PROTECTIVE EFFECT OF ASCORBIC ACID AGAINST OXIDATIVE DAMAGE INDUCED BY HYDROGEN PEROXIDE IN CULTURED HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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ABSTRACT

Hydrogen peroxide is one of the reactive oxygen species for cellular injury. It is overproduced during oxidative stress and is known to damage proteins, nucleic acids and cell membranes. The present study was aimed to study the protective effect of ascorbic acid against the toxic doses of hydrogen peroxide using lipid peroxidation and cytokinesis blocked micronucleus assay. Hydrogen peroxide was studied at 50, 100 and 200 μ M and was found to increase a dose dependent increase in lipid peroxidation and micronuclei frequency. The treatment of 100 and 200 μ M of hydrogen peroxide separately along with 20, 40 and 80 μ M of ascorbic acid showed a dose dependent decrease in the lipid peroxidation and micronuclei frequency. The results suggest a protective effect of ascorbic acid against the hydrogen peroxide induced oxidative damage in cultured human peripheral blood lymphocytes.

KEY WORDS

Hydrogen peroxide, Ascorbic acid, Lipid peroxidation, Micronucleus, Reactive oxygen species

INTRODUCTION

Hydrogen peroxide (Reactive oxygen species) is overproduced during oxidative stress and is known to damage proteins, nucleic acids, cell membranes and have been implicated in cancer, aging, and also in several chronic neurodegenerative diseases (1). Hydrogen peroxide is known to cause oxidative DNA damage primarily through the hydroxyl radical that results from the Fenton reaction (2, 3, 4).

H₂O₂ has been reported to be associated with the induction of cancer in animals (5), mutagenic and carcinogenic (6). It is believed to be involved in the initiation and promotion of carcinogenesis (7). It has also been reported to cause DNA damage in the form of chromosomal aberrations (8), sister

chromatid exchanges (9), single strand breaks (10) and double strand breaks (11). In the present study we have estimated the lipid peroxidation in terms of quantifying malondialdehyde (MDA). Lipid peroxidation products of poly unsaturated fatty acids (PUFAs) are considered of importance for genotoxic effects (12, 13). Reactive oxygen species can induce lipid peroxidation and per oxidative fatty-acid fragments and radicals can *vice versa* lead to the formation of reactive oxygen species (13). Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are the two most prominent lipid peroxidative products (14). The method we have established for lipid peroxidative assay in present study give negligible reaction with 4-hydroxynonenal. In the presence of hydrochloric acid malondialdehyde reacts with two molecules of 1-methyl-2-phenylindole to yield a stable chromophore with intense maximal absorbance at 586 nm (15). The assay performed in hydrochloric acid based medium enables the specific measurement of MDA in the presence of 4-hydroxyalkenals, because in the presence of hydrochloric acid, 4-hydroxyalkenals undergo an irreversible cyclization reaction (15). The present study was aimed to study the protective effect of ascorbic acid against hydrogen peroxide induced oxidative damage in cultured human peripheral blood lymphocytes using lipid peroxidation and cytokinesis blocked micronucleus assay.

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MATERIALS AND METHODS

Chemicals: Hydrogen peroxide (CAS 7722-84-1); Ascorbic acid (CAS No: 50-81-7); (Central Drug House, India); 1-methyl-2-phenylindole (Sigma); acetonitrile (SRL, India); Methanol (Qualigens, India); HCl (Qualigens, India); TRIS (SRL, India); RPMI 1640, Phytohaemagglutinin-M, antibiotic-antimycotic mixture (In vitrogen), Dimethyl sulphoxide (DMSO), Giemsa stain (Merck), 5-Bromodeoxyuridine (SRL), Cytochalasin-B (Sigma).

Human lymphocyte culture: Duplicate peripheral blood cultures were treated according to Carballo et al (16). Briefly, about 0.5 ml of heparinized blood samples were obtained from a healthy female donor and were placed in a sterile glass culture tube containing 7 ml of RPMI-1640, supplemented with 1.5 of ml fetal calf serum, 0.1 ml of antibiotic-antimycotic mixture and 0.1 ml of phytohaemagglutinin. The tubes were placed in an incubator at 37°C for 24 h.

Lipid peroxidation assay: The present method of lipid peroxidation assay is based on the reaction of malonaldehyde with two molecules of 1-methyl-2-phenylindole at 45°C at 586 nm (15). The estimation of lipid peroxidation by Suryawanshi et al (17) was based on the same reaction. After 24h, the treatment of hydrogen peroxide (H_2O_2) at final concentration of 50, 100 and 200 μ M was given separately and kept for another 24 h at 37°C in an incubator. Simultaneously the activity of 100 and 200 μ M of H_2O_2 was examined in the presence of 20, 40 and 80 μ M of ascorbic acid separately and respectively to see the effect on the lipid peroxidation. Untreated and negative controls were also run simultaneously.

Preparation of buffers: Reagent 1 (R1): 10mM 1-methyl-2-phenylindole in acetonitrile was dissolved in 30 ml of acetonitrile and finally 10 ml of methanol was added to make 40 ml of volume. Reagent 2 (R2): 37% HCl.

Procedure for treated cells:

1. After incubation of 24 hr, the treated blood cultures were centrifuged at 3000 g for 20 min and the supernatant was collected.
2. To a fresh tube about 1300 μ l of R1 was taken in a microcentrifuge tube.
3. About, 1 ml of supernatant was diluted 10 times with Tris buffer (20 μ M; pH 7.4) and about 200 μ l of diluted supernatant was taken from each of the treated culture along with the 200 μ l of distilled water separately and vortexed.
4. To each tube 300 μ l of R2 was added and vortexed.

5. The tubes were incubated at 45°C for 40 min.
6. The tubes were cooled on ice and centrifuged at 15000g for 10 min at 4°C.
7. The reading was noted at 586 nm on a digital photo colorimeter (Metzer)

Cytokinesis – blocked micronucleus assay (CBMN): The presence of MN in a binucleated cell (BNC) was assayed by blocking the cells at the cytokinesis stage by the method of Fenech and Morley (18). Lymphocyte culture was set as described earlier in the text. After 24 h, the treatment of hydrogen peroxide (H_2O_2) at final concentration of 50, 100 and 200 μ M was given separately and kept for another 48 h at 37°C in an incubator. Simultaneously the activity of 100 and 200 μ M of H_2O_2 was examined in the presence of 20, 40 and 80 μ M of ascorbic acid separately and respectively to see the effect on the micronucleus frequency. Untreated and negative controls were also run simultaneously. Cyt-B (3 μ g/5 ml culture) was added to the culture at 44h after the initiation. After a total of 72h incubation the cells were centrifuged at 800g for 5 min. Supernatant was discarded and the cell pellets were treated with a hypotonic solution. Cells were fixed with freshly prepared methanol: acetic acid (3:1) for 10 min and then centrifuged at approximately 800g for 5 min. After air drying the slides were stained with 5% Giemsa stain in phosphate buffer (pH 6.8) for 10 min. BNCs surrounded by well preserved cytoplasm were scored for the presence of MN. At least 2000 cytokinesis-blocked (CB) binucleated human lymphocytes with preserved cytoplasm were scored. MN was identified with the criteria followed by Fenech et al (19).

Statistical analysis: Statistical analysis was performed by χ^2 test for micronucleus test. Student's 't' test was performed for lipid peroxidation assay using statistica soft Inc. Regression analysis was also performed to see the dose effect of ascorbic acid on H_2O_2 induced lipid peroxidation and micronucleus using statistica soft Inc.

RESULTS

The Table 1, shows the mean absorbance value after the treatment of 50, 100 and 200 μ M of H_2O_2 on cultured human peripheral blood lymphocytes. The values were significant as compared to untreated ($P < 0.01$). The treatment of 50, 100 and 200 μ M of H_2O_2 were associated with the mean absorbance value of 0.12, 0.16, and 0.18 respectively (Table1; Fig1). The untreated and negative controls were associated with the mean absorbance value of 0.02 and 0.03 respectively. The regression analysis was also performed to study the dose treatment effect of H_2O_2 on the mean absorbance value on

Table 1: Mean absorbance value at 586 nm after hydrogen peroxide treatment for the estimation of lipid peroxidation

Treatments	Mean absorbance value (586 nm)
H ₂ O ₂ (μM)	
50	0.12 ^a
100	0.16 ^a
200	0.18 ^a
H ₂ O ₂ (μM)+ AA (μM)	
100+ 20	0.10 ^b
100+ 40	0.08 ^b
100+ 80	0.07 ^b
200+20	0.12 ^b
200+40	0.11 ^b
200+80	0.09 ^b
AA (μM)	
20	0.03
40	0.03
80	0.04
Untreated	0.02
Negative control	0.03

^aP<0.01 with respect to untreated; ^bP<0.05 with respect to H₂O₂ treatment

cultured human peripheral blood lymphocytes. An increase in the slope of linear regression line ($P < 0.11$) was observed as the dose of the H₂O₂ treatment was increased (Fig 2). When 100mM of H₂O₂ treatment was given along with 20, 40 and

Fig 2: Effect of hydrogen peroxide on the absorbance value at 586 nm

80mM of ascorbic acid separately, a significant dose dependent decrease in the absorbance value at 586 nm was observed i.e. 0.10, 0.08 and 0.07 respectively (Table 1; Fig.1). Similar results were obtained when 200mM of H₂O₂ treatment was given along with 20, 40 and 80mM of ascorbic acid separately i.e. 0.12, 0.11 and 0.09 respectively (Table 1; Fig.1). Regression analysis was also performed to see the effect of different doses of ascorbic acid on the absorbance at 586 nm along with 100 and 200μM of H₂O₂. A decrease in the slope of linear regression line was observed when 100μM ($P < 0.05$)

Fig 1: Effect of ascorbic acid on the absorbance value after the treatment with hydrogen peroxide

HP1 = H₂O₂ 50 μM; HP2 = H₂O₂ 100 μM; HP3 = H₂O₂ 200 μM; AA1 = 20 μM; AA2 = 40 μM; AA3 = 80 μM; U = Untreated; NC = Negative Control (200 μL distilled water)

Fig 3: Effect of ascorbic acid on the absorbance value at 586 nm after the treatment along with 100 μ M of hydrogen peroxide

and 200 μ M ($P < 0.04$) was treated with 20, 40 and 80 μ M of ascorbic acid respectively (Fig 3 and 4).

Micronuclei induction: The treatments of 50, 100 and 200 μ M of H_2O_2 were associated with 52, 56 and 63 micronuclei / 10^3

Fig 4: Effect of ascorbic acid on the absorbance value at 586 nm after the treatment along with 200 μ M of hydrogen peroxide

cells respectively, that were significant from the untreated (9.5 MN/ 10^3 cells) (Table 2; Fig 5). Regression analysis was also performed to see the dose effect of H_2O_2 treatment on the frequency of MN. An increase in the slope of linear regression line was observed ($P < 0.004$) (Fig 6). When 100 μ M of H_2O_2

Table 2: Evaluation of the effects of ascorbic acid on hydrogen peroxide induced micronucleus on human peripheral blood lymphocytes

Treatment	BN Cells scored	Distribution of BN cells according to number of MN				MN / 10 ³ cells
		0	1	2	3	
H ₂ O ₂ (μM)						
50	2000	1931	72	13	2	52 ^a
100	2000	1903	82	12	3	56 ^a
200	2000	1891	92	14	3	63 ^a
H ₂ O ₂ (μM) + Ascorbic acid (μM)						
100 + 20	2000	1923	71	6	0	41.5 ^b
100 + 40	2000	1932	65	3	0	35.5 ^b
100 + 80	2000	1939	60	1	0	31 ^b
200 + 20	2000	1907	85	7	1	51 ^b
200+ 40	2000	1927	70	3	0	38 ^b
200 + 80	2000	1938	61	1	0	31.5 ^b
Ascorbic acid (μM)						
20	2000	1975	20	2	0	12
40	2000	1972	22	2	0	13
80	2000	1970	24	2	0	14
Untreated	2000	1982	17	1	0	9.5
Negative control (DMSO, 5 μl/ml)	2000	1981	18	1	0	10

^a $P < 0.001$ (significant as compared to untreated); ^b $P < 0.01$ (significant as compared to H_2O_2 treatment).

treatment was given along with 20, 40 and 80 μM of ascorbic acid separately, a significant dose dependent decrease in MN/ 10^3 cells was observed i.e. 41.5, 35.5 and 31 MN/ 10^3 cells respectively (Table 2; Fig 5). Similar results were obtained when 200 μM of H_2O_2 treatment was given along with 20, 40 and 80 μM of ascorbic acid separately i.e. 51, 38 and 31.5 MN/ 10^3 cells respectively (Table 2; Fig 5). The untreated and negative controls were associated with 9.5 and 10 MN/ 10^3 cells respectively (Table 2; Fig. 5). Regression analysis was also performed to see the effect of different doses of ascorbic acid on micronuclei induction by 100 and 200 μM of H_2O_2 . A decrease in the slope of linear regression line was observed when 100 μM ($P < 0.03$) and 200 μM ($P < 0.07$) was treated with 20, 40 and 80 μM of ascorbic acid respectively (Fig 7 and 8).

DISCUSSION

In the present study a clear dose dependent significant increase in the concentration of MDA was obtained after H_2O_2 treatment. The results of the present investigation reveal that ascorbic acid is potent enough to reduce the oxidative damage caused by H_2O_2 on cultured human peripheral blood lymphocytes. In *in vivo* conditions H_2O_2 may be generated directly by divalent reduction of O_2 or indirectly by univalent reduction of $\text{O}_2^{\cdot-}$. H_2O_2 is the primary product of the reduction of O_2 by numerous oxidases, such as xanthine oxidase, uricase, D-amino acid oxidase, and α -hydroxy acid oxidase localized in peroxisomes (20). H_2O_2 is decomposed to H_2O and O_2 by a reaction catalysed by redox active metal complexes of which catalase and peroxidase are the most

Fig 6: Effect of hydrogen peroxide on the frequency of micronuclei

effective (20). H_2O_2 also reacts with $\text{O}_2^{\cdot-}$ to initiate Haber-Weiss reaction producing $\cdot\text{OH}$ in the presence of Fe^{+2} .

$\cdot\text{OH}$ is responsible for DNA damage (9). In our present study a dose dependent increase in DNA damage was observed by H_2O_2 treatment. Genotoxicity testing provides human a risk assessment. An increase in the genotoxic damage is associated with an increased overall risk of cancer (21, 22). The micronucleus is a well known marker of genotoxicity and any reduction in the frequency of the genotoxic endpoint gives an indication of the antigenotoxicity of a particular compound (23). The results of the present study showed that the ascorbic

Fig 5: Effect of ascorbic acid on the frequency of micronucleus induced by hydrogen peroxide

HP1 = H_2O_2 50 μM ; HP2 = H_2O_2 100 μM ; HP3 = H_2O_2 200 μM ; AA1 = 20 μM ; AA2 = 40 μM ; AA3 = 80 μM ; U = Untreated; NC = Negative Control (200 μL distilled water)

Fig 7: Effect of ascorbic acid on the frequency of micronuclei induced by 100 µM hydrogen peroxide

Fig 8: Effect of ascorbic acid on the frequency of micronuclei induced by 200 µM hydrogen peroxide

acid is potent enough to reduce the genotoxic damage of H_2O_2 thereby reducing the chances of developing cancers, as the high concentration of H_2O_2 leads to the cancer induction in animals due to the DNA damage (5). Ion radicals generated during oxidative stress lead to a chain reaction called lipid peroxidation. Aldehydes generated from lipid peroxidation form DNA adducts and lipid hydroperoxides (LOOH) results in extensive single and double strand breaks (24). Various constituents of fruits and vegetables possess anti-oxidant activity that protects the membranes during the oxidative stress (25). Antioxidant vitamins are able to inactivate highly reactive molecules such as free radicals, which are generated during

various biochemical processes in the cells (26). Vitamins act as antioxidants and free radical scavengers, thereby acting as anticarcinogenic, anticlastogenic and antimutagenic agents. Of these vitamin C and α -tocopherol are among the best known antioxidants used in *in vivo* animal models (26). Ascorbic acid is a non enzymatic antioxidant and is therefore potentially involved in protecting cells against oxidative stress. It also acts as a free radical scavenger and its presence assists various other mechanisms in decreasing numerous disruptive free radical processes (27). The co-administration of ascorbic acid with H_2O_2 decreased lipid peroxidation. The results indicate the beneficial effects of ascorbic acid to overcome oxygen dependent cytotoxicity in animals. Although the detail mechanism of the action of ascorbic acid is not fully understood, it is believed that the ascorbic acid as an anti oxidant might stimulate the 7- α -hydroxylation of lipids and cholesterol nuclei thus enhancing their degradation to bile acids, which could be excreted from the body (27, 28). Vitamin C is an antioxidant, it possesses substantial nucleophilic character and it has been suggested that, ascorbate might protect against electrophilic attack on cellular DNA and cell membranes by intercepting reactive agents (29) or that ascorbyl anion radical, with the high extent of unpaired electron delocalization, is responsible for the scavenging of free radicals or ascorbic acid might act as a chain breaking inhibitor of the peroxidation process by scavenging intermediary peroxy and alkoxyl radical (30,31). However, ascorbic acid has been shown to be cytotoxic at higher concentrations, but the selected doses lie within the range of humans (32).

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