ORIGINAL ARTICLE

Effect of vitamins A and C on antioxidant status of breast-cancer patients undergoing chemotherapy

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SUMMARY

What is known and Objective: Reactive oxygen/nitrogen species generated by antineoplastic agents are prime suspects for the toxic side-effects of acute or chronic chemotherapy. The present study was undertaken to test whether vitamins C and E (VCE) supplementation protect against some of the harmful effects of commonly used anticancer drugs in breast-cancer patients.

Methods: In a randomized 5-month study, the activity of various antioxidant enzymes (superoxide dismutase, catalase, glutathione-S-transferase and glutathione reductase) and the levels of malondialdehyde and reduced glutathione were measured in forty untreated breast-cancer patients (stage II) and compared with those of healthy controls. The degree of DNA damage was also assessed in the peripheral lymphocytes of the patients by alkaline single cell gel electrophoresis. The untreated patients were then randomly assigned to either treatment with chemotherapy alone (5-fluorouracil 500 mg/m² i.v. day 1, doxorubicin 50 mg/m² i.v. day 1 and cyclophosphamide 500 mg/m² i.v. day 1, every 3 weeks for six cycles) or to the same chemotherapy regimen supplemented with VCE (vitamin C 500 mg tablet and vitamin E 400 mg gelatin capsule). On completion of the treatments, both the groups were studied again for the levels of the markers measured prior to treatment.

Results and Discussion: The untreated group showed significantly lower levels of antioxidant enzymes (P < 0.001) and reduced glutathione (P < 0.001), and more extensive lipid peroxidation (P < 0.001) and DNA damage than healthy controls. Similar but less pronounced patterns were observed in the patients receiving chemotherapy alone. The group of patients receiving VCE supplementation had all the marker levels moving towards normal values. Activities of superoxide dismutase, catalase, glutathione-S-transferase and glutathione reductase, and the levels of reduced glutathione were significantly increased (P < 0.01) while, the levels of malondialdehyde and DNA damage were significantly (P < 0.01) reduced in the VCE supplemented group relative to those of patients receiving chemotherapy alone as well as relative to the pretreatment levels.

What is new and Conclusion: Co-administration of VCE restored antioxidant status, lowered by the presence of breast-cancer and chemotherapy. DNA damage was also reduced by VCE. The results suggest that VCE should be useful in protecting against chemotherapy-related side-effects and a randomized control trial to evaluate the effectiveness of VCE in breast-cancer patients using clinical outcomes would be appropriate.

Keywords: antioxidants, breast-cancer, chemotherapy, DNA damage, lipid peroxidation, vitamin C, vitamin E

WHAT IS KNOWN AND OBJECTIVE

Chemotherapy is one of the mainstays in the treatment of cancer. However, although chemo-
therapy improves the survival of cancer patients, they cause significant toxicity. Besides acute toxicity such as nausea, alopecia, oral mucositis (1) and bone marrow depression, long-term side-effects can reduce the quality of life of these patients. Free radical mediated DNA cross-linking is one of the ways through which most of the chemotherapeutic drugs (such as doxorubicin, bleomycin, vincristine, cyclophosphamide and hydroxyurea) exert their cytotoxic effects (2).

Antioxidants counteract free radicals and prevent tissue and organ damage (3). Administration of dietary or pharmaceutical supplemental antioxidants, concurrently with cytotoxic regimens, has been reported to cause modest decreases in treatment-related side-effects (4, 5). However, concern has been expressed (6, 7) that the protective action of supplemental antioxidants on chemotherapy induced oxidative damage might not be restricted to normal tissues, but can extend to tumour cells as well.

Although antioxidants may play a role in the primary prevention of cancer by reducing the oxidative modification of DNA (8), they may also interfere with the action of chemotherapeutic agents that act solely via the production of reactive oxygen species, and induction of apoptosis (3). Considerable in vitro and animal data have shown the protective effect of vitamin C and other antioxidants against damage by radiation and chemotherapy (9, 10). It seems likely that they would reduce treatment-related toxicity but there is no data to show that this is indeed the case (5, 11).

Vitamin E may be an important nutrient for enhancing antineoplastic activity by preventing the peroxidation of lipids (12). In animal studies, combinations of high doses of vitamin E with chemotherapy have been reported to have beneficial effects, detrimental effects, and no effect (13). Vitamin C is a water-soluble antioxidant. The interaction of vitamin C with chemotherapy and radiotherapy remains unclear although high concentrations of vitamin C have been reported to increase the toxicity of some chemotherapeutic drugs in animals (13).

The lowered physiological antioxidant defense during chemotherapy may lead to increased oxidative stress and free radical-mediated organ damage. Vitamin C and vitamin E are some of the most important natural antioxidants, which act synergistically to neutralize free radicals (14). In the present study, we investigated the effects of VCE supplementation on in vivo antioxidant status and DNA damage in breast-cancer patients.

**METHODS**

Forty women aged between 35 and 65 years and with histological diagnosis of breast carcinoma, participated in a prospective, randomized 5-month study. Only patients with stage II of TNM classification, and who had not undergone any previous treatment for malignancy were recruited from both the out-patient and hospital inpatients populations under the care of the Department of surgery, J.N Medical College, Aligarh, U.P, India. Forty age- and sex-matched healthy volunteers formed the control group. Patients were subjected to detailed history and examination. None of the patients had concomitant diseases such as diabetes mellitus, liver disease or rheumatoid arthritis, and none of was using oral contraceptives, hormones or vitamin supplements. At the same time, confounding variables such as smoking and menopausal status were also considered. The clinical characteristics of patients are presented in Table 1. The study was approved by the ethics committee of A M Univer-

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<th>Table 1. Clinical characteristics of breast cancer patients</th>
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<tr>
<td>Patients with breast carcinoma</td>
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<td>Age range (years)</td>
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<td>Age at menarche (years)</td>
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<td>Chemotherapy alone</td>
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<td>(Fluorouracil, doxorubicin and cyclophosphamide)</td>
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<td>Chemotherapy + VCE</td>
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T, tumour size in diameter; T2 2–4 cm; N, regional lymph node metastasis; N1, metastasis in a single ipsilateral regional lymph node of <3-cm diameter; M, distant metastasis; M0, no evidence of distant metastasis.
sity and informed consent was obtained from all the participants.

Blood specimens were drawn from the patients and control subjects at 10:00 a.m. and plasma was collected by centrifugation at 2500g for 10 min at 4 °C. The plasma of the untreated patients and controls was subjected to the measurement of activities of various antioxidant enzymes - superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione reductase (GR) and the levels of malondialdehyde (MDA) and reduced glutathione (GSH). The degree of DNA damage was assessed in the peripheral lymphocytes by alkaline single cell gel electrophoresis. The untreated patients were then randomly assigned to treatment with chemotherapy alone (5-fluorouracil 500 mg i.v. day 1, doxorubicin 50 mg/m² i.v. day 1 and cyclophosphamide 500 mg/m² i.v. day 1, every 3 weeks for six cycles) or to the same chemotherapy regimen and VCE supplementation (vitamin E, tocopheryl acetate 400 mg as Evion 400 IU gelatinous capsule manufactured by Merck Limited, India and vitamin C as Limecee 500 mg tablet manufactured by Piramal Healthcare Limited, India) once a day during and after completion of the treatment regimens, both the groups were studied again for the levels of MDA and GSH, activities of SOD, CAT, GST and GR, and the extent of DNA damage.

Biochemical estimations

Determination of superoxide dismutase activity. Superoxide dismutase activity in the plasma was assayed by the method of Marklund and Marklund based on the ability of superoxide dismutase to inhibit the auto-oxidation of pyrogallol at 412 nm (15). Results were expressed as enzyme units/mg protein. One enzyme unit is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation per 3 mL of assay mixture.

Determination of catalase activity. Catalase activity was determined by following the rate of decomposition of H₂O₂ at 240 nm (16). Results were expressed as enzyme units/mg protein. One unit is defined as nmoles of H₂O₂ consumed min/mg/protein.

Determination of glutathione-s-transferase activity. Plasma GST activity was assayed by the method of Habig et al. using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate (17). Calculation was made using milli molar extinction coefficient value of 9.6/mM/cm results expressed as enzyme units/mg protein. One enzyme unit is defined as nmole CDNB conjugate formed per minute per mg protein.

Determination of glutathione reductase activity. Glutathione reductase activity was assessed according to the method of Carlberg and Mannervik (18). This enzyme catalyzes the NADPH dependent reduction of glutathione disulfide to glutathione. Oxidation of NADPH was followed spectrophotometrically at 340 nm after 30-s intervals for 3 min. Results were expressed as enzyme units/mg protein. One enzyme unit is defined as nmole of NADPH oxidized per minute per mg protein.

Determination of lipid peroxidation products. Plasma MDA level was estimated by measuring the concentration of thiobarbituric acid reactive substances (19). The pink-coloured chromogen formed by the reaction of 2-thiobarbituric acid with the breakdown products of lipid peroxidation was read at 535 nm. Results were expressed as nanomoles of MDA formed per mg protein.

Determination of reduced glutathione concentration. The level of reduced glutathione was determined by the method of Jollow et al. (20) by using sulfosalicylic acid and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). Results were expressed as micromoles GSH/mg protein.

Estimation of protein content. The protein content in all the samples was estimated according to the method of Lowry et al., using bovine serum albumin as standard (21). The blue colour developed was measured at 660 nm. The result was expressed as mg protein/mL plasma.

DNA damage studies by single cell gel electrophoresis (Comet assay)

Comet assay was performed under alkaline conditions essentially according to the procedure of
Singh et al. (22) with slight modifications. Fully frosted microscopic slides precoated with 10% normal melting agarose at about 50 °C (dissolved in Ca++ and Mg++ free PBS) were used. Around 10,000 cells (isolated from blood) were mixed with 75 μL of 1.0% low melting point agarose (LMPA) to form a cell suspension, pipetted over the first layer, and covered immediately by a cover slip. The slides were placed on a flat tray and kept on ice for 10 min to solidify the agarose. The cover slips were removed, a third layer of 0.5% LMPA (75 μL) was pipetted, and cover slips placed over it and allowed to solidify on ice for 5 min. The cover slips were removed and the slides were then immersed in cold lysing solution containing 2.5 M NaCl, 100 mM EDTA, pH 10 and 1% Triton X100 (added just prior to use) for a minimum of 1 h at 4 °C. After lysis, DNA was allowed to unwind for 30 min in alkaline electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was performed at 4 °C in field strength of 0.7 V/cm and 300 mA current. The slides were then neutralized with cold 0.4 M Tris, pH 7.5, stained with 75 μL EtBr (20 μg/mL) and covered with a cover slip. The slides were finally placed in a humidified chamber to prevent drying of the gel and analyzed the same day. Slides were scored using an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK) attached to an Olympus (CX41) fluorescent microscope and a COHU 4910 (equipped with a 510–560 nm excitation and 590 nm barrier filters) integrated CC camera. Comets were scored at 100× magnification. Images from 50 cells (25 from each replicate slide) were analyzed. The parameter taken to assess cellular DNA damage was tail length (migration of DNA from the nucleus, μm) and was automatically generated by Komet 5.5 image analysis system.

**Statistical analysis**

All the data are expressed in mean ± standard error of mean. The statistical significance of any observed effects was evaluated using one-way ANOVA. The significance probability P ≤ 0.01.

**RESULTS AND DISCUSSION**

Free radical species generated by antineoplastic agents are thought to account for much of their toxic effects. The body’s defense against oxidative stress is primarily dependent upon an orchestrated synergism between several endogenous (SOD, CAT, GST, GR and GSH) and exogenous (vitamins A, E and C) antioxidants.

In the present study the plasma levels of GSH (Fig. 2) and the activities of all antioxidant enzymes- SOD, CAT, GST and GR (Table 2) were significantly lower (P < 0.001) while the extent of lipid peroxidation (Fig. 1) and DNA damage (Fig. 3) were significantly higher (P < 0.001) in the untreated breast-cancer patients than in the control subjects. This observed diminution in the levels of enzymatic and non-enzymatic antioxidants and the increased level of MDA and DNA damage in the untreated patients may be attributed to the severe oxidative stress generated by chemotherapy. Impaired SOD and CAT activity together with decreased levels of GSH, which acts as one of the guarding factors against oxidative stress (23) might make the tissue vulnerable to oxidative toxicity and...
their detoxifying capabilities and make the system more prone to H₂O₂ toxicity. The increased generation of reactive oxygen species also induces DNA strand breaks causing oxidative modification (24) and DNA damage as is evident by the increased tail length in the untreated patients as compared to controls (14.2 ± 1.9 µm vs. 1.3 ± 0.01 µm). Damaged DNA also results in impaired ability of cells to repair or prevent disease (25).

Dietary supplementation with antioxidant vitamins C and E (the most important naturally-occurring antioxidant vitamins) may be helpful in modulating the oxidative stress generated during the course of chemotherapy. The antioxidant effect of the ascorbic acid (vitamin C) is related to its capacity to lose a hydrogen atom and to form a relatively stable ascorbate free radical. The main protective effects of vitamin C against cancer could be due, mainly to its antioxidant effect (26). Ascorbic acid spares GSH and together with vitamin E prevents the oxidation of glutathione (27). Administration of vitamin E and ascorbate is known to significantly reduce the concentrations of lipid peroxides and enhance GSH levels (28). By preventing lipid peroxidation, vitamin E maintains rapid proliferation of cancer cells, which is essential for chemotherapy. Administration of vitamin E

lipid peroxidation as is reflected in increased levels of MDA. Also, because GSH is a substrate for GST and glutathione peroxidase (GPx), it might hamper has been reported to have an important protective effect against chemotherapy-induced peripheral nerve damage (29). In addition, vitamins E and C in

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combination, have been reported to reduce the oxidative damage induced by certain anesthetic drugs (30) and exercise (31). In the present study, we found that VCE supplementation markedly increased \((P < 0.01)\) the levels of all free radical scavenging enzymes, SOD, CAT, GST and GR (Table 2) as well as of the non-enzymatic antioxidant, GSH (Fig. 2). There was also a significant reduction \((P < 0.01)\) in the levels of MDA (Fig. 1) after treatment in subjects receiving VCE compared to the pretreatment levels as well as levels in patients treated with chemotherapy alone. The extent of DNA damage correlated with the changes observed for the antioxidant parameters. Co-administration of VCE during chemotherapy reduced free radical–mediated DNA damage as is evident by a significant reduction in the tail length seen in the VCE supplemented group \((5.9 \pm 0.5 \mu m)\) compared to the untreated patients \((14.2 \pm 1.9 \mu m)\) as well as patients treated with chemotherapy alone \((9.4 \pm 0.9 \mu m)\) (Fig. 3).

**WHAT IS NEW AND CONCLUSION**

Co-administration of VCE restored antioxidant status, lowered by the presence of breast-cancer and chemotherapy. DNA damage was also reduced by VCE. The results suggest that VCE should be useful in protecting against chemotherapy-related side-effects and a randomized control trial to evaluate the effectiveness of VCE in breast-cancer patients using clinical outcomes would be appropriate.

**REFERENCES**

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