:

Group I — Rats served as control;
Group II — Rats were received arsenic as sodium arsenite in drinking water at a concentration of 100 ppm;
Group III — Rats were given arsenic along with ascorbic acid (200 mg/kg body weight dissolved in distilled water and given by oral gavage once a day);
Group IV — Rats were given arsenic along with α-tocopherol (400 mg/kg body weight dissolved in mineral oil and given by oral gavage once a day);
Group V — Rats were given arsenic along with ascorbic acid (200 mg/kg body weight) and α-tocopherol (400 mg/kg body weight).

The animals were maintained on commercial rat feed. Each group consisted of six animals and had access to food and water *ad libitum*. On completion of 30 days, the animals were killed by cervical decapitation. Blood was collected in heparinized tubes. Plasma was separated and hemolysate was prepared. Liver and kidney were excised immediately and immersed in physiological saline. Ten percent homogenate was prepared in fresh tissues with 0.01 M Tris–HCl buffer (pH 7.4).

Preparation of hemolysate was achieved by the method of Quist.[12] Blood collected with EDTA was centrifuged at 2000×*g* for 20 minutes at 4°C. The packed cells were washed with saline to remove the buffy coat. An aliquot of packed cells was then washed three times with isotonic buffer (0.31 M Tris–HCl, pH 7.4). One milliliter of washed cells was lysed using 9.0 mL of hypotonic buffer (0.015 M Tris–HCl buffer, pH 7.2). The lysed cells were centrifuged for 30 minutes at 15 000×*g*. The supernatant (haemolysate) was used for the assay of enzymes. The pellet was repeatedly washed with hypotonic buffer until a clear supernatant was obtained. The resulting erythrocyte membrane pellet was suspended in 0.01 M Tris–HCl buffer, pH 7.4, and used for the assay of lipid peroxidation (LPO).

The protein content was determined according to Lowry *et al.*,[13] using bovine serum albumin as standard. LPO was assayed by the method of Ohkawa *et al.*,[14] in which the malondialdehyde released served as the index of LPO. The total sulfhydryls estimation (TSH) was done according to the method of Sedlack and Lindsay.[15] Reduced glutathione (GSH) was assayed by the method of Moron *et al.*,[16] based on the reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) which is readily reduced by sulfhydryls forming a yellow substance. The optical density of this yellow substance is measured at 412 nm. The method of Omaye *et al.*[17] was followed for the estimation of ascorbic acid. Vitamin E was estimated by the method of Desai.[18] This method reduces ferric ions to ferrous ions in the presence of tocopherol and forms a pink complex with bathophenanthroline.

The activity of superoxide dismutase (SOD) was assayed based on Marklund and Marklund.[19] One unit of SOD activity is defined as the enzyme that inhibits the rate of autooxidation of pyrogallol by 50%. Catalase (CAT) activity was assayed by the method Claiborne.[20] The activity of glutathione peroxidase (GPX) was determined using hydrogen peroxide as substrate in the presence of reduced glutathione.[21] Glutathione reductase activity (GR) was assayed by measuring the disappearance of NADPH at 340 nm as described by Carlberg and Mannervik.[22] The activity of glucose-6-phosphate dehydrogenase activity was assayed spectrophotometrically by the method of Ellis and Kirkman.[23]

Table 1 Level of lipid peroxidation and the status of antioxidants in blood of control and experimental rats

Parameters	*Group I (control)*	*Group II (arsenic)*	*Group III (arsenic + ascorbic acid)*	*Group IV (arsenic + α-tocopherol)*	*Group V (arsenic + ascorbic acid + α-tocopherol)*
LPO	2.82 ± 0.27	4.07 ± 0.55[a]	3.22 ± 0.31[a,b]	3.0 ± 0.33[b]	2.85 ± 0.27[b,c]
TSH	1.97 ± 0.20	0.91 ± 0.18[a]	1.71 ± 0.19[a,b]	1.83 ± 0.16[b]	1.92 ± 0.19[b]
GSH	2.31 ± 0.21	1.52 ± 0.2[a]	2.13 ± 0.20[b]	2.24 ± 0.19[b]	2.28 ± 0.22[b]
Ascorbic acid	1.41 ± 0.15	0.82 ± 0.13[a]	1.34 ± 0.14[b]	1.29 ± 0.12[b]	1.38 ± 0.13[b]
α-Tocopherol	1.16 ± 0.12	0.71 ± 0.1[a]	1.04 ± 0.11[b]	1.12 ± 0.09[b]	1.13 ± 0.12[b]
SOD	3.82 ± 0.37	2.17 ± 0.31[a]	3.27 ± 0.34[a,b]	3.69 ± 0.37[b]	3.77 ± 0.4[a,b]
CAT	79.4 ± 8.12	40.35 ± 6.11[a]	62.87 ± 6.08[a,b]	67.93 ± 7.52[a,b]	76.45 ± 7.16[b,c]
GPX	6.52 ± 0.67	3.84 ± 0.45[a]	5.53 ± 0.61[b]	6.15 ± 0.64[b]	6.43 ± 0.64[b,c]
GR	1.25 ± 0.12	0.89 ± 0.13[a]	1.15 ± 0.10[b]	1.18 ± 0.09[b]	1.23 ± 0.11[b]
G6PD	1.51 ± 0.16	0.98 ± 0.14[a]	1.37 ± 0.15[b]	1.41 ± 0.12[b]	1.48 ± 0.14[b]

Each value is expressed as mean ± SD for six rats in each group. LPO nmol MDA released/mg protein; TSH μg/mg protein; GSH μg/mg protein; ascorbic acid μg/mg protein; α-tocopherol μg/mg protein; SOD units/min per mg protein; CAT μmol H_2O_2 consumed/min per mg protein; GPX μmol GSH oxidized/min per mg protein; GR nmol NADPH oxidized/min per mg protein and G6PD units/min per mg protein. Superscript letters represent $P < 0.05$. [a]As compared with group I. [b]As compared with group II. [c]As compared with group III. [d]As compared with group IV.

Table 2 Level of lipid peroxidation and the status of antioxidants in liver of control and experimental rats

Parameters	*Group I (control)*	*Group II (arsenic)*	*Group III (arsenic + ascorbic acid)*	*Group IV (arsenic + α-tocopherol)*	*Group V (arsenic + ascorbic acid + α-tocopherol)*
LPO	2.58 ± 0.30	3.78 ± 0.42^{a}	$2.84 \pm 0.33^{a,b}$	$2.76 \pm 0.31^{a,b}$	2.61 ± 0.28^{b}
TSH	15.16 ± 1.89	7.58 ± 1.26^{a}	$11.51 \pm 1.62^{a,b}$	$12.08 \pm 1.73^{a,b}$	$14.73 \pm 1.76^{b,c,d}$
GSH	4.05 ± 0.42	2.10 ± 0.33^{a}	$3.12 \pm 0.38^{a,b}$	$3.48 \pm 0.37^{a,b}$	$3.94 \pm 0.41^{b,c}$
Ascorbic acid	2.85 ± 0.26	1.44 ± 0.21^{a}	2.79 ± 0.25^{b}	2.65 ± 0.27^{b}	2.83 ± 0.26^{b}
α-Tocopherol	1.77 ± 0.19	0.82 ± 0.11^{a}	1.68 ± 0.18^{b}	1.73 ± 0.19^{b}	1.76 ± 0.16^{b}
SOD	8.38 ± 0.91	4.94 ± 0.76^{a}	$6.29 \pm 0.64^{a,b}$	$6.81 \pm 0.68^{a,b}$	$8.22 \pm 0.93^{b,c,d}$
CAT	62.5 ± 6.33	41.56 ± 5.21^{a}	$50.24 \pm 5.87^{a,b}$	$54.56 \pm 5.93^{a,b}$	$61.52 \pm 6.86^{b,c}$
GPX	5.41 ± 0.43	3.26 ± 0.33^{a}	$4.93 \pm 0.38^{a,b}$	5.18 ± 0.41^{b}	5.3 ± 0.39^{b}
GR	0.31 ± 0.02	0.16 ± 0.05^{a}	$0.26 \pm 0.03^{a,b}$	$0.28 \pm 0.02^{a,b}$	$0.29 \pm 0.03^{b,c}$
G6PD	2.03 ± 0.23	1.28 ± 0.19^{a}	$1.83 \pm 0.18^{a,b}$	$1.89 \pm 0.19^{a,b}$	$1.96 \pm 0.17^{b,c}$

Each value is expressed as mean ± SD for six rats in each group. LPO nmol MDA released/mg protein; TSH μg/mg protein; GSH μg/mg protein; ascorbic acid μg/mg protein; α-tocopherol μg/mg protein; SOD units/min per mg protein; CAT μmol H_2O_2 consumed/min per mg protein; GPX μmol GSH oxidized/min per mg protein; GR nmol NADPH oxidized/min per mg protein and G6PD units/min per mg protein. Superscript letters represent $P < 0.05$. [a]As compared with group I. [b]As compared with group II. [c]As compared with group III. [d]As compared with group IV.

Statistical analysis

Values are means ± SD for six rats in each group, and significance of the differences between mean values was determined by one-way analysis of variance (ANOVA) followed by the Duncan test for multiple comparison. Values of $P < 0.05$ were considered to be significant.

Results

Table 1 depicts the levels of LPO in erythrocyte membrane, levels of ascorbic acid and GSH in whole blood, levels of α-tocopherol and TSH in plasma and the activities of antioxidant enzymes in hemolysate of control, arsenic-exposed and ascorbic acid- and α-tocopherol-treated rats. In arsenic-intoxicated rats, the level of lipid peroxide was found to be considerably high (44%), whereas the levels/activities of antioxidants were remarkably low compared to controls. Arsenic exposure decreased the level of TSH by 58%, GSH by 34%, ascorbic acid by 41% and α-tocopherol by 38% and decreased the activity of SOD by 43%, CAT by 49%, GPX by 41%, GR by 29% and G6PD by 35%. Arsenic-administrated rats simultaneously treated with ascorbic acid alone, α-tocopherol alone and combination of ascorbic acid and α-tocopherol for 30 days, demonstrated a significant decrease in the level of LPO ($P < 0.05$) and an increase in the levels of non-enzymatic antioxidants and the activities of enzymatic antioxidants ($P < 0.05$) when compared to arsenic-exposed rats.

Arsenic intoxication imposes deleterious effects on the redox status of liver as evidenced by a drastic increase in the level of LPO and a decrease in the levels/activities of antioxidants. The status of antioxidants was found to be significantly lowered in arsenic-treated rats, the decrease being 50% for TSH, 48% for GSH, 49% for ascorbic acid, 53% for α-tocopherol, 41% for SOD, 33% for CAT, 40% for

Table 3 Level of lipid peroxidation and the status of antioxidants in kidney of control and experimental rats

Parameters	*Group I (control)*	*Group II (arsenic)*	*Group III (arsenic + ascorbic acid)*	*Group IV (arsenic + α-tocopherol)*	*Group V (arsenic + ascorbic acid + α-tocopherol)*
LPO	2.13 ± 0.19	3.14 ± 0.34^{a}	$2.44 \pm 0.21^{a,b}$	2.32 ± 0.28^{b}	2.16 ± 0.24^{b}
TSH	9.96 ± 0.78	5.47 ± 0.65^{a}	$7.69 \pm 0.96^{a,b}$	$8.82 \pm 0.85^{a,b}$	$9.78 \pm 1.12^{b,c}$
GSH	2.35 ± 0.21	1.56 ± 0.19^{a}	$2.06 \pm 0.23^{a,b}$	2.15 ± 0.20^{b}	2.26 ± 0.24^{b}
Ascorbic acid	1.72 ± 0.16	0.91 ± 0.1^{a}	1.67 ± 0.18^{b}	1.60 ± 0.15^{b}	1.70 ± 0.18^{b}
α-Tocopherol	1.19 ± 0.15	0.67 ± 0.08^{a}	$1.05 \pm .11^{b}$	1.12 ± 0.03^{b}	1.17 ± 0.14^{b}
SOD	4.94 ± 0.47	3.25 ± 0.35^{a}	$4.07 \pm 0.46^{a,b}$	4.28 ± 0.49^{b}	$4.81 \pm 0.53^{b,c}$
CAT	42.3 ± 4.85	24.6 ± 3.11^{a}	34.87 ± 3.74^{b}	38.65 ± 4.01^{b}	$41.4 \pm 4.22^{b,c}$
GPX	4.19 ± 0.43	2.06 ± 0.35^{a}	$3.34 \pm 0.39^{a,b}$	$3.51 \pm 0.42^{a,b}$	$4.08 \pm 0.43^{b,c,d}$
GR	0.28 ± 0.02	0.16 ± 0.02^{a}	$0.22 \pm 0.02^{a,b}$	$0.24 \pm 0.03^{a,b}$	$0.26 \pm 0.03^{b,c,d}$
G6PD	1.66 ± 0.15	1.07 ± 0.14^{a}	$1.50 \pm 0.12^{a,b}$	$1.58 \pm 0.13^{a,b}$	$1.62 \pm 0.12^{b,c}$

Each value is expressed as mean ± SD for six rats in each group. LPO nmol MDA released/mg protein; TSH μg/mg protein; GSH μg/mg protein; ascorbic acid μg/mg protein; α-tocopherol μg/mg protein; SOD units/min per mg protein; CAT μmol H_2O_2 consumed/min per mg protein; GPX μmol GSH oxidized/min per mg protein; GR nmol NADPH oxidized/min per mg protein and G6PD units/min per mg protein. Superscript letters represent $P < 0.05$. [a]As compared with group I. [b]As compared with group II. [c]As compared with group III. [d]As compared with group IV.

GPX, 48% for GR and 37% for G6PD. Supplementation of ascorbic alone and α-tocopherol alone to arsenic-intoxicated rats showed a significant decrease in the level of LPO and a concomitant increase in the levels of non-enzymatic antioxidants and the activities of enzymatic antioxidants. Simultaneous administration of ascorbic acid and α-tocopherol to arsenic-treated rats restored the observed changes to near normal (Table 2).

Table 3 shows the LPO and antioxidant status in the kidney of arsenic-exposed and vitamins-supplemented rats. The level of LPO was found to be significantly increased by 60% in arsenic-treated rats compared to control rats. The levels/activities of non-enzymatic antioxidants were found to be significantly decreased. The decrease being 50% for TSH, 48% for GSH, 49% for ascorbic acid and 48% for α-tocopherol and the activity of SOD by 41%, CAT by 36%, GPX by 40%, GR by 58% and G6PD by 37%. Coadministration of arsenic along with ascorbic acid and α-tocopherol reverted these changes as comparable to control rats.

Discussion

LPO is regarded as one of the basic mechanisms of tissue damage caused by free radicals.[24] LPO is initiated by the abstraction of a hydrogen atom from the side chain of polyunsaturated fatty acids in the membrane lipids. It is known to occur in biological membranes, with potential injurious consequences. Arsenic is shown to stimulate the release of free iron through the activation of haeme oxygenase, the rate-limiting enzyme in haeme degradation.[25] The free iron may thereby be involved in Fenton-type reactions leading to enhanced LPO. In the present study, cotreatment with ascorbic acid and α-tocopherol reduced the level of lipid peroxides in arsenic-exposed rats. This may be due to their inherent antioxidant property. Ascorbic acid and α-tocopherol are able to quench the LPO chain and protect the membrane from the attack of free radicals.

During arsenic exposure, remarkable depletion of TSH, GSH, ascorbic acid and α-tocopherol has been observed. Thiols are thought to play a pivotal role in protecting cells against reactive oxygen species. Moreover, thiols act as a target site for arsenic-induced cellular damage. Lowered levels of TSH represent increased production of free radicals and binding of arsenic with thiols. GSH, a non-protein thiol, is involved in many cellular processes including the detoxification of endogenous and exogenous compounds.[26] GSH plays an important role in detoxification of arsenic through different mechanisms; facilitating arsenic uptake by the cells,[27] modulation of arsenic methylation reaction[28] and stimulation of excretion of methylated arsenic compounds.[29] Furthermore, the intracellular level of GSH is inversely correlated with the cytotoxicity of arsenic.[30]

The antioxidants GSH, ascorbic acid and α-tocopherol are interrelated by recycling processes. α-Tocopherol present in the cell membrane counteracts with free radicals and ultimately gets transformed into tocopheroxyl radicals.[31] Recycling of tocopheroxyl radicals to α-tocopherol is achieved by reaction with ascorbic acid. The dehydroascorbic acid formed in this reaction is reduced to ascorbic acid by non-enzymatic reaction with GSH.[32] The increase in the levels of TSH and GSH after administration with ascorbic acid and α-tocopherol may be due to the conversion of oxidized glutathione to reduced glutathione and thereby maintaining cell viability.

Subcellular membranes and associated thiol-bearing enzymes represent sensitive sites for arsenic, causing perturbation of cellular function.[33] Reactive oxygen species can themselves reduce the activities of enzymes.[34]

SOD and CAT are the most important defence enzymes against toxic effects of oxygen metabolism. SOD accelerates the dismutation of superoxide to H_2O_2 which can be said as primary defence, as it prevents further generation of free radicals. CAT catalyzes the removal of H_2O_2 formed during the reaction catalyzed by SOD. A decrease in the activity of SOD can be owed to an enhanced superoxide production during arsenic metabolism.[35] The superoxide radical also inhibits the activity of catalase.[36] The paucity of NADPH production during arsenic exposure decreases the catalase activity.[37] An increase in the activity of SOD was observed in arsenic-exposed rats after treatment with ascorbic acid and α-tocopherol. This may be due to the direct reaction of ascorbic acid and α-tocopherol with superoxide, hydroxyl radicals, peroxyl and alkoxyl radicals.

GPX reduces lipid hydroperoxides into lipid alcohols; this enzyme is coupled with G6PD and GR, which supply NADPH and GSH, respectively. Sodium arsenite induces the oxidation of NADPH.[38] This causes an increase in the NADP/NADPH ratio and thereby decreases the activity of GR which is also effectively inhibited by arsenic.[39] This in turn reduces the conversion of oxidized glutathione into reduced glutathione. GSH serves as a substrate for GPX. The decrease in the activity of GPX is due to the decrease in the level of GSH and increase in the level of LPO during arsenic exposure.[40] The observed decrease in the activity of G6PD in arsenic-treated rats may be ascribed to an increase in the production of oxidized glutathione. The increase in the activities of GPX, G6PD and GR after supple-

mentation with antioxidant vitamins may be due to the increase in the level of GSH which in turn increases the activities of related enzymes.

These results suggest that ascorbic acid and α-tocopherol can prevent the formation of LPO by overall enhancement of enzymatic and non-enzymatic antioxidant defence mechanisms in arsenic-administered rats. Ascorbic acid and tocopherol, possibly through recycling mechanism, contribute to decreased GSH utilization. In conclusion, the combination of these antioxidants may be therapeutically beneficial in the treatment against arsenic-induced toxicity to a considerable extent.

Acknowledgement

This work was supported by grants from Ministry of Environment and Forests, Government of India, New Delhi.

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