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Protective role of tocopherol and ascorbic acid in taxol-treated human erythrocytes in vitro

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Abstract

Taxol is a well-known anticancer agent. It is used for the treatment of several kinds of cancer, including breast cancer, lung cancer and ovarian cancer. In spite of being a good chemotherapeutic agent, taxol has several side effects. Drug-induced haemolytic anaemia is one of the most common side effects of taxol. This study investigated the haemolytic effect of taxol on normal erythrocytes and the protective effect of natural antioxidants ascorbic acid and tocopherol in the presence of taxol. We evaluated the osmotic fragility and the activity of enzymes superoxide dismutase and catalase of erythrocytes in the presence of taxol alone and taxol in combination with tocopherol and ascorbic acid. Taxol-induced haematological perturbation significantly caused haemolysis and reduced the activities of superoxide dismutase and catalase in erythrocytes. The antioxidants tocopherol and ascorbic acid demonstrated a protective effect when added to taxol. The combination of tocopherol with taxol significantly protected the osmotic lysis of erythrocytes and increased the activities of superoxide dismutase but had less effect on catalase. Ascorbic acid showed significant protection of erythrocytes from osmotic lysis but didn't show any significant effect on superoxide and catalase. The results suggest that both antioxidants, especially tocopherol, could exhibit a protective effect against taxol-induced haematological toxicity.

Keywords

Taxol, haemolytic anaemia, tocopherol, ascorbic acid, erythrocytes

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Introduction

Cancer is one of the major public health problems worldwide and is the second leading cause of death in the United States,[1] causing the death of almost 6 million people every year. There are many treatments available for cancer, the most common of them are chemotherapy, radiotherapy and surgery. Medication or chemotherapy is one type of cancer treatment used alone or in combination with other treatments.[2] Although chemotherapy is one of the most common treatments, it is associated with many side effects. One of them is anaemia, which has an adverse impact on quality of life of the patient.[3] More than 50% of all cancer patients encounter anaemia, regardless of the treatment received, and approximately 20% of all patients undergoing chemotherapy suffer from anaemia and require red blood cell transfusion as their haemoglobin concentrations declined below 10 g/dL.[4] Drug-induced anaemia may be caused by stem cell death, blockage or delay of haematopoietic factors, oxidative damage to mature haematopoietic cells, long-term myelodysplasia, immune-mediated haematopoietic cell destruction, and microangiopathy and plasma volume expansion with dilutional anaemia and osmotic imbalance of erythrocyte.[5] Chemotherapeutic agents used for phase I and II trials in cancer treatment have often shown oxidative stress and haemolytic anaemia. Oxidative

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stress is believed to enhance haemolytic anaemia caused by anticancer drugs.[6]

Taxol is a well-known anticancer drug that was isolated from the bark of the Pacific Yew tree. It promotes polymerization and stabilization of tubulin to microtubules by interfering with mitosis.[7] Taxol is effective in the treatment of patients with refractory ovarian cancer, breast cancer, malignant melanoma and other solid tumors.[8] It disrupts the tubulin polymerization process and arrests the cells at G2/M phase.[9] In addition to taxol's chemotherapeutic properties, there are many side effects associated with its use.[10] When taxol is used to treat solid tumors, patients experience myelosuppression, that is, bone marrow suppression which includes neutropenia, anaemia and thrombocytopenia.[11,12] Studies confirm the taxol treatment causes increased level of superoxide, hydrogen peroxide and nitric oxide (NO), causing the oxidative stress in the cells.[13]

Antioxidants are known to be effective in scavenging free radicals from the blood and other cells so they may reduce the side effect of chemotherapy on the erythrocytes by protecting them from oxidative damage.[12,14] This oxidative damage caused by the drug may be ameliorated by tocopherol and ascorbic acid.[15,16]

In the present study, we evaluated the effect of taxol on healthy erythrocytes and in combination with antioxidants tocopherol and ascorbic acid in vitro.

Materials and methods

Reagents

Pure taxol, tocopherol and ascorbic acid were purchased from Sigma Aldrich, USA. Dimethyl sulphoxide (DMSO) was of Sisco Research Laboratories Pvt. Ltd (SRL). NaCl, Na_2PO_4, 30% H_2O_2 and NaH_2PO_4 were taken from Panreac Quimica. The dilution of different antioxidants and anticancer was prepared by dissolving them in sufficient amount of agents in DMSO. NaCl, Na_2HPO_4 and NaH_2PO_4 were used to prepare the phosphate-buffer saline in autoclaved double distilled water. For superoxide dismutase (SOD), assay kit was obtained from Biodiagnostic Cairo, Egypt.

Methods

Blood sampling

Five millilitres of venous blood were obtained with informed consent from healthy volunteers (20 volunteers of age group 20–35 years). Each sample was heparinized and used. All laboratory determinations were run in duplicate, and the mean value was used.

Osmotic fragility assay

The osmotic fragility assay[17] was carried out to study the osmotic haemolysis of erythrocytes caused by taxol. Briefly, the heparinized blood was incubated with 1 μg/ml concentration of taxol at 37°C for 30 min. Aliquots of saline solutions, with concentrations ranging from 10 to 1 g/L were prepared. The treated erythrocytes were then transferred to the tubes containing decreasing concentrations of saline solutions. After mixing carefully, the cell suspensions were left to equilibrate for 30 min and then centrifuged at 3000 r/min for 5 min. The absorbance of supernatants was taken at 540 nm, standardized against a blank assay (10 g/L saline supernatant corresponds to 0% haemolysis). The recorded optical density (OD) of the supernatant reflects the degree of haemolysis of the erythrocytes.[18] The lysis percentage was calculated by dividing the OD of the supernatant obtained from a particular saline concentration by the OD of the standard (1 g/L) representing 100% haemolysis. Osmotic fragility curves were constructed by plotting the lysis percentage against the concentration of saline solutions. The MEF25, MEF50 and MEF75 (mean erythrocyte fragility) values, which are the saline concentrations at which 25%, 50% and 75%, respectively, red blood cells haemolysed (at standard pH and temperature) were obtained from the curve.

Treatment conditions

To study the osmotic fragility of erythrocytes in the presence of anticancer agent taxol and antioxidants (tocopherol and ascorbic acid), we tried three different conditions, pretreatment condition where blood was treated with tocopherol or ascorbic acid for 30 min prior to the treatment of taxol. The second condition was co-treatment where the taxol was co-treated with tocopherol or ascorbic acid, and third condition was post-treatment where erythrocytes were first treated with taxol then tocopherol or ascorbic acid.

Statistical analysis

The results are reported as means $\pm$ standard error of the mean. Statistical analysis was performed with Student's t test and multiple regression analysis. A $p < 0.05$ was considered statistically significant.

Preparation of erythrocyte lysate

The erythrocyte lysate was prepared by washing erythrocytes with 0.9% NaCl for four times, then the washed erythrocytes were made up to 2.0 ml in cold sterilized water. A 25-fold dilution of lysate was made using sterilized water.

SOD assay

The activity of the enzyme SOD has been investigated using SOD assay kit available commercially. The assay was based on the method described by Nishikimi et al.[19] Briefly, the 500–500 μl lysate was treated with taxol, tocopherol, ascorbic acid, taxol with tocopherol and taxol with ascorbic acid in separate microcentrifuge tubes for 30 min. The final concentration of each drug was 1 μg/ml. The

Table 1. Pre-treatment of tocopherol (1 μl/ml) and ascorbic acid (1 μl/ml) with taxol (1 μl/ml), resulting in mean erythrocyte fragility (MEF).

SNo.	Pre-treatment condition	Mean erythrocyte fragility (MEF)		
		MEF25	MEF50	MEF75
1	Control	0.600 ± 0.0096	0.545 ± 0.005	0.4825 ± 0.0095
2	Taxol	0.7775 ± 0.005	0.7025 ± 0.0056	0.6335 ± 0.0057
3	Tocopherol	0.63 ± 0.0058	0.545 ± 0.00577	0.4725 ± 0.0096
4	Ascorbic acid	0.62 ± 0.0058	0.545 ± 0.00577	0.475 ± 0.0057
5	Taxol + tocopherol	0.7075 ± 0.00815	0.1880 ± 0.01893	0.1500 ± 0.00957
6	Taxol + ascorbic acid	0.775 ± 0.005	0.685 ± 0.005	0.6550 ± 0.0035

1 represents control (without any treatment); 2 represents erythrocytes treated with taxol for 30 min; 3 represents erythrocytes treated with tocopherol for 30 min; 4 represents erythrocytes treated with ascorbic acid for 30 min; 5 represents erythrocytes pretreated with tocopherol before treated with taxol; and 6 represents erythrocytes pretreated with ascorbic acid before treated with taxol.

working reagent was prepared by adding 10 ml of mM phosphate buffer (pH 8.5), 1 ml of nitroblue tetrazolium solution and 1 ml of Nicotinamide Adenine Dinucleotide immediately before use. Phenazine methosulphate (PMS) solution was diluted 1000 times before use. Then 100 μl of the treated lysate was mixed with 1 ml of working reagent, and after mixing, 100 μl of PMS solution was added to the tube. The absorption was recorded at 560 nm for 5 min.

Catalase assay

Catalase activity was determined in erythrocyte lysate using Aebi's method.[20] Briefly, the 100 μl of the treated lysate was added to a cuvette containing 2.90 ml H_2O_2 solution (0.036% in 50 mM potassium buffer). Absorption was measured at 240 nm for 180 s. The time required for the A_{240} to decrease from 0.45 to 0.40 absorbance units was recorded. And 3 ml of the buffer was used in blank.

Osmotic fragility assay

The osmotic fragility of the red blood cells in the presence of taxol alone or in combination with tocopherol and ascorbic acid was measured by scoring haemolysis in saline solution. The concentrations of saline solution causing 25%, 50% and 75% lysis of red blood cells have been evaluated and designated as MEF25, MEF50 and MEF75, respectively.

The control MEF values were 0.600 ± 0.0096, 0.545 ± 0.005 and 0.4825 ± 0.0095, which means in control, the concentration of saline causing the 25%, 50% and 75% lysis of red blood cells were 0.600 ± 0.0096, 0.545 ± 0.005 and 0.4825 ± 0.0095, respectively.

The MEF25, MEF50 and MEF75 values of red blood cells were 0.7775 ± 0.005, 0.7025 ± 0.0056 and 0.6335 ± 0.0057, respectively, when the red blood cells were treated with taxol, but when they were pre-treated with tocopherol, the MEF25, MEF50 and MEF75 values were 0.7075 ± 0.00815, 0.1880 ± 0.01893 and 0.1500 ± 0.00957, respectively (Table 1). The results indicate the protective role of tocopherol (Figure 1).

When the cells were pre-treated with ascorbic acid, the values were 0.775 ± 0.005 (MEF25), 0.685 ± 0.005 (MEF50) and 0.6550 ± 0.0035 (MEF75). Ascorbic acid didn't show the significant protection of red blood cells from taxol (Table 1).

When the red blood cells were treated with taxol combined with tocopherol (co-treatment; Figure 2), the damaging of red blood cells (MEF50) was reduced by 0.4750 ± 0.00957 compared with the red blood cells treated with taxol alone (0.7025 ± 0.0056). On the other hand, when the red blood cells were treated with taxol combined with ascorbic acid, the damaging of red blood cells (MEF50) was reduced by 0.5975 ± 0.002 compared with the red blood cells treated with taxol alone.

Taxol combined with tocopherol and ascorbic acid demonstrated MEF50 (Table 2). In this case, the tocopherol and ascorbic acid reduced the damaging effect of taxol by concentrations.

In post-treatment (antioxidants were added to the erythrocytes after taxol exposure for 30 min), the MEF25, MEF50 and MEF75 values were same as taxol alone, which indicates the damage caused by taxol in red blood cells cannot be reversed by post-treatment with tocopherol and ascorbic acid (data not shown here).

Enzyme assays

SOD activity. Mean SOD activity (units/ml) of erythrocytes were 191.8 ± 7, 33.4 ± 9.2, 195 ± 6.7, 181.5 ± 3.7, 119.4 ± 4.7 and 105.0 ± 7.5 in case of control, taxol, tocopherol, ascorbic acid, taxol in combination with tocopherol and taxol in combination with ascorbic acid, respectively, which indicates the SOD activity was significantly reduced by taxol, but tocopherol and ascorbic acid reduce the damaging effect of taxol when added along with it (Figure 3).

Catalase assay

The activity of catalase in control, taxol, tocopherol, and ascorbic acid, taxol with tocopherol and taxol with ascorbic

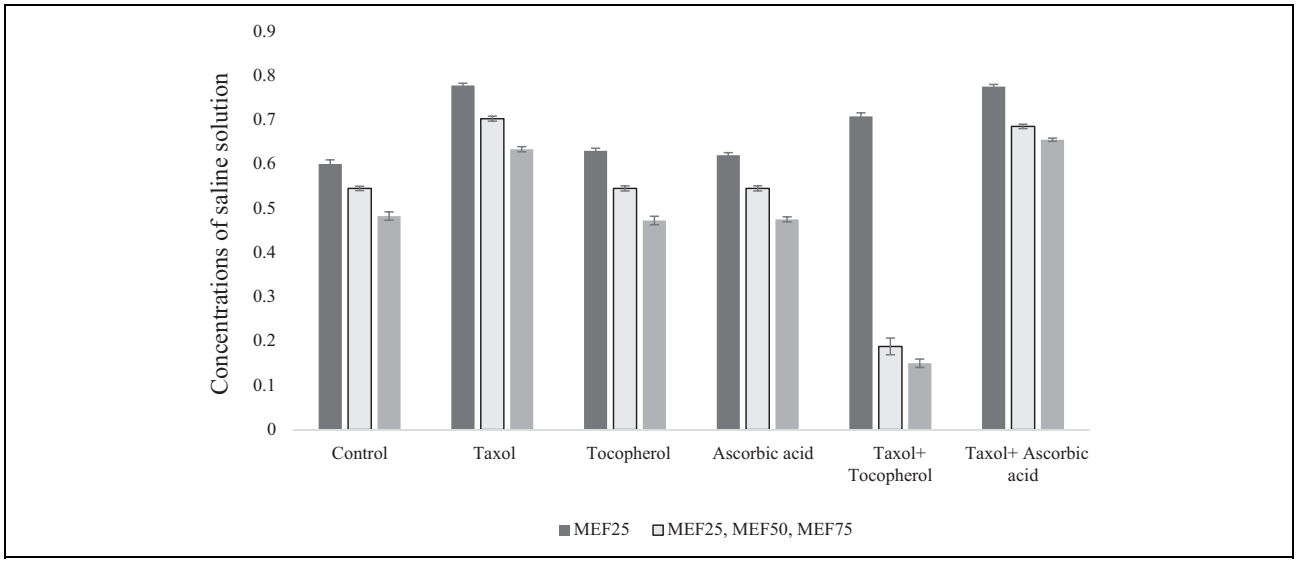

Figure 1. Pre-treatment of tocopherol (1 μl/ml) and ascorbic acid (1 μl/ml) with taxol (1 μl/ml), resulting in mean erythrocyte fragility (MEF). The results indicate that the pre-treatment of red blood cells with tocopherol protects the osmotic damage caused by taxol, especially in case of MEF50 and MEF75.

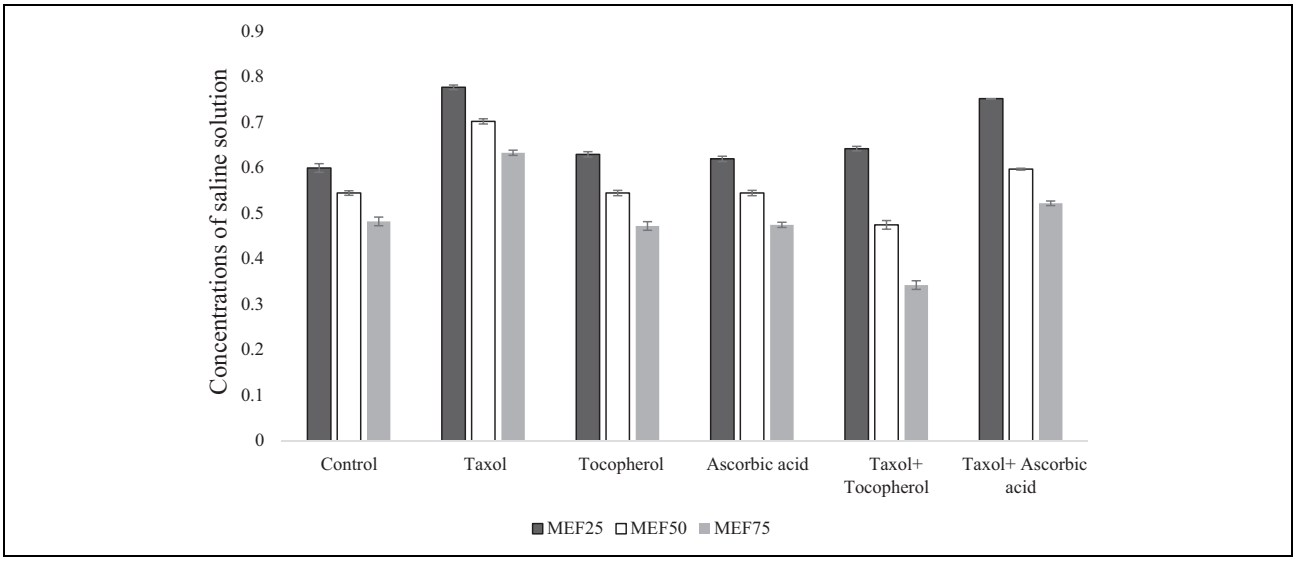

Figure 2. Co-treatment of tocopherol (1 μl/ml) and ascorbic acid (1 μl/ml) with taxol (1 μl/ml), resulting in mean erythrocyte fragility (MEF). The results indicate that the damaging effect of taxol can be slightly reduced by treatment of the red blood cells with tocopherol along with taxol.

acid on erythrocyte lysate was recorded as 394.28 ± 24, 184 ± 13, 399.42 ± 32, 378 ± 23, 250.9 ± 15 and 212.3 ± 12, respectively (Figure 4). The results indicate the toxic effect of taxol on the catalase activity of red blood cells, and this effect was reduced when the red blood cells were treated with tocopherol or ascorbic acid along with the taxol.

Discussion

Drug-induced haematological disorders can be caused by many commonly used drugs, and drug-induced anaemia may be because of oxidative stress, immune haemolysis, megaloblastic change or dyshaemopoiesis with sideroblastosis.[5,21] Chemotherapy has been suggested to cause an oxidative stress to human antioxidative defence systems[22] and is responsible for the acute haemolytic anaemia in patients.[23] The acute haemolytic anaemia caused the reduction in the concentration of haemoglobin below 10 g/L, leading to blood transfusion in the cancer patients.[24] In several reports, it is shown that many chemotherapeutic agents induce haemolysis in human as well as in primates especially the alkaloids.[25–27] Paclitaxel is responsible for the

Table 2. Co-treatment of tocopherol (1 μl/ml) and ascorbic acid (1 μl/ml) with ta (1 μl/ml), resulting in mean erythrocyte fragility (MEF).

		Mean erythrocyte fragility (MEF)		
SNo.	Co-treatment condition	MEF25	MEF50	MEF75
1	Control	0.600 ± 0.0096	0.545 ± 0.005	0.4825 ± 0.0095
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4	Ascorbic acid	0.62 ± 0.0058	0.545 ± 0.00577	0.475 ± 0.0057
5	Taxol + tocopherol	0.6425 ± 0.005	0.475 ± 0.00957	0.3425 ± 0.0095
6	Taxol + ascorbic acid	0.7525 ± 0	0.5975 ± 0.002	0.5225 ± 0.005

1 represents control (without any treatment); 2 represents erythrocytes treated with taxol for 30 min; 3 represents erythrocytes treated with tocopherol for 30 min; 4 represents erythrocytes treated with ascorbic acid for 30 min; 5 is erythrocytes treated with taxol and tocopherol for 30 min; and 6 represents erythrocytes treated with taxol and ascorbic acid for 30 min.

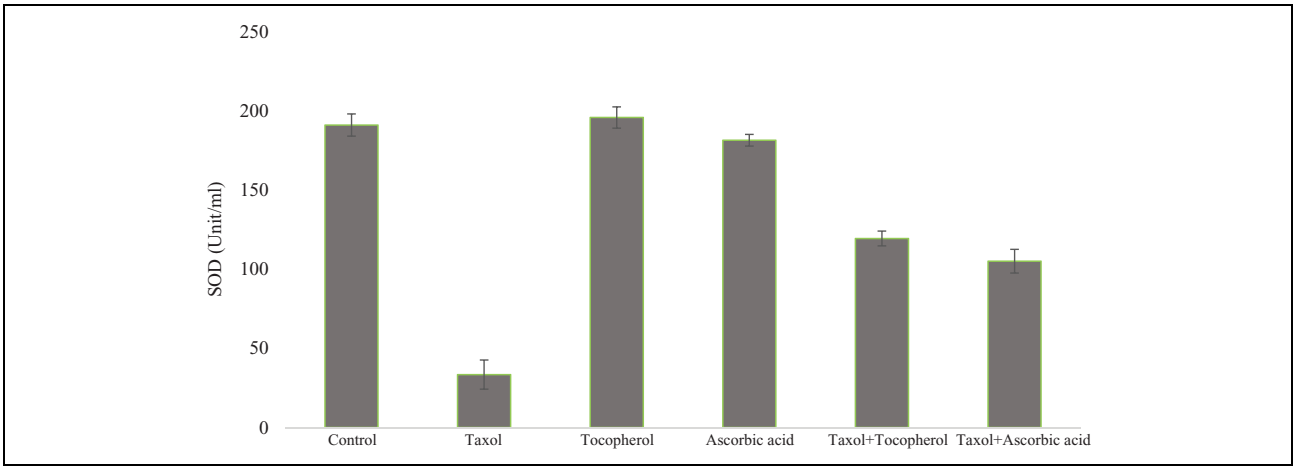

Figure 3. Superoxide dismutase (SOD) activity in control, taxol treated, tocopherol treated, ascorbic acid treated, taxol with tocopherol and taxol with ascorbic acid-treated human erythrocyte. The results clearly indicate that the red blood cells treated with taxol have very less SOD activity in comparison with the control, but tocopherol or ascorbic acid reduced the damaging effect of taxol on the erythrocytes.

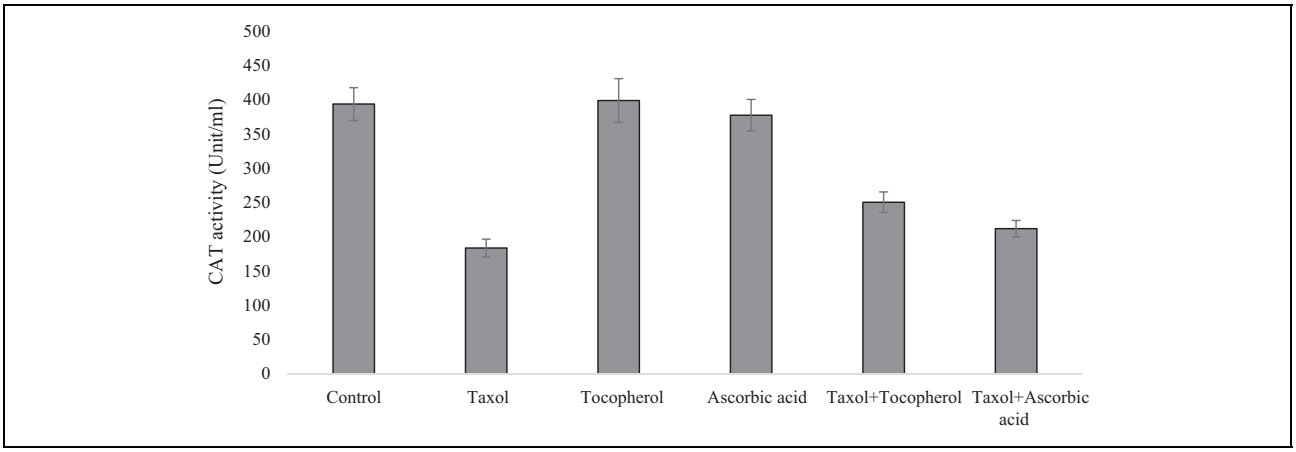

Figure 4. Catalase activity in control, taxol treated, tocopherol treated, ascorbic acid treated, taxol with tocopherol and taxol with ascorbic acid-treated human erythrocyte. The results demonstrate the toxic effect of taxol on catalase activity of red blood cells, but the protective effect of tocopherol and ascorbic acid when added along with taxol on catalase in red blood cells.

systematic oxidative stress and erythrocyte oxidative injury, leading to the development of anaemia in advanced breast cancer patients.[9] Other studies indicated the increased levels of superoxide, H_2O_2 and nitric oxide in the cells treated with the taxol.[13] The antioxidants play very important role in protecting cells from oxidative damage. Tocopherol is a constituent of an erythrocytic membrane, is a potent antioxidant and is known to neutralize the toxic effects of reactive oxygen species generated by many chemicals.[28–30]

Our data demonstrated that taxol induces oxidative stress in the human erythrocytes. Taxol causes the osmotic lysis and reduction in the major antioxidant enzymes SOD and catalase. The same study shows that the haemotoxic effect of taxol can be reduced by treating cells with tocopherol and ascorbic acid.

In osmotic fragility studies, the results indicate that when cells were treated with taxol, the erythrocytic damage due to osmotic stress was high, but when the cells were treated with taxol in the presence of tocopherol and ascorbic acid (pre-treatment and co-treatment), the effect was reduced, especially in the case of pre-treatment. Both the pre-treatment and co-treatment confirmed that the tocopherol has the potential to protect the erythrocytes from osmotic lysis. The results show that the red blood cells when treated with taxol for 30 min before addition of tocopherol and ascorbic acid, there was no effect of tocopherol and ascorbic acid to restore the damage caused by taxol, suggesting that tocopherol and ascorbic acid have protective role rather than treatment effect.

SOD is the first line of defence enzyme against the deleterious effects of oxyradicals.[31,32] Our studies indicate that taxol reduced SOD activity approximately five times compared to control. This reduction in the activity of SOD was ameliorated by tocopherol and ascorbic acids.

Catalase enzyme plays very important role in scavenging hydrogen peroxide in erythrocytes.[33] In our studies, we found that there were no significant changes in the catalase activity when the cells were treated with tocopherol or ascorbic acid. Taxol led to a reduction in catalase activity, and tocopherol and ascorbic acid were not able to change this. These results indicate that the activity of catalase is permanently decreased by taxol.

In conclusion, our data indicate that taxol causes acute haemolysis in erythrocytes. The activity of SOD and catalase is also reduced by taxol treatment, but the damaging effect of taxol can be minimize by adding both antioxidants with taxol, particularly tocopherol, which protected cells from oxidative damage caused by taxol.

Declaration of conflicting interests

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