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## Report

# Vitamin C suppresses the cisplatin toxicity on blood platelets

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**The effects of vitamin C on the oxidative stress in blood platelets induced by cisplatin were studied. In the presence of vitamin C we measured in blood platelets the production of thiobarbituric acid reactive substances (TBARS), the generation of superoxide radicals ($O_2^{\cdot -}$), other reactive oxygen species ($H_2O_2$, singlet oxygen and organic radicals) and catalase activity. Vitamin C at a low concentration (0.1 mM), like cisplatin (20 $\mu$M), induced blood platelet oxidative stress: an increase of TBARS, chemiluminescence and generation of superoxide radicals. After treatment of blood platelets with vitamin C at a high concentration (3 mM), chemiluminescence ($p>0.05$), the levels of $O_2^{\cdot -}$ ($p<0.01$) and TBARS ($p<0.002$) were reduced. We have shown that vitamin C at a high concentration (3 mM) had a protective effect against oxidative stress in platelets caused by cisplatin (20 $\mu$M). It diminished platelet lipid peroxidation and reactive oxygen species generation induced by cisplatin. In the presence of vitamin C, the catalase activity was suppressed. [© 2000 Lippincott Williams & Wilkins.]**

*Key words*: Blood platelet, cisplatin, oxidative stress, vitamin C.

## Introduction

Cisplatin (*cis*-diamminedichloroplatinum II, CDDP) is especially useful in the treatment of epithelial malignancies; however, the use of cisplatin is accompanied by numerous and frequently severe toxicities including hematological toxicity.[1–3] We have shown that cisplatin exerted an inhibition effect on blood platelet activation.[4,5] In particular, cisplatin reduced adenine nucleotides and protein secretion, and inhibited platelet aggregation and eicosanoid synthesis.[4,5] Our earlier results revealed that cisplatin not only induces platelet lipid peroxidation,[6–8] but also has stimulatory action on the production of reactive oxygen species (ROS),[9–11], which may damage platelet membrane receptors and affect signal transduction pathways in blood platelets.

Sugihara *et al.*[12] have previously demonstrated that different antioxidants including $\alpha$-tocopherol and mannitol decreased cisplatin-induced oxidative stress in rat kidney cortex. Our results indicated that selenite (1 $\mu$M) also has a protective effect against cisplatin-induced changes in platelet functions.[11,13,14] The role of vitamin C in chemotherapy has been also known for a long time. Vitamin C diminished the nephrotoxicity effect of cisplatin.[15] In the present study we investigated whether vitamin C had a protective effect on platelet lipid peroxidation, the level of ROS and catalase activity in blood platelets treated with cisplatin.

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## Materials and methods

### Materials

Cisplatin, luminol, L-ascorbic acid and cytochrome *c* were purchased from Sigma (St Louis, MO). All other reagents were of analytical grade and were provided by commercial suppliers.

### Isolation of blood platelets

Pig blood was collected into ACD solution (citric acid/citrate/dextrose; 5:1; v/v) and platelets were isolated by differential centrifugation of blood (20 min at 200 *g*). Platelet-rich plasma was separated and centrifuged for 20 min at 1000 *g* to sediment platelets. The resulting pellet was resuspended in $Ca^{2+}$/$Mg^{2+}$-free Tyrode's buffer (140 mM NaCl, 10 mM glucose and 15 mM Tris–HCl, pH 7.4) and the platelets were

subsequently washed 3 times with the same buffer. The entire washing procedure was performed in plastic tubes and carried out at room temperature. Blood platelets were suspended in $Ca^{2+}$/$Mg^{2+}$-free Tyrode's buffer at a final concentration of $5 \times 10^8$ platelets/ml. The platelet suspensions were incubated (0–60 min at 37°C) with: (i) vitamin C at final concentrations of 0.1, 0.5, 1, 1.5, 2, 2.5 and 3 mM, (ii) cisplatin at a final concentration of 20 $\mu$M, and (iii) vitamin C (3 mM) plus cisplatin (20 $\mu$M) added together or cisplatin added after 10 min preincubation with vitamin C. The platelets were counted by the photometric method as described by Walkowiak *et al.*[16]

### Chemiluminescence measurements

The level of ROS species ($O_2^{\cdot -}$, $H_2O_2$, singlet oxygen and organic radicals) in control blood platelets and platelets incubated with vitamin C or/and cisplatin was recorded using the chemiluminescence method as described by Król *et al.*[17] The chemiluminescence signals were evaluated by means of a Berthold LB950 automatic luminescence analyzer after the addition of 20 $\mu$l of 2 mM luminol solution in buffered saline. Results were expressed as the integral over the total measuring time per 15 min and presented as percent of control values obtained for control platelets.

### $O_2^{\cdot -}$ generation in blood platelets

Generation of superoxide anion ($O_2^{\cdot -}$) in control platelets and in platelets incubated with vitamin C or/and cisplatin was measured by means of superoxide dismutase-inhibitable reduction of cytochrome *c*, as described by Jahn and Hansch.[18] Briefly, an equal volume of $Ca^{2+}$/$Mg^{2+}$-free Tyrode's buffer, containing cytochrome *c* (160 $\mu$M), was added to a 1 ml suspension of platelets. After incubation, the platelets were sedimented by centrifugation at 2000 *g* for 5 min and the supernatants were transferred to cuvettes. Reduction of cytochrome *c* was measured spectrophotometrically at 550 nm. To calculate the molar concentration of $O_2^{\cdot -}$ an extinction coefficient for cytochrome *c* of 18700 $M^{-1}$ $cm^{-1}$ was used.

### TBARS production in blood platelets

Incubation of blood platelets in buffer (control and incubated with vitamin C or/and cisplatin at 37°C) was stopped by cooling the samples in an ice-bath. Samples of platelets were transferred to an equal volume of 20% (v/v) cold trichloroacetic acid in 0.6 M HCl and centrifuged at 1200 *g* for 15 min. One volume of clear supernatant was mixed with 0.2 volumes of 0.12 M thiobarbituric acid in 0.26 M Tris at pH 7.0 and immersed in a boiling water bath for 15 min. Absorbance at 532 nm was measured and results were expressed as nmol of MDA.[19]

### Enzyme assays

Catalase (EC 1.11.1.6) activity was measured spectrophotometrically according to Bartosz[20] using 30% $H_2O_2$ as substrate in a 3 ml reaction mixture containing 50–200 $\mu$g of protein in 0.05 M phosphate buffer (pH 6.8) at 25°C.

### Statistical analysis

The statistical analysis was done by several tests. In order to eliminate uncertain data, both the Q-Dixon and Grubbs tests were performed. All the values in this study were expressed as mean $\pm$ SD. The statistically significant differences between variations were found (Snedecor–Fisher test) so the differences between means were assessed by applying the Cochran–Cox test. Regression lines were calculated by means of the least-squares method.

## Results

The results of the present study demonstrate that the effectiveness of ascorbic acid on platelet responses is strongly dependent on its dose. At a low concentration vitamin C stimulated the luminol-dependent chemiluminescence (Figure 1). With an increase of the concentration this stimulatory effect of vitamin C was suppressed (Figure 1). A dose-dependent decrease of chemiluminescence in platelets is shown in Figure 1 ($p < 0.001$). In the presence of the highest dose of vitamin C (3 mM) about 10% ($p > 0.05$) of chemiluminescence in platelets was registered, whereas in the presence of vitamin C at the concentration of 0.1 mM, chemiluminescence reached about 38% ($p < 0.05$) (Figure 1).

Our studies demonstrated that vitamin C had a different influence on the generation of superoxide anion ($O_2^{\cdot -}$) in platelets measured by the superoxide dismutase-inhibitable reduction of cytochrome *c*. After incubation of platelets with vitamin C at the concentration of 0.5 mM the generation of $O_2^{\cdot -}$ significantly increased in a time-dependent manner ($p < 0.01$) (Figure 2), whereas vitamin C at the highest concentration (3 mM) caused the inhibition of $O_2^{\cdot -}$ generation in a time-dependent manner ($p < 0.01$) (Figure 2).

We noticed that vitamin C had a different action on platelet lipid peroxidation measured by the thiobarbi-

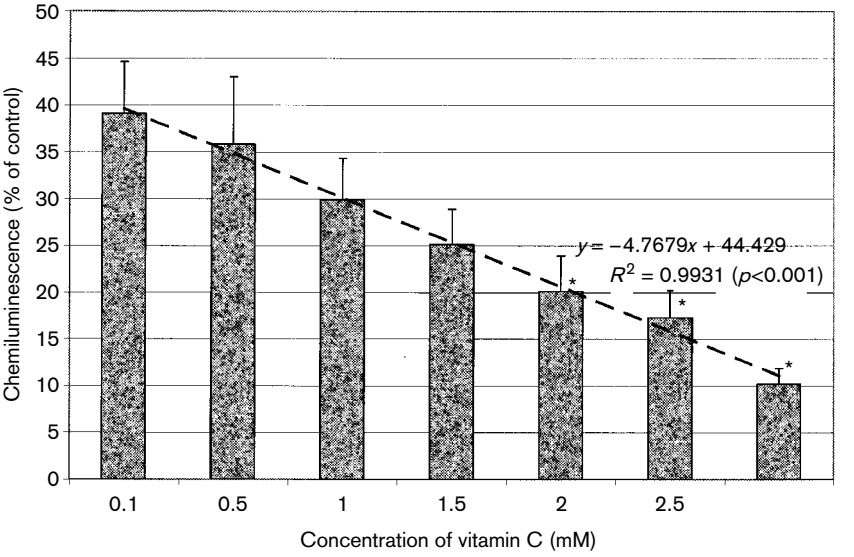


**Figure 1.** Chemiluminescence of blood platelets stimulated (5 min) with vitamin C at different concentrations ($n$=6, $p<0.05$, $^{*}p>0.05$). Regression line was calculated by means of the least-squares method.

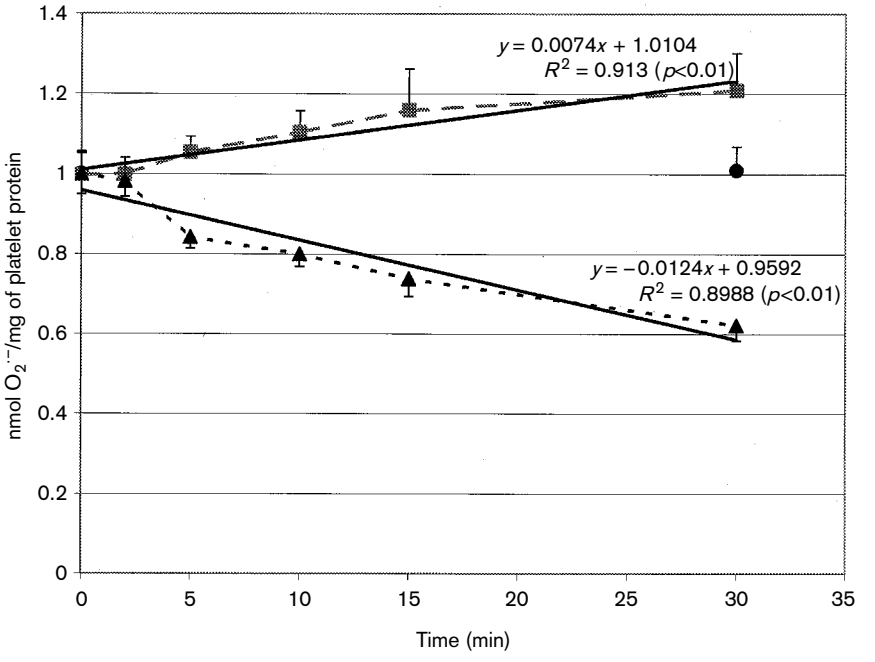


**Figure 2.** The effects of vitamin C [(■) 0.5 and (▲) 3 mM; 0–30 min; 37°C; (◆) control] on the level of $O_2^{\cdot -}$ in blood platelets. Each experimental point is a mean of five independent measurements. Regression lines were calculated by means of the least-squares method.

turic acid technique and expressed as TBARS (Figure 3). We showed that vitamin C at the concentration of 0.5 mM induced lipid peroxidation in pig blood platelets. Contrary, vitamin C at the highest used dose decreased the level of TBARS (Figure 3). These effects were time dependent ($p<0.002$), as shown in Figure 3.

Cisplatin (20 $\mu$M) had a significant stimulatory effect on the chemiluminescence of platelets ($p<0.05$) (Figure 4). After pre-exposure of blood platelets with vitamin C (3 mM, 10 min), the stimulatory effect of cisplatin on this process was markedly diminished (about 60%) ($p<0.02$) (Figure 4). Treatment of cells simultaneously with cisplatin and vitamin C decreased the chemiluminescence (about 40%) ($p<0.05$) (Figure

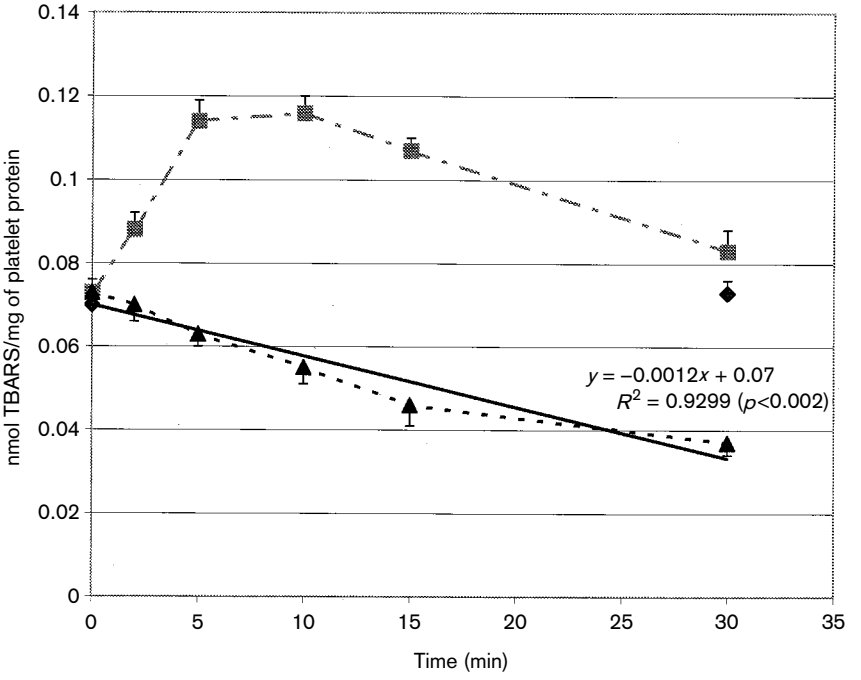


**Figure 3.** The effects of vitamin C [(■) 0.5 and (▲) 3 mM; 0–30 min; 37°C; (◆) control] on the production of TBARS in blood platelets. Each experimental point is a mean of five independent measurements. Regression lines were calculated by means of the least-squares method.

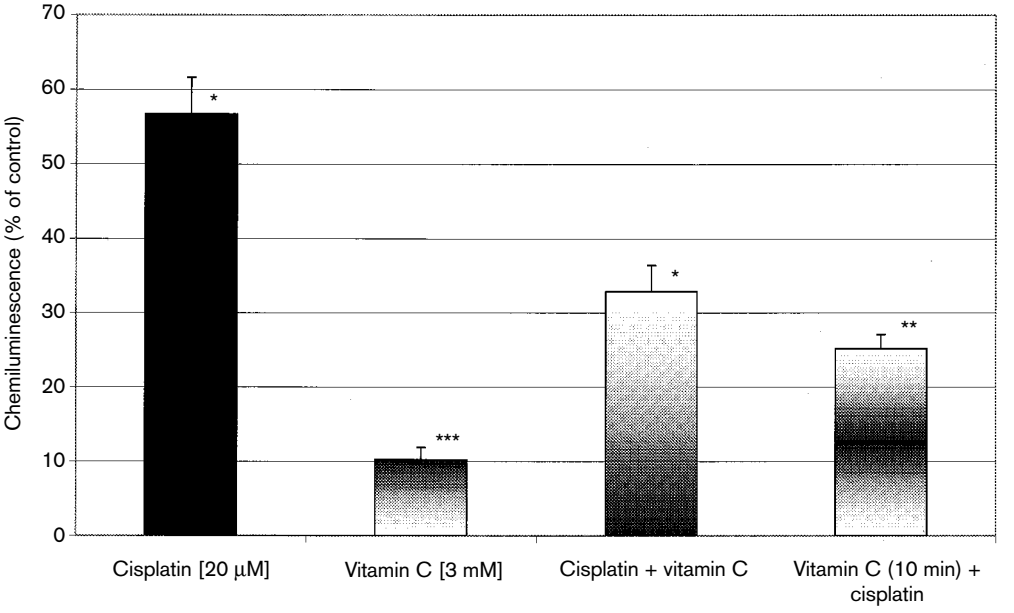


**Figure 4.** The effects of cisplatin alone (20 $\mu$M, 5 min), vitamin C alone (3 mM, 5 min), cisplatin and vitamin C added to blood platelets together (5 min) or cisplatin (5 min) added after preincubation of blood platelets with vitamin C (10 min) on chemiluminescence in pig blood platelets. Results, expressed as percent of control, are means $\pm$ SD of six experiments (*$p < 0.05$; **$p < 0.02$; ***$p > 0.05$).

4). Cisplatin (20 $\mu$M) caused the production of $O_2^{\cdot -}$ in blood platelets ($p < 0.001$) (Figure 5). The level of $O_2^{\cdot -}$ was reduced (about 25%) in blood platelets treated simultaneously with cisplatin (20 $\mu$M) and vitamin C (3 mM) (Figure 5), but after preincubation of platelets with vitamin C (3 mM, 10 min), the generation of $O_2^{\cdot -}$ was decreased by about 35% (Figure 5). The stimulatory effect of cisplatin (20 $\mu$M)

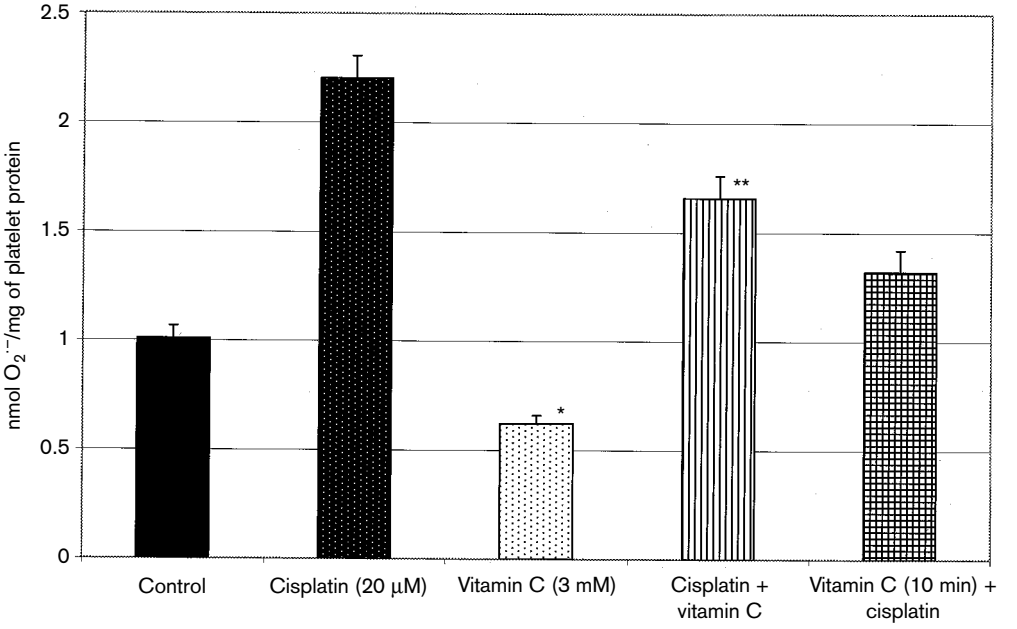


**Figure 5.** The effects of cisplatin alone (20 $\mu$M, 5 min), vitamin C alone (3 mM, 5 min), cisplatin and vitamin C added to blood platelets together (5 min) or cisplatin (5 min) added after preincubation of blood platelets with vitamin C (10 min) on the level of $O_2^{\cdot -}$ in pig blood platelets. Results, expressed as percent of control, are means $\pm$ SD of six experiments ($p<0.05$; *$p<0.02$; **$p<0.001$).

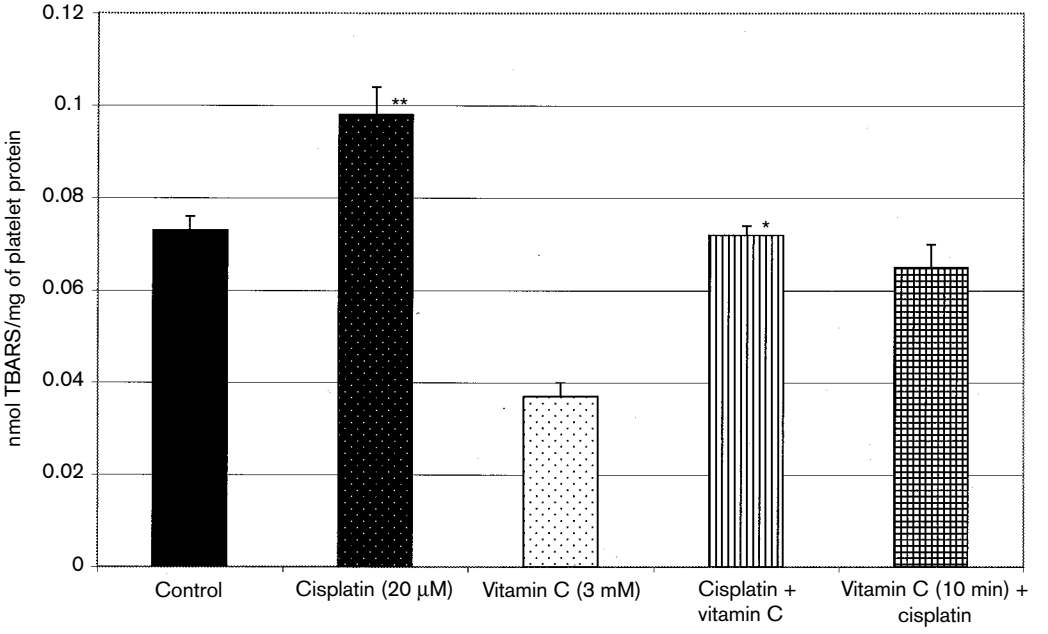


**Figure 6.** The effect of cisplatin alone (20 $\mu$M, 5 min), vitamin C alone (3 mM, 5 min), cisplatin and vitamin C added to blood platelets together (5 min) or cisplatin (5 min) added after preincubation of blood platelets with vitamin C (10 min) on the level of TBARS in pig blood platelets. Results, expressed as percent of control, are means $\pm$ SD of six experiments ($^{*}p<0.05$; *$p<0.01$; **$p<0.001$).

on platelet lipid peroxidation was also reduced by vitamin C (Figure 6).

Treatment of blood platelets with cisplatin (for 5–60 min) resulted in the decreased activity of catalase in a dose- and time-dependent manner (Tables 1 and 2). We noticed that vitamin C alone did not change the catalase activity (data are not presented); however, after pre-exposure of platelets to vitamin C (3 mM,

10 min) the inhibitory effect of cisplatin (20 $\mu$M, 30 min) on the activity of catalase was decreased to about 20% (Table 3).

**Table 1**. The dose-dependent inhibitory effects of cisplatin (30 min, 37°C) on the catalase activity in pig blood platelets

| Concentration of cisplatin ($\mu$M) | Activity of catalase (U/min/mg) |
|---|---|
| 0 | 78±15 |
| 0.1 | 72±11 |
| 1 | 66±10 |
| 10 | 51±11 |
| 20 | 29±9 |

Results, expressed as percent of control, are means±SD of six experiments ($p<0.05$). Regression line was calculated by means of the least-squares method. The $R$ value was −0.995 ($p<0.001$); $y=-2.05x+70.4$.

**Table 2**. The time-dependent effects of cisplatin (20 $\mu$M, 37°C) on the catalase activity in pig blood platelets

| Time of incubation (min) | Activity of catalase (U/min/mg) |
|---|---|
| 0 | 78±15 |
| 5 | 67±12 |
| 15 | 39±10 |
| 30 | 29±9 |
| 60 | 28±5 |

Results, expressed as percent of control, are means±SD of six experiments ($p<0.05$).

**Table 3**. The effect of vitamin C (3 mM, 10 min, 37°C) and cisplatin (20 $\mu$M, 30 min, 37°C) on the catalase activity in pig blood platelets

| | Activity of catalase (U/min/mg) | Percent of inhibition |
|---|---|---|
| Control | 78±15 | – |
| Cisplatin | 29±9 | 63±6 |
| Vitamin C* | 76±12 | 3±0.5 |
| Cisplatin + vitamin C | 62±9 | 20±2.5 |

Results, expressed as percent of control, are means±SD of five experiments ($p<0.05$, *$p>0.05$).

## Discussion

The present results indicate that vitamin C is capable of lowering the toxicity of cisplatin. Cisplatin is a widely used antineoplastic agent; however, its toxicity may become an important dose-limiting factor. Blood platelets may play a valuable role in the pathomechanism of hemostasis in cancer. It is known that platelets from patients with cancer exhibit different qualitative abnormalities.[21,22] During chemotherapy, drugs may change biological functions of blood platelets.[4,5] Although the exact biochemical mechanisms responsible for the hematological toxicity of cisplatin have not yet been defined, the involvement of oxidative stress is indicated by several lines of evidence. After incubation of platelets with cisplatin, platelet lipid peroxidation and generation of ROS in blood platelets were demonstrated.[6–8,11] Cisplatin depleted glutathione (GSH) and protein thiols in platelets,[10,23] and inhibited the activity of antioxidative enzymes such as superoxide dismutase and GSH peroxidase.[7] Our present results showed that cisplatin also inhibited the platelet catalase activity (Tables 1 and 2).

Radical scavengers and antioxidants may be used to protect against cisplatin-induced cytotoxicity. It has been shown that vitamin C has protective effects against cisplatin.[24–27] Ascorbic acid is a significant antioxidant of human plasma. This ability is given by a relatively rapid reaction with many ROS, especially with peroxyl radicals. Ascorbic acid forms a low reactive semidehydroascorbate radical with radicals. It can be regenerated *in vivo* by the enzyme system by dismutase reaction through dehydroascorbate back into ascorbate.[28] Ascorbic acid is a significant antioxidant in the absence of transition metal ions, while in their presence its pro-oxidative properties are preferred. *In vivo* recommended high doses of vitamin C need not be harmful for a healthy human, whereas they need not be useful in some diseases.[28] Our results suggest that vitamin C at high doses may play an important role in the protecting of blood platelets against cisplatin-induced oxidative stress (Table 4). The results presented in this study demonstrate that vitamin C has beneficial effect on the level of ROS (measured by chemiluminescence and reduction of

**Table 4**. Inhibition of cisplatin (20 $\mu$M)-induced oxidative stress in blood platelets caused by 3 mM vitamin C (5 min)

| | Inhibition (%) | | |
|---|---|---|---|
| | Chemiluminescence | $O_2^{\cdot -}$ | TBARS |
| Cisplatin + vitamin C (added to blood platelets together) | 41.9±3.9 | 24.7±3.5 | 26.5±4.7 |
| Vitamin C (10 min) + Cisplatin | 55.5±4.8 | 40.0±5.1 | 33.7±3.9 |

The numbers represent oxidative stress as percentage of that recorded for blood platelets stimulated by cisplatin and are mean values±SD.

cytochrome *c*) and lipid peroxidation (expressed as TBARS) in blood platelets (Figures 1–3). A decrease of chemiluminescence correlates with an increase of vitamin C concentration (Figure 1).

In platelets treated with vitamin C (3 mM) the oxidative stress induced by cisplatin was distinctly diminished (Figures 4–6 and Table 4). Vitamin C completely inhibited production of free radicals induced by cisplatin in a cell-free system[29] and in renal tissue.[15] Moreover, in the mammalian system vitamin C modulated the mutagenic effects produced by cisplatin.[30] It seems that vitamin C at the high doses as antioxidant may decrease or eliminate negative consequences of toxic side effects of cisplatin on blood platelets (Table 4) and their hemostatic function. The beneficial aspects and pharmacological significance of a combined action of these two compounds should be taken into account.

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