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Vitamin C Prevents Oxidative Damage

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Ascorbate – deficiency leads to extensive oxidative damage of proteins and protein loss in the guinea pig tissue microsomes as evidenced by sodium dodecyl sulfate polyacrylamide gel electrophoresis, accumulation of carbonyl, bityrosine as well as by tryptophan loss. Oxidative damage is reversed by ascorbate therapy. Oxidative damage in ascorbate deficiency also leads to lipid peroxidation in guinea pig tissue microsomes as evidenced by accumulation of conjugated dienes, malondialdehyde and fluorescent pigment. Lipid peroxides disappear after ascorbate therapy but not by vitamin E. The observations substantiate the previous *in vitro* findings that ascorbate specifically prevents oxidative degradation of microsomal membranes. The results indicate that vitamin C may exert a powerful protection against degenerative diseases associated with oxidative damage and play a critical role in wellness and health maintenance.

Keywords: vitamin C, microsomal membranes, lipid peroxidation, oxidative degradation of proteins, ascorbate deficiency

INTRODUCTION

Free radical damage constitutes a constant and significant threat for aerobic organisms. There is a growing literature[1-5] providing evidence that oxidative damage such as lipid peroxidation, protein oxidation and DNA damage play a role in most health problems including cardiovascular diseases, cancer and aging and that antioxidants have a critical role in wellness, health maintenance and the prevention of degenerative diseases. Considerable *in vitro* studies, both enzymatic and non-enzymatic, have been carried out to understand the mechanism of reactive oxygen species (ROS) – mediated oxidative damage and its prevention.[1-5] However, most of these studies require the presence of free iron in the incubation medium, a condition which has less relevance in the *in vivo* situation, because in the normal physiological condition iron does not exist in the free state but remains bound with proteins. In fact, the mechanism of oxidative damage *in vivo* and the preventive role of specific antioxidants for maintenance of good health are yet to be known. Using a model *in vitro* system, which is more relevant to the *in vivo* situation, we have demonstrated[6,7] that NADPH initiates lipid peroxidation and oxidative degradation of microsomal proteins in the

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absence of free iron. Lipid peroxidation and protein degradation are mediated by cytochrome P450 and specifically prevented by ascorbic acid.[6,7] Other scavengers of ROS including superoxide dismutase, catalase, glutathione and vitamin E are ineffective.[6,7] Ascorbate also prevents free iron-independent cytochrome P450 – mediated protein oxidation when NADPH is replaced by superoxide[6]. Probably, the oxidant is a perferryl moiety, namely, cytochrome P450 $Fe^{3+} \bullet O_2^{\bullet}$. The protein degradation occurs irrespective of tissues and it is a two-step process: (i) oxidation of proteins and (ii) rapid proteolytic degradation of the oxidized proteins.[6] Ascorbic acid prevents protein oxidation and thereby subsequent proteolytic degradation probably by interacting with cytochrome P450 $Fe^{3+} \bullet O_2^{\bullet}$. The *in vitro* observations on the protective effect of ascorbic acid against ROS-mediated oxidative damage have been substantiated by the *in vivo* results obtained with guinea pigs. The results may be relevant to human nutrition, because guinea pigs, like humans, are also incapable of synthesizing ascorbic acid.

MATERIALS AND METHODS

Ascorbic acid, malondialdehyde bisdiethylacetal, Coomassie Brilliant Blue were purchased from Sigma Chemical Company, USA. Malondialdehyde was freshly prepared from the malondialdehyde bisdiethylacetal by hydrolysis with 1% H_2SO_4. ∝-Tocopherol was a gift from Ciba Geigy Limited, Switzerland. HPLC grade n-hexane, methanol and water were purchased from Spectrochem Private Limited, India. All other reagents were of analytical grade.

Preparation of microsomes, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), estimation of protein, measurements of carbonyl, bityrosine and tryptophan loss were carried out by the methods described before[6] using freshly isolated microsomes directly without incubation with NADPH and *in vitro* oxidation.

Production of ascorbic acid deficiency was done as before.[8] Ascorbate deficiency was diagnosed by loss of body weight, apparent immobilization of hind legs and periosteal hematomas observed on the 20th day of ascorbate-deficiency. Pair-fed controls received 15 mg ascorbic acid per guinea pig per day. Tissue ascorbic acid was estimated by HPLC.[8] Ascorbate therapy was made by oral administration of ascorbate at a dose of 50 mg per guinea pig per day for 2 days followed by 15 mg per guinea pig per day for the next 8 days. After ascorbate therapy, the animals were indistinguishable by general appearance from the pair-fed controls.

For the estimation of conjugated dienes, freshly isolated microsomes (1 mg protein) without any *in vitro* oxidation were extracted with a mixture of methanol:n-hexane (1:3) and the absorbance of the n-hexane layer was recorded at 234 nm in a Hitachi U-2000 spectrophotometer, taking extinction coefficient at 234 nm = 25 mM^{-1}.

Measurements of malondialdehyde was made by thiobarbituric acid test[8] using freshly isolated microsomes without any further treatment. Fluorescent pigment was measured in freshly isolated microsomes (0.5 mg protein per ml of 0.1 M sodium phosphate buffer, pH 7.4) by recording fluorescence at 355 nm excitation and 450–500 nm emission[8] in a Hitachi Fluorescence Spectrophotometer model F3010.

RESULTS

In ascorbate-deficient guinea pigs (albino, male 350–400 g) fed ascorbate – deficient diet for 20 days, the average (n = 4) tissue contents of ascorbic acid fell to about one-tenth of the normal (pair-fed) values in the liver (0.13 mM), kidney (0.05 mM), lung (0.12 mM), brain (0.08 mM) and one-twentieth in the adrenal gland (0.35 mM). Plasma content of ascorbic acid was practically nill. Figure 1 shows that ascorbate- deficient guinea pigs suffer extensive and generalized loss of microsomal proteins irrespective of tissues, as

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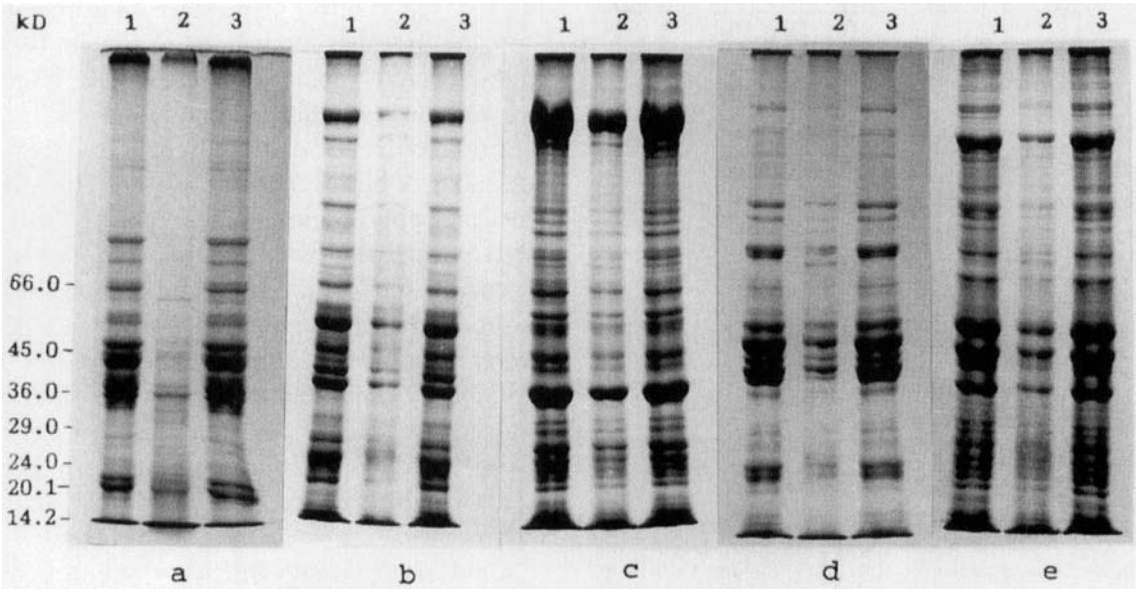

FIGURE 1 SDS-PAGE of guinea pig tissue microsomes. (a) liver; (b) kidney; (c) lung, (d) adrenal gland and (e) brain. Each lane contained microsomes equivalent to 15 mg of tissue. Proteins were stained with Coomassie Blue R-250. Lane 1, pair-fed control; Lane 2, after 20 days of ascorbate – deficiency; Lane 3, after ascorbate therapy for 10 days on and from 20th day of deficiency at a dose of 50 mg per guinea pig per day for 2 days followed by 15 mg per guinea pig per day for the next 8 days.

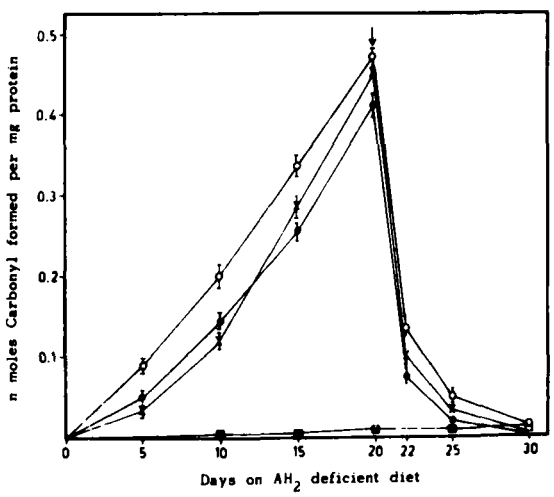

FIGURE 2 Production of carbonyl in ascorbate-deficient guinea pig tissue microsomes and effect of ascorbate therapy. O, liver; ●, kidney; X, lung; □, pair-fed controls that contained practically no carbonyl. Arrow (↓) indicates start of ascorbate therapy on the 20th day of deficiency. Carbonyl was estimated by the 2,4-dinitrophenyl hydrazine method.[7] Each point represents mean ± SD from four animals.

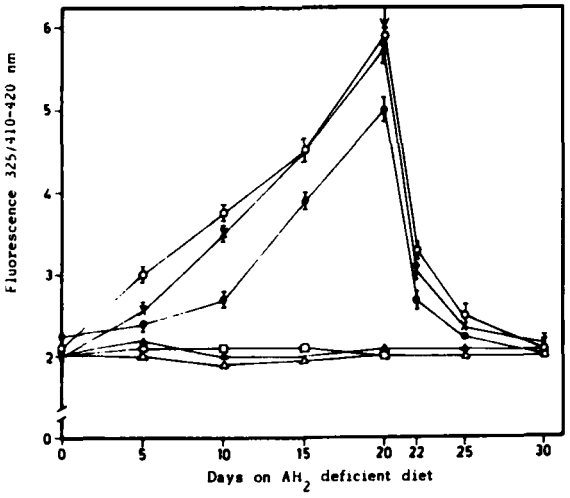

FIGURE 3 Formation of bityrosine in ascorbate-deficient guinea pig tissue microsomes and effect of ascorbate therapy. O, liver; ●, Kidney; X, lung. The bottom lines represent pair-fed controls: □, liver; ▲, kidney; △, lung. Arrow (↓) indicates start of ascorbate therapy. Fluorescent intensity was measured at 325 nm excitation and 410–420 nm emission.[7] Each point represents mean ± SD from four animals.

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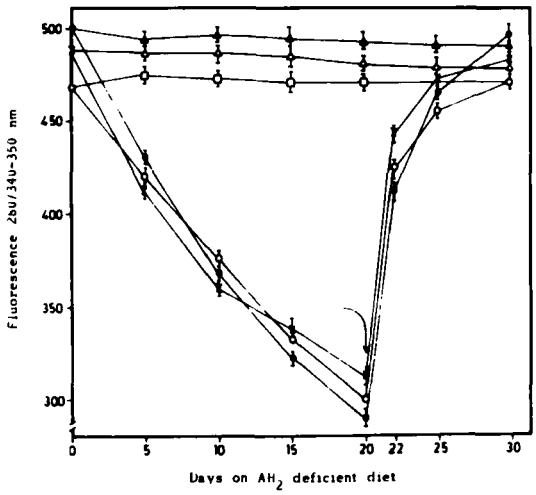

FIGURE 4 Tryptophan loss in ascorbate-deficient guinea pig tissue microsomes and effect of ascorbate therapy. O, liver; ●, kidney; X, lung. The top horizontal lines represent pair-fed controls: □, liver; ▲ kidney; △, lung. Arrow (↓) indicates start of ascorbate therapy. Fluorescent intensity was measured at 280 nm excitation and 340–350 nm emission.[7] Each point represents mean ± SD from four animals.

evidenced by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Densitometric scanning indicates that after 20 days of ascorbic acid deficiency, there is a loss of microsomal protein bands of about 65% in the liver (Figure 1a), 48% in the kidney (Figure 1b), 61% in the lung (Figure 1c), 72% in the adrenal gland (Figure 1d) and 60% in the brain (Figure 1e). In separate experiment using Lowry method,[6] it has been observed that there is about 68% loss of proteins in the liver microsomes of ascorbate-deficient guinea pigs compared to that of pair-fed controls. Comparative results obtained with pair-fed controls indicate that the protein loss is not due to inanition. *In vitro* data[6] indicate that the microsomal protein loss is due to oxidative degradation. That ascorbate-deficiency leads to oxidation of microsomal proteins is evidenced by the accumulation of carbonyl (Figure 2), bityrosine (Figure 3) and also by tryptophan loss (Figure 4). Oxidized proteins disappear after ascorbic acid therapy (Figures 2–4), which also reverts the SDS–PAGE to that characteristic of the pair-fed controls (Figure 1). Densitometric scanning indicates a reversion of protein bands to the extent of about 95% in the liver (Figure 1a), 93% in the kidney (Figure 1b), 91% in the lung (Figure 1c), 95% in the adrenal gland (Figure 1d) and 87% in the brain (Figure 1e). The reversion probably occurs by preventing further oxidative damage and allowing the animal to regenerate the lost proteins. The protein loss in ascorbate-deficient animals is not due to lack of protein synthesis.[9] Ascorbate could not be replaced by oral vitamin E at a dose of 15 mg ∝-tocopherol acetate per guinea pig per day for 10 days, which resulted in accumulation of 1.20 ± 0.15 n moles of vitamin E per mg protein of liver microsomes.[8]

Oxidative damage in ascorbate-deficient guinea pigs is also evidenced by lipid peroxidation. Ascorbic acid deficiency leads to accumulation of conjugated dienes, malondialdehyde (MDA) and fluorescent pigment in the

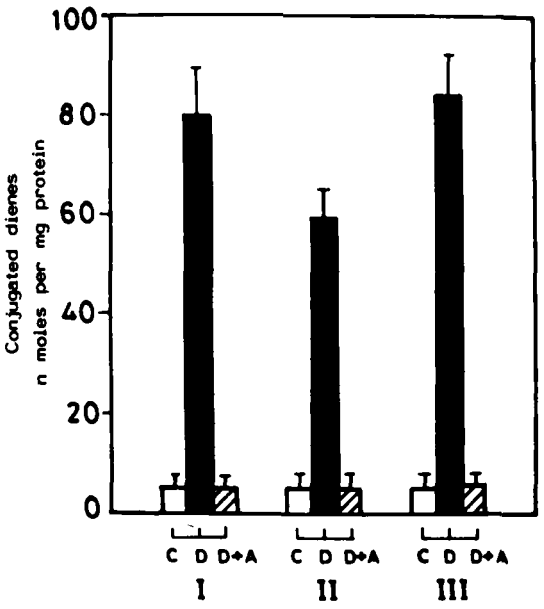

FIGURE 5 Increase in conjugated dienes in ascorbate-deficient guinea pig tissue microsomes and effect of ascorbate therapy. Liver, Figure 5I; Kidney, Figure 5II; Lung, Figure 5III. C, pair-fed controls; D, after 20 days of ascorbate-deficiency; D + A, ascorbate-deficient for 20 days followed by ascorbate therapy for 10 days as in Figure 1. Each point represents mean ± SD from four animals.

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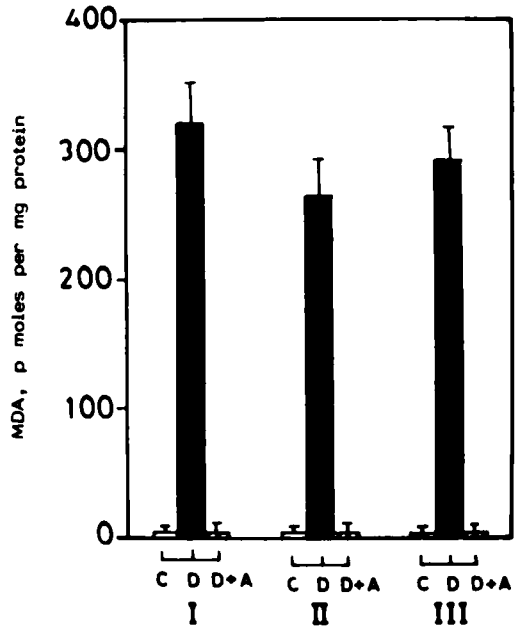

FIGURE 6 Accumulation of malondialdehyde (MDA) in ascorbate-deficient guinea pig tissue microsomes and effect of ascorbate therapy. Liver, Figure 6I; kidney, Figure 6II; lung Figure 6III. C, pair-fed controls, D, after 20 days of ascorbate-deficiency and D + A, ascorbate-deficient followed by ascorbate therapy as in Figure 5. Each point represents mean ± SD from four animals.

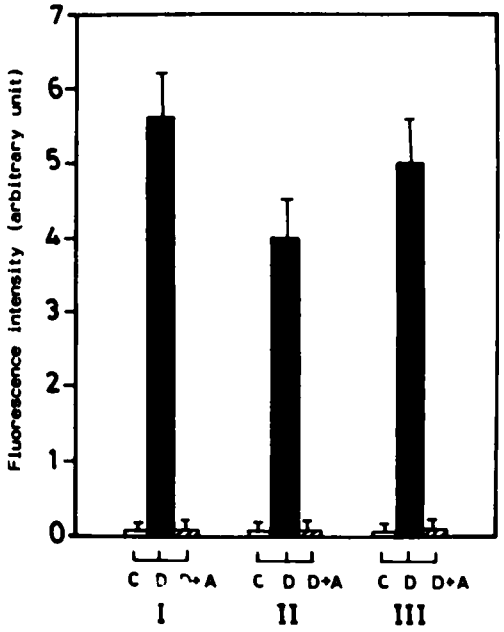

FIGURE 7 Accumulation of fluorescent pigment in ascorbate-deficient guinea pig tissue microsomes and effect of ascorbate therapy. Liver, Figure 7I; kidney, Figure 7II; lung, Figure 7III. C, pair-fed controls; D, after 20 days of ascorbate deficiency; D + A, ascorbate-deficient followed by ascorbate therapy as in Figure 5. Each point represents mean ± SD from four animals.

microsomal membranes of different tissues. After 20 days of ascorbate-deficiency, the increase in conjugated dienes in nmoles per mg protein over that of pair-fed controls (taking extinction coefficient at 234 nm = 25 mM^{-1}) was 74 ± 10 (n = 4) in the liver, 54 ± 6 in the kidney and 77 ± 8 in the lung (Figure 5). The levels of MDA in pmoles per mg microsomal protein were 320 ± 36 (n = 4) in the liver; 265 ± 30 in the kidney; and 290 ± 32 in the lung (Figure 6). It is known that MDA readily reacts with free amino groups of proteins and membrane phospholipids to yield fluorescent pigment.[8] Figure 7 shows that the accumulation of fluorescent pigment per mg microsomal protein was 5.6 ± 0.6 (arbitrary unit) (n = 4) in the liver; 4.0 ± 0.5 in the kidney and 5.0 ± 0.6 in the lung. Accumulations of MDA, conjugated dienes and fluorescent pigment were reversed after ascorbic acid therapy at a dose of 50 mg ascorbate per guinea pig per day for two days followed by 15 mg per guinea pig per day for the next 8 days (Figures 5–7).

DISCUSSION

The observations presented in this paper together with the previous *in vitro* results[6–8,10] definitely indicate that ascorbic acid prevents oxidative damage of the microsomal membranes. We have shown[10] before that ascorbic acid also prevents oxidative degradation of collagen by ROS produced by superoxide generated from activated macrophages. The content of superoxide dismutase in the extracellular fluid is negligible.[11] Only a small amount of glycosylated tetrameric superoxide dismutase is present in the extracellular fluid.[12] Therefore, the prevention of collagen breakdown by ascorbic acid imparts a specific effect of the vitamin for the protection of

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collagen in the extracellular matrix of mammalian tissues. All these results indicate a potential role of ascorbic acid for the protection of mammalian tissues against oxidative damage both at the intracellular and extracellular levels. We consider that scurvy symptoms in acute ascorbic acid deficiency is apparently a premortal syndrome of severe oxidative damage leading to disintegration of tissues at all levels. This offers an explanation to the biochemical mechanism of scurvy, which has still remained a riddle since the delineation of scurvy symptoms by James Lind[13] in 1753. Until now an explanation given for the cause of scurvy is that ascorbate deficiency leads to improper hydroxylation of collagen resulting in inhibited collagen synthesis. This explanation has been subjected to criticism, because there is little evidence for direct participation of ascorbic acid in collagen synthesis. The function of ascorbate for the stimulation of collagen hydroxylation is to keep the nonheme iron[14] of prolyl-4-hydroxylase in the active Fe(II) – state, which during catalytic action occasionally undergoes oxidation to the inactive Fe(III) – state.[14] Although ascorbic acid appears to be highly effective, it can be replaced by other reducing agents.[15] Barnes[16] came to the conclusion that hydroxylation of collagen during scurvy is only slightly impaired. Lehninger[17] raised the question whether hydroxylation of collagen proline is at all a major biological function of ascorbic acid. Recently Meister[18] considers that ascorbic acid performs a specific function for the prevention of scurvy, which has not yet been discovered. Since very small concentrations of ascorbate specifically prevent oxidative degradation of microsomal proteins and also collagen, we consider that a major function of ascorbic acid in the prevention of scurvy is the prevention of oxidative degradation of proteins both at the intracellular and extracellular levels.

Scurvy does not precipitate immediately after ascorbic acid deficiency. There is a wide gap between full health and scurvy and the interim period can be considered to be a state of marginal or subclinical ascorbic acid deficiency. Although frank scurvy is nowadays rare, subclinical ascorbic acid deficiency is common, particularly in the elderly.[19] We have shown that in subclinical ascorbic acid deficiency oxidative damage occurs as evidenced by lipid peroxidation and accumulation of aggregated proteins in the microsomal membranes despite the presence of adequate levels of antioxidants including ∝-tocopherol, reduced glutathione, protein thiols and scavenging enzymes, namely, superoxide dismutase (SOD), catalase and glutathione peroxidase.[8,20] We postulate that progressive oxidative damage in persistent subclinical vitamin C deficiency may result in the oxidation of low density lipoproteins leading to atherosclerosis, oxidative damage of heart tissues leading to myocardial injury, and also oxidative damage of DNA leading to mutation and cancer.

The protective role of vitamin C against oxidative damage has evolutionary significance. Fishes lack L-gulonolactone oxidase, the terminal enzyme in the pathway of ascorbic acid biosynthesis.[21,22] L-gulonolactone oxidase evolved in the amphibians when the vertebrates were exposed to an environment of about thirty times higher concentration of oxygen than their aquatic counterpart.[23] Apparently, vitamin C has provided the terrestrial vertebrates a strong defence against oxidative damage, which would have otherwise been toxic and fatal. The guinea pig, primates, humans and other animals, those dependent on dietary ascorbic acid,[21] become susceptible to oxidative damage in vitamin C deficiency.

Although vitamin C has left an inextricable impression in the minds of the public and many researchers with the controversial claims that megadoses of the vitamin can cure most illness including common cold and cancer, results obtained from our laboratory and others[24–29] suggest that vitamin C may exert a powerful protection against degenerative diseases associated with oxidative damage.

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