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Protection by α-Tocopherol but not Ascorbic Acid from Hydrogen Peroxide Induced Cell Death in Normal Human Breast Epithelial Cells in Culture

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α-Tocopherol and ascorbic acid have been suggested to play a role in breast cancer prevention due to their antioxidative capacity. Increased exposure to endogenous and exogenous sex steroids is a known risk factor for breast cancer. We have studied the effects of α-tocopherol and ascorbic acid on hydrogen peroxide induced cell death in sex hormone treated normal breast epithelial cells in culture. We found that α-tocopherol but not ascorbic acid alone protected the cells. The effect of α-tocopherol increased when ascorbic acid was added to the cultures. The hydrogen peroxide degradation rate decreased in cultures treated with α-tocopherol alone and in combination with ascorbic acid compared to cells grown in medium or with ascorbic acid only. Oestradiol and progesterone treatment did not influence the results. Possible beneficial effects of combining various antioxidants, endogenous as well as exogenous, on human breast tissue need to be investigated further both *in vivo* and *in vitro*.

Keywords: α-Tocopherol, ascorbic acid, oestradiol, progesterone, breast

INTRODUCTION

Oxidative damage to DNA may contribute to cancer development in various organs, including the breast.[1,2] Man is continuously exposed to both endogenous and exogenous oxidants. Endogenous oxidants are formed as a result of aerobic metabolism. Examples of exogenous sources of oxidants include background radiation, cigarette smoke, and environmental pollution. The defence against oxidative stress consists of antioxidants synthesised in our body and antioxidants derived from diet. Antioxidant micronutrients have been suggested to play a role in cancer prevention, but the results are, however, inconsistent. Several epidemiological studies have shown a beneficial effect of vitamins C and E in breast cancer prevention,[3–5] while others have shown that the vitamins have no

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protective effect.[6–8] The interactions and combined benefits of the various antioxidants derived from diet, as well as the endogenous antioxidants are, still unclear.

There is a great variation in breast cancer rates among countries, possibly due to differences in environmental, dietary and/or reproductive factors. In Sweden, approximately 10% of all women will develop breast cancer during their lives.

Increased exposure to endogenous and exogenous sex steroids is a known risk factor for breast cancer.[9] However, knowledge about the effects of oestradiol and progesterone on normal breast tissue and the mechanisms behind the increased breast cancer risk is still limited. The promotive effect of oestradiol and/or progesterone on cell division in breast epithelial cells has been suggested to be one risk factor.[10,11] Another risk factor is the action of oestradiol as a chemical carcinogen by its reactive metabolites.[12]

We have recently demonstrated that oestradiol and progesterone affect several antioxidative enzymes, resulting in increased cell death after hydrogen peroxide exposure in normal breast epithelial cells in culture.[13] We have also shown that the *in vivo* levels of glutathione increase in breast tissue late in the menstrual cycle when the levels of oestradiol and progesterone are high.[14]

The present work investigates possible protective effects of α-tocopherol and ascorbic acid in normal human breast epithelial cells in culture exposed to hydrogen peroxide. We also investigate whether oestradiol and progesterone influence the effects of α-tocopherol and ascorbic acid.

MATERIALS AND METHODS

Chemicals Insulin, hydrocortisone, isoproterenol, ethanolamine, phosphoryl ethanolamine, epidermal growth factor, transferrin, t-butyl hydroperoxide, n-octyl sodium sulphate, d-α-tocopherol succinate (T3126), α-tocopherol and ascorbic acid were purchased from Sigma (St. Louis, MO, USA). Bovine pituitary extract was obtained from GIBCO BRL Paisley (Scotland, UK), and 17β-oestradiol and progesterone from Apoteksbolaget (Stockholm, Sweden). Other chemicals were obtained from standard sources.

Cell culture Normal human breast epithelial cells (AG1134) were purchased from the Coriell Institute for Medical Research (Camden, NJ, USA). The cells were cultured from breast tissue obtained from a 28 year old woman undergoing reduction mammoplasty and the cells were well characterised as normal human mammary epithelial cells.[15,16] They were cultured in MCBD 170 medium (GIBCO BRL, Paisley, Scotland, UK) supplemented with insulin (5 µg/ml), hydrocortisone (0.5 µg/ml), isoproterenol (10^{-5} M), ethanolamine (10^{-5} M), phosphoryl ethanolamine (10^{-4} M), epidermal growth factor (5 ng/ml), bovine pituitary extract (70 µg/ml) and transferrin (5 µg/ml). Culturing was performed at 37°C in humidified air with 5% CO_2. The cells were subcultured and seeded into 35 or 60 mm dishes (Costar, Cambridge, NJ, USA) using 0.05% trypsin supplemented with 0.02% ethylenediaminetetra acetate (EDTA). 17β-Oestradiol (10^{-8} M), progesterone (10^{-8} M) and α-tocopherol succinate (7.5 µM) were added in growth medium 24 h after subcultivation; the cultures were treated for seven days before experiments. The α-tocopherol succinate was dissolved in ethanol with a final ethanol concentration of 0.2%. Ascorbic acid (60 µM) was added 24 h before experiments. The medium in all cultures was changed every second day.

Cell viability Normal human breast epithelial cells (20,000 cells/cm^2) were exposed to 1.5 mM hydrogen peroxide in HBSS supplemented with 10 mM 4-(-2-hydroxylethyl)-1-piperazine-ethanesulphonic acid (HEPES), pH 7.4 at 37°C for 2 h. After treatment with hydrogen peroxide, the cultures were rinsed in HBSS and incubated for 24 h in fresh medium without added hormones. Viability, measured as lactate dehydrogenase (LDH) activity in the cellular pellet and in the medium, was expressed as percentage of LDH activity in the cellular pellet.[17]

α-Tocopherol content After treatment for seven days, α-tocopherol was analysed by addition of 400 µl solution containing one part methanol : ethanol : isopropanol (20 : 19 : 1) and one part 0.1 M sodium dodecyl sulphate supplemented with 2.27 mM 2,6-di-*tert*-butyl-*p*-creosol and 300 µl hexane to a 35 mm Petri dish, as previously described.[18] The sample was mixed for 2 min and centrifuged at 8000 × *g* for 10 min, after which the hexane phase was withdrawn and dried under nitrogen gas. The residue was dissolved in 250 µl methanol : ethanol : isopropanol (20 : 19 : 1) and analysed within 2 h using an HPLC fitted with a Kromasil 100-5C18 column (250 × 4.6 mm; Hichrom, Reading, UK). The mobile phase consisted of methanol : ethanol : isopropanol (20 : 19 : 1) and 20 mM lithium perchlorate. The flow rate was 0.7 ml/min. α-Tocopherol was detected by a BAS LC-4B electrochemical detector (BAS, West Lafayette, IN, USA) fitted with a glassy carbon electrode at +0.5 V. The concentration of α-tocopherol was calculated from a standard curve using α-tocopherol dissolved in ethanol : isopropanol (95 : 5) as a standard. The concentration of the α-tocopherol standard was calculated from its absorption at 290 nm ($\varepsilon = 3467\,M^{-1}\,cm^{-1}$). The detection limit was 0.95 nM α-tocopherol.

α-Tocopherol succinate content α-Tocopherol succinate was determined by measuring the amount of α-tocopherol before and after base hydrolysis.[19] The cells were treated as described in the previous paragraph and divided into two portions. One was analysed for α-tocopherol and the other was treated with 200 µl of 4 M KOH overnight. Two hundred microlitres 4 M HCl and 300 µl hexane were added. The sample was then treated and analysed by HPLC by the same procedure as described above.

Ascorbic acid content After 24 h treatment, 35 mm Petri dishes were rinsed in phosphate buffered saline with 300 µl 0.5 M perchloric acid supplemented with 1 mM EDTA. The samples were kept on ice and centrifuged at 2000 × *g* for 10 min. The supernatant was used for ascorbic acid analysis using an HPLC and a Kromasil 100-5C18 column (250 × 4.6 mm; Hichrom, Reading, UK) as previously described.[20] The mobile phase was purged with helium gas and consisted of 5% methanol in 0.1 M sodium phosphate buffer containing 0.1 mM EDTA, pH 2.5 and 0.2 mM octyl sodium sulphate. The flow rate was 0.5 ml/min. An electrochemical detector fitted with an Au-electrode at +0.6 V was used for detection of ascorbic acid and the concentration was calculated according to a standard curve using freshly prepared ascorbic acid as a standard.

Degradation of hydrogen peroxide Human breast epithelial cells were grown in 60 mm dishes and exposed to 1.5 mM H_2O_2 in HBSS (4 ml/60 mm dish) at 37°C. Aliquots (50 µl) were taken from the cell supernatant and the H_2O_2 concentration was assayed using *p*-hydroxyphenylacetic acid (pHPA) as a probe. In the presence of horseradish peroxidase, H_2O_2 oxidises pHPA to its fluorescent dimer. Fluorescence intensity was measured at λex = 315 nm/λem = 410 nm using an RF-540 fluorescence spectrophotometer, Shimadzu.[21,22]

Statistical analysis Data are presented as mean ± SD. Groups were compared using ANOVA, (Statview 4.5) and Fisher's post-hoc test; $p < 0.05$ was considered statistically significant.

RESULTS

The hormone treatment did not alter the growth rate of the cells as measured by cell count and protein content. Pre-treatment with α-tocopherol succinate (7.5 µM) increased the levels of α-tocopherol in the lipid phase in all cultures. There were no differences in hormone treated cells and controls (Table I). α-Tocopherol was not detected in cells grown in medium only or in medium with added sex hormones. After a 24 h incubation the α-tocopherol succinate was completely accumulated into the breast epithelial cells as α-tocopherol, the levels of cellular α-tocopherol

TABLE I Ascorbic acid and α-tocopherol content (μM/10^6 cells) in normal human breast epithelial cells in culture

Hormone treatment	Ascorbic acid	α-Tocopherol
None	16.3 (±0.64)	6.3 (±0.2)
Oestradiol	15.1 (±0.14)	6.3 (±0.3)
Oestradiol + Progesterone	15.2 (±0.12)	6.1 (±0.2)

Normal human breast epithelial cells cultured in the presence or absence of sex steroids for seven days. α-Tocopherol succinate (7.5 μM) was added for seven days and ascorbic acid (60 μM) was added 24 h before measurement by HPLC, as described in the Methods section. Values are mean ± SD ($n = 4$). There were no differences between the groups.

succinate were below the detection limit. Concentrations between 1–10 μM α-tocopherol succinate were non-toxic and did not inhibit proliferation, measured by protein content, compared to control cells. When the concentration was raised to 15 μM, proliferation was inhibited.

Some cells were treated with ascorbic acid dissolved in water. Concentrations between 10–75 μM were non-toxic; 100 μM caused changes in cell morphology (blebbing). No differences in proliferation were detected during the ascorbic acid treatment, either in hormone or non-hormone treated cells. After pre-treatment with 60 μM ascorbic acid for 24 h, there were no differences in the content of ascorbic acid between hormone treated cells and controls (Table I). Ascorbic acid was not detectable in cells grown without the vitamin. After 24 h, 78 ± 2% ($n = 3$) of the ascorbic acid remained in the medium.

Viability of cells not exposed to hydrogen peroxide was 94–97%. Viability after exposure to hydrogen peroxide was significantly lower ($p = 0.0016$) in cultures pre-treated with oestradiol (10^{-8} M) or a combination of oestradiol and progesterone (10^{-8} M) than in non-pre-treated cultures (Figures 1–3, white columns). Adding ascorbic acid to the cultures did not increase the viability of the cells. Treatment with α-tocopherol resulted in a clear increase of viability both in hormone and non-hormone treated cells ($p < 0.0001$). When the cells were treated with a combination of ascorbic acid and α-tocopherol, there was a slight additional viability compared to α-tocopherol treatment only ($p < 0.05$). Data are shown in Figures 1–3.

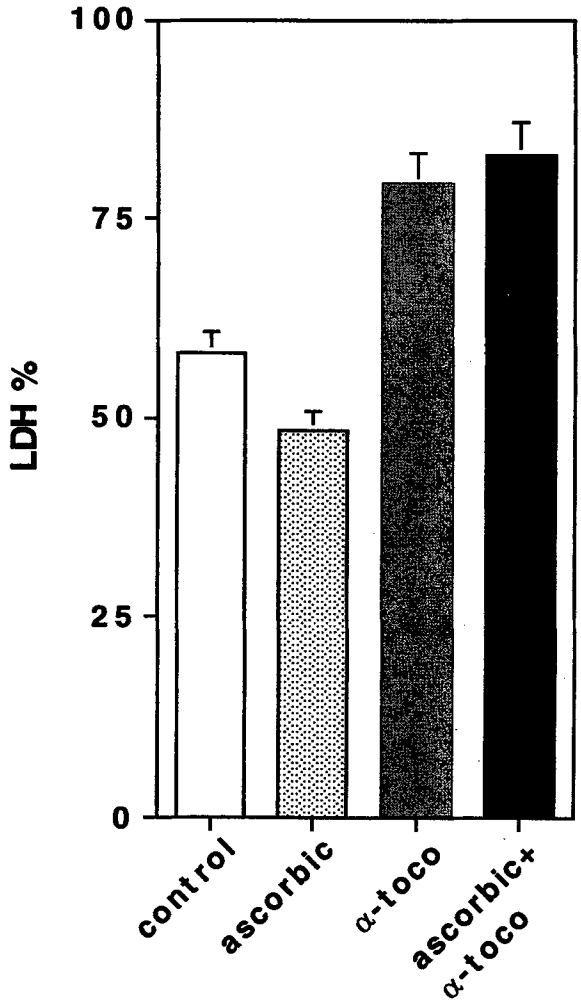

FIGURE 1 Viability of normal breast epithelial cells (20,000/cm^2) cultured in medium only and exposed to 1.5 mM H_2O_2 for 2 h. Effects of seven-day pre-treatment with 7.5 μM α-tocopherol or 24 h pre-treatment with 60 μM ascorbic acid or a combination of the two vitamins. Results are expressed as percentage of LDH activity remaining in the cells, and the values represent means ± SD ($n = 4$–6). Cells cultured with 17β-oestradiol and progesterone had a decreased viability compared to cells grown in medium only ($p = 0.0016$), (white columns in Figures 1–3). α-Tocopherol treatment increased the viability compared to controls in all groups ($p < 0.0001$). Ascorbic acid added to α-tocopherol resulted in an additional increase in viability ($p < 0.05$). Cells not exposed to H_2O_2 had a viability of 94–97% (ANOVA, Statview 4.5).

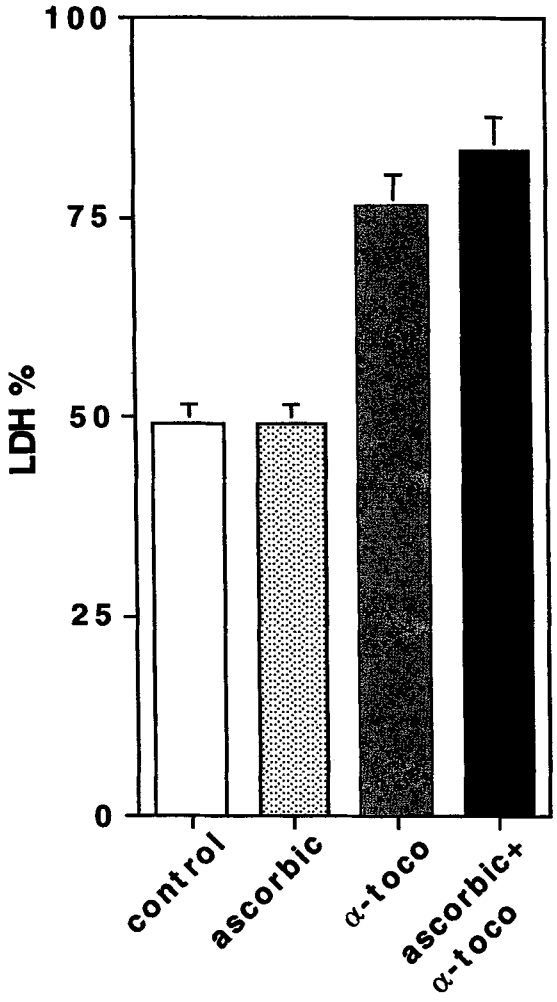

FIGURE 2 Viability of normal breast epithelial cells (20,000/cm^2) cultured with 17β-oestradiol (10^{-8}M) and treated in the same way as in Figure 1.

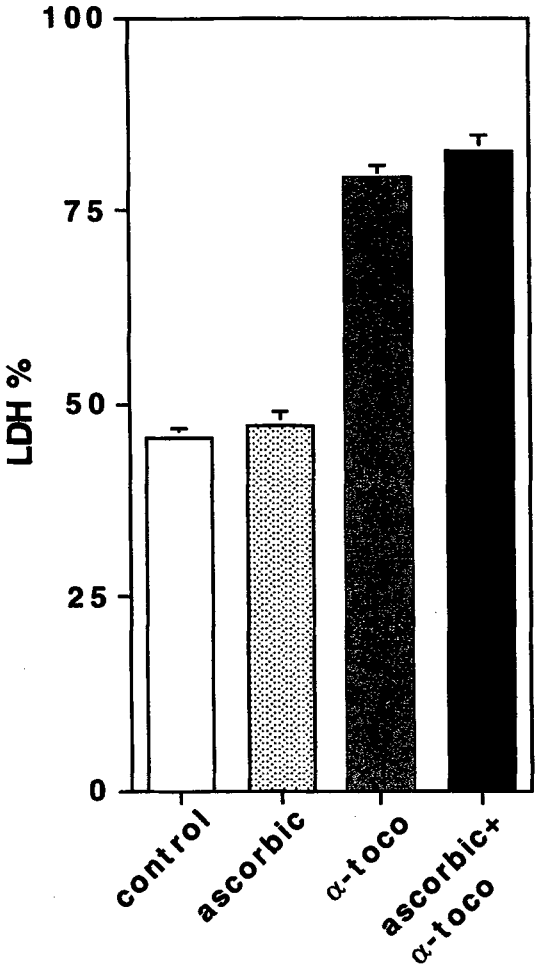

FIGURE 3 Viability of normal breast epithelial cells (20,000/cm^2) cultured with 17β-oestradiol (10^{-8}M) and progesterone (10^{-8}M) in combination and treated in the same way as in Figure 1.

The hydrogen peroxide degradation rates were decreased in cells treated with α-tocopherol only and with α-tocopherol and ascorbic acid in combination, compared with cells grown in medium only or with ascorbic acid only, ($p < 0.0001$). Data are shown in Figure 4.

DISCUSSION

In this experiment, we show that α-tocopherol, but not ascorbic acid, protects normal breast epithelial cells from hydrogen peroxide induced cell death and decreases the degradation rate of hydrogen peroxide. This effect was not dependent on sex hormones. In cells treated with a combination of α-tocopherol and ascorbic acid, the protective effect was enhanced.

In order to induce cell death and to be able to measure differences between treatments the cells must be exposed to non-physiological and high concentrations of H_2O_2. With a high level of H_2O_2 the time of exposure could be short with minor interference on cell adhesion.

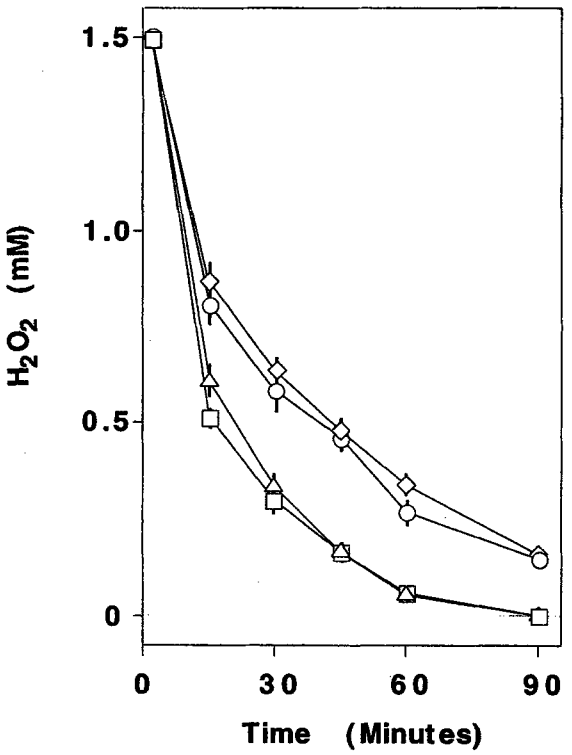

FIGURE 4 Hydrogen peroxide degradation rate in normal human breast epithelial cells cultured in medium only (squares), ascorbic acid (triangles), α-tocopherol (circles) and α-tocopherol + ascorbic acid (rhombi). Aliquots (50 µl) were taken from the cell supernatant and the H_2O_2 concentration was assayed using *p*-hydroxyphenylacetic acid (pHPA) as a probe. The hydrogen peroxide degradation rates were decreased in cells treated with α-tocopherol only and with α-tocopherol and ascorbic acid in combination, compared with cells grown in medium only or with ascorbic acid only ($p < 0.0001$).

As recommended, the breast epithelial cells were cultured, at start, in a medium without added ascorbic acid and α-tocopherol,[15,16] which is the standard procedure in all cell culture systems. Hence the results demonstrate the effect of physiological vitamin concentrations, rather than the effect of *extra* added vitamins in the cell culture system.

Whether ascorbic acid acts as an antioxidant or a pro-oxidant *in vivo* is still unclear. *In vitro*, it can act both as an antioxidant and pro-oxidant.[23,24] Ascorbic acid exerts its pro-oxidant effect mainly by reducing iron ions, which in turn could enhance the generation of hydroxyl radicals. However, *in vivo* most transition metal ions are attached to binding proteins and are not available for these reactions. Although others have shown that ascorbic acid has an antioxidative effect *in vitro*,[25] it had no protective effect against hydrogen peroxide induced cell death in our experiments. Generation of hydroxyl radicals by interaction of free iron ions could contribute to the toxicity of hydrogen peroxide in normal human breast epithelial cells. This is supported by our previous findings, where the iron chelating agent desferrioxamine protected against hydrogen peroxide induced cell death in this cell type.[13]

In addition to its own antioxidant and/or pro-oxidant effects, ascorbic acid is also suggested to regenerate α-tocopherol from the α-tocopheryl radical.[26] This could be a possible explanation for the slight increase in viability seen in the cultures treated with a combination of α-tocopherol and ascorbic acid.

Treatment with α-tocopherol succinate alone had a significant protective effect on oxidative mediated cell death in our experiments. After short-term treatment (4 h), it has been shown that cellular accumulation of intact α-tocopherol succinate rather than cellular α-tocopherol exert the cytoprotection.[19] In our experiments, the cells were treated with α-tocopherol succinate for a total of seven days. The medium, supplemented with α-tocopherol succinate, was changed every second day resulting in a 48 h incubation. We could also show that already after 24 h the levels of α-tocopherol succinate were below the detection limit. This suggests that the accumulated cellular α-tocopherol and not the α-tocopherol succinate itself is responsible for the cytoprotection. The H_2O_2 degradation rate also decreased in cells cultured with α-tocopherol. This lipid-soluble antioxidant is found in cell membranes and is a major contributor to the protection against lipid peroxidation.[27] A possible explanation for our findings is that the incorporated

α-tocopherol in the cell membranes protects from cell damage both by scavenging the H_2O_2 and preventing its entrance into the cells. All vitamin E (tocopherols and tocotrianols) in the human body is derived from diet and α-tocopherol is the most biologically active form of vitamin E. *In vitro*, it has been shown that α-tocopherol succinate inhibits proliferation in breast cancer cell lines.[28] However, animal experiments as well as epidemiological studies have yielded mixed results concerning vitamin E and breast cancer risk.[6] Fibrocystic breast disease and breast tissue hyperplasia are known risk factors for breast cancer and several years ago it was suggested that breast tenderness and size of breast cysts decreased in women taking vitamin E.[29]

In our experiments, there was an inhibition of proliferation when the concentration of α-tocopherol was raised. This suggests that α-tocopherol has a beneficial effect in normal breast cells both as an inhibitor of proliferation and as an antioxidant. Vitamin E has extremely low toxicity[30] and since oestradiol and progesterone do not influence the effects of the vitamin, there could be a safe and beneficial effect of vitamin E treatment both in pre-menopausal and post-menopausal women. However, additional studies of diet as well as supplements, serum, and tissue levels of the different components, are needed in order to prove a possible reduction in breast cancer rates. Moreover, measuring the overall antioxidant ability of an organ or cell system is probably more important than investigating one particular nutrient or antioxidant.

In summary, this *in vitro* study showed that α-tocopherol had a protective effect on cell death in normal human breast epithelial cells exposed to hydrogen peroxide. The protective effect was enhanced when ascorbic acid was added to the cells. Ascorbic acid alone had no protective effect. Oestradiol and progesterone did not influence the effects of the vitamins. Possible beneficial effects of combining various antioxidants, endogenous as well as exogenous, on human breast tissue need to be investigated further both *in vivo* and *in vitro*.

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