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THE EFFECT OF CIS-DIAMMINEDICHLOROPLATINUM, SELENITE AND A CONJUGATE OF CISPLATIN WITH SELENITE [$(NH_3)_2Pt(SeO_3)$] ON OXIDATIVE STRESS IN BLOOD PLATELETS MEASURED BY CHEMILUMINESCENCE METHOD

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The effects of cisplatin, sodium selenite and a conjugate of these compounds [$(NH_3)_2Pt(SeO_3)$] on the generation of free radicals in blood platelets measured by chemiluminescence method were investigated *in vitro*. In platelets incubated with cisplatin (20 μM, 5 min) a dose-dependent increase of chemiluminescence was observed ($p < 0.05$). Contrary to the stimulatory action of cisplatin (20 μM) on the production of free radicals, selenite (1 μM) and its conjugate with cisplatin (20 μM) had only slight effects on the oxidative stress in platelets ($p < 0.05$). The observed increase of chemiluminescence after cisplatin action correlated with a decrease of platelet free thiols present in reduced glutathione. Pre-treatment of blood platelets with buthionine sulfoximine (50 μM, 1 h, 37°C) leading to a decrease of glutathione reduced the cisplatin — induced generation of free radicals in these cells ($p < 0.05$).

Key words: *blood platelets, cisplatin, selenite, free radicals, chemiluminescence*

INTRODUCTION

Blood platelets, anucleated elements formed from megakaryocytes, are important components of blood with a significant role in haemostasis. They offer an attractive model system for studying some aspects of the cytotoxicity of cisplatin (cis-diamminedichloroplatinum II, CDDP) independent of the action on DNA. Cisplatin belongs to the most effective antineoplastic compounds among all platinum drugs. The antitumor activity of cisplatin is attributed mainly to its ability to form adducts with DNA (1, 2). On the other hand, a variety of adverse effects can accompany the use of this drug. Side effects such

as nephrotoxicity, bone marrow toxicity, ototoxicity or haematological toxicity are significant in preventing the use of high doses of cisplatin (3). Cisplatin causes haematological toxicity inducing oxidative stress and changing the function of blood cells; it has an inhibitory effect on platelet activation (4—6). The detailed molecular mechanism by which cisplatin can damage blood cells is not completely understood, though it has been established that cisplatin induces cell membrane lipid peroxidation (7—9), causes a decrease in -SH groups and forms a complex with glutathione (1, 6, 10—12). Our earlier results revealed an important role of thiol groups in the mechanism of cisplatin action. Depletion of GSH by L-buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylocysteine synthetase reduces the oxidative stress (13).

Satoh *et al.* (14) have previously demonstrated, that selenite coadministration enables the use of higher doses of CDDP due to reduced toxicity without affecting the antitumor activity of the drug. A decrease in selenium (Se) intake *via* the diet has been reported to enhance the toxicity of cisplatin and it is possible that the difference in the sensitivity of patients to CDDP observed in clinical cases may be partly due to a discrepancy in dietary selenium intake. The enhancement of Se level in the cells may play a role in reducing the toxicity of cisplatin. Our preliminary results indicated that selenite at a non-toxic concentration (1 μM) has a protective effect against CDDP-induced inhibition of platelet activation (5, 15).

The aim of the investigations was to evaluate the cytotoxic properties of cisplatin, selenium and novel Se-Pt conjugate $[(NH_3)_2Pt(SeO_3)]$ on platelets by measuring the level of free thiols in blood platelets and free radicals generation using the chemiluminescence method. In this paper we also compare the obtained results with the known action of cisplatin and selenite at non-toxic concentrations on blood platelets.

MATERIAL AND METHODS

Cisplatin, sodium selenite, 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB), L-buthionine sulfoximine and luminol were obtained from Sigma Chemical Co (USA). Conjugate of selenium with cisplatin $[(NH_3)_2Pt(SeO_3)]$ synthesised in the Institute of Pur Chemicals, Lachema, Brno (batch no 290592) was a gift obtained from Prof. V. Kleinwachter (Institute of Biophysics, Czech Academy of Sciences, Brno). All other chemicals were of A. R. grade from POCh (Gliwice, Poland).

Isolation of blood platelets

Pig blood was collected into ACD solution (citric acid/citrate/dextrose) 5 : 1 v/v. Platelets were isolated by differential centrifugation of blood (20 min, at 200 × g). The platelet-rich plasma was

then centrifuged for 20 min at 1000 × g to sediment platelets. The resulting pellet was resuspended in the modified Ca^{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 three times with the same buffer. The platelet suspensions (1—10 mg proteins/ml) were incubated (5 min—60 min, at 37°C) with:

— cisplatin at the final concentrations of 0.01; 0.1; 1; 10 and 20 μM
— Se-Pt at final concentration of 20 μM
— selenite (1 μM) plus cisplatin (20 μM)

In some experiments blood platelets were preincubated with BSO (50 μM, 1 h, 37°C) and then treated with cisplatin at the concentration of 20 μM (5 min, 37°C).

Total platelet protein and number of platelets used for chemiluminescence (5×10^8 platelets/ml) was determined by modified Lowry method (16).

Chemiluminescence measurements

The level of free radicals in control blood platelets and platelets incubated with cisplatin, Se-Pt, sodium selenite or selenite plus CDDP was recorded using the chemiluminescence method. Generation of free radicals in platelets preincubated with L-buthionine sulfoximine (50 μM, 1 h, 37°C) and then treated with cisplatin was measured by the same method. The chemiluminescence signals were evaluated by means of a Berthold LB950 automatic luminescence analyser after the addition of 20 μl of 2 mM luminol solution in buffered saline. Results were expressed as the integral over the total measuring time (15 min) and presented as % of control values obtained for control platelets.

Isolation of acid soluble- and acid precipitable fractions

To frozen control, CDDP or Se-Pt-treated platelets (1 ml of platelet suspension) 1 ml of precipitating solution (85% H_3PO_4-0.5 ml; 10% EDTA-1 ml; NaCl-15g) to precipitate proteins were added. Acid-soluble (glutathione) and acid-insoluble (proteins) platelet fractions were separated according to Ando and Steiner (17) and the amount of -SH groups were estimated with DTNB.

Determination of the free-SH groups

A. In acid-soluble fraction:

To 0.5 ml of acid soluble fraction 3.2 ml of 0.32 M Na_2HPO_4 and 0.25 ml of 4 mM DTNB in 1% sodium citrate were added. After 15 min. incubation at room temperature, the absorbance at 412 nm was measured.

B. In acid-precipitable fraction:

To the pellet 5 ml H_2O and 3 ml 10% SDS were added. After solubilization 0.5 ml samples were taken and free -SH groups were determined as described above. Standard -SH curve was preparated for glutathione (GSH) at the concentration of 20—100 nmol.

Statistical analysis was done using the Student's test for paired data.

RESULTS

The cytotoxic effects of cisplatin, selenite and a novel compound — conjugate $[(NH_3)_2Pt(SeO_3)]$ on the level of free radicals in pig blood platelets measured by chemiluminescence method were studied. The tested compounds showed different influence on the generation of free radicals. Incubation of blood platelets with cisplatin (5 min) stimulated the increase of the luminol — dependent chemiluminescence in platelets (*Fig. 1*). The action of CDDP on platelet chemiluminescence was dose — dependent ($p < 0.05$) (*Fig. 1*).

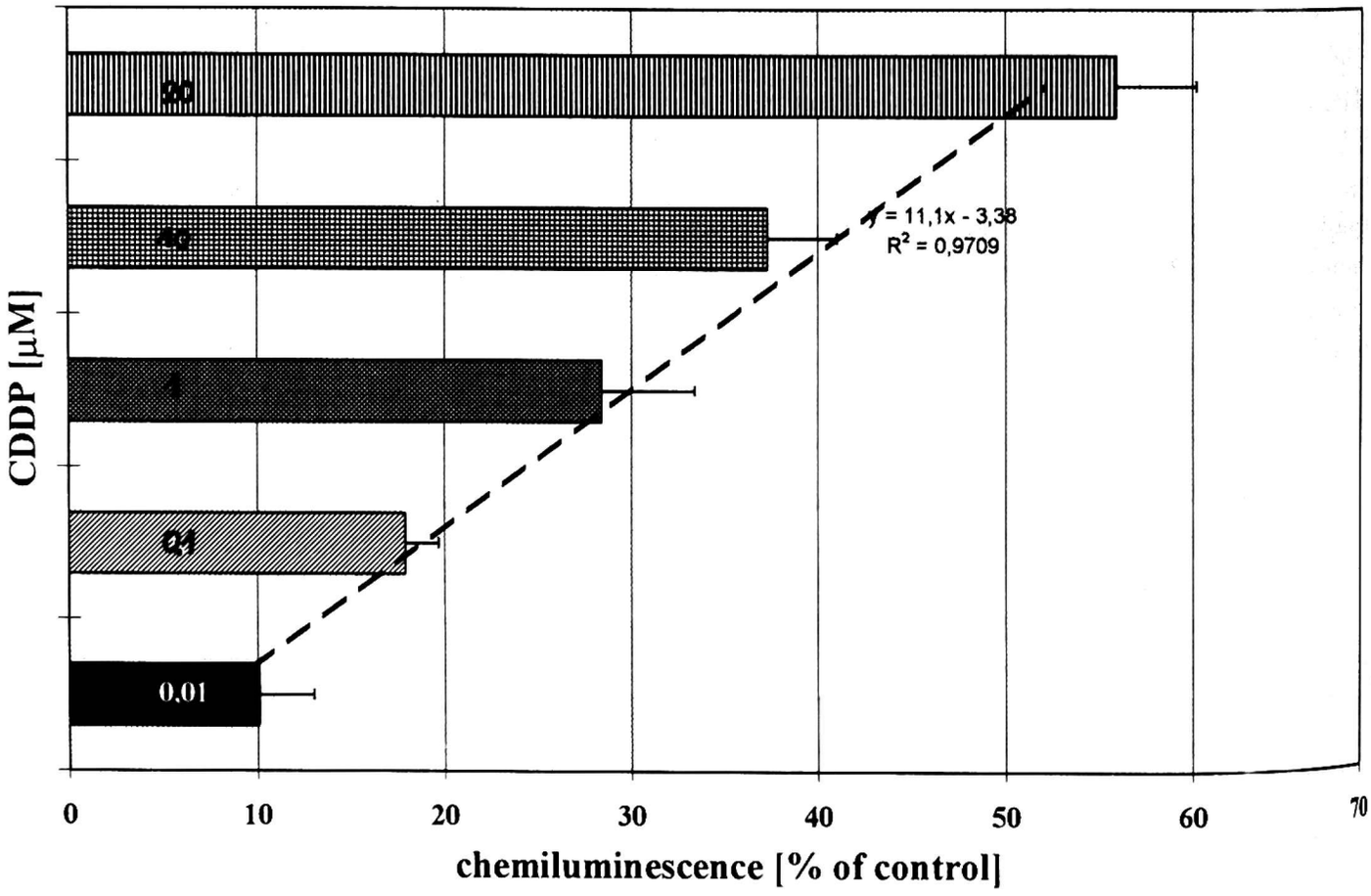

Fig. 1. Chemiluminescence of blood platelets stimulated with cisplatin at different concentrations (0.01; 0.1; 1; 10 and 20 μM; 5 min) ($n = 7$; $p < 0.05$). Regression line was calculated by means of least squares method. The R value was 0.985.

Contrary to the stimulatory action of cisplatin, sodium selenite at the used concentration (1 μM) and a novel conjugate Se-Pt (20 μM) had unsignificant effects on the chemiluminescence of platelets ($p > 0.05$) (*Fig. 2*). After preexposure of platelets to selenite (1 μM, 10 min), the stimulatory effect of CDDP on this process fell markedly ($p < 0.05$) (*Fig. 3*). Treatment of blood

platelets chemiluminescence — induced by CDDP alone ($p < 0.05$) (*Fig. 3*). The presence of selenite in the incubation medium distinctly reduced the effect of cisplatin on free radicals generation in blood platelets (about 90%) (*Fig. 3*).

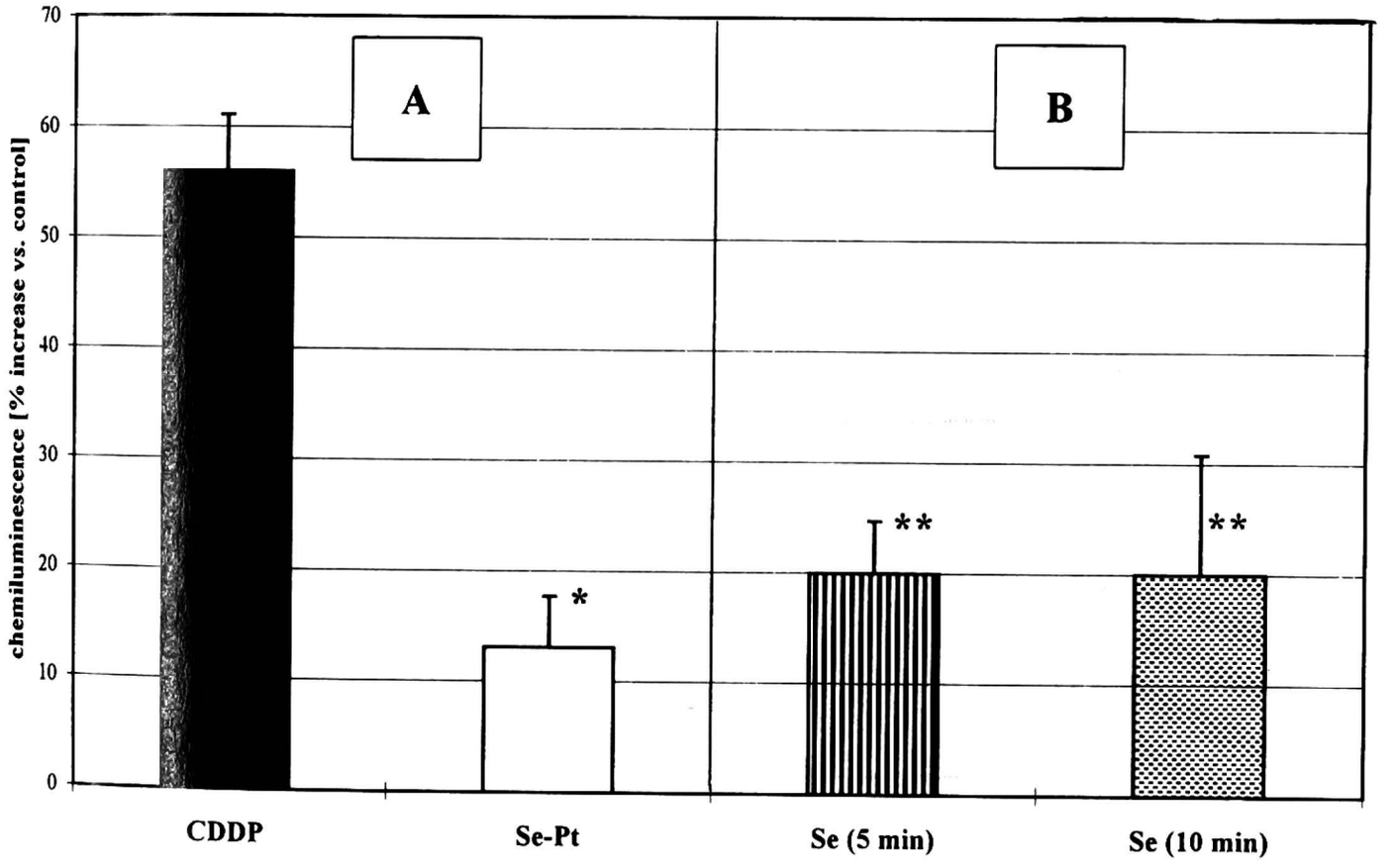

Fig. 2. The effects of (A) cisplatin alone (20 μM; 5 min), its conjugate with selenite (Se-Pt, 20 μM; 5 min) and (B) selenite (1 μM; 5 and 10 min) on chemiluminescence in pig blood platelets. Results, expressed as percent of control, are means ± SD of five experiments, * $p < 0.05$ Se-Pt — treated platelets versus CDDP — treated platelets, ** $p > 0.05$ Se — treated platelets versus control platelets.

Contrary to the stimulatory action of cisplatin, sodium selenite at the used concentration (1 μM) and a novel conjugate Se-Pt (20 μM) had unsignificant effects on the chemiluminescence of platelets ($p > 0.05$) (*Fig. 2*). After preex-posure of platelets to selenite (1 μM, 10 min), the stimulatory effect of CDDP on this process fell markedly ($p < 0.05$) (*Fig. 3*). Treatment of blood platelets with cisplatin and simultaneously with sodium selenite decreased the chemiluminescence — induced by CDDP alone ($p < 0.05$) (*Fig. 3*). The presence of selenite in the incubation medium distinctly reduced the effect of cisplatin on free radicals generation in blood platelets (about 90%) (*Fig. 3*).

Our studies demonstrated that in platelets pre-treated with BSO (50 μM) and incubated with cisplatin (20 μM, 5 min) the decrease of GSH level caused by L-buthionine sulfoximine was accompanied by reduced chemiluminescence (*Fig. 4*).

After incubation of blood platelets with CDDP the amount of GSH and free thiols in proteins was significantly decreased (*Fig. 5* and *6*). After 1 h of incubation of platelets with CDDP, glutathione was decreased to about 2.6 ± 0.3 nmol/mg of platelet protein (by 36.6%) whereas the level of GSH was only slightly decreased (to about 3.6 ± 0.24 nmol/mg of platelet protein) in blood platelets treated by Se-Pt (*Fig. 5*). In the case of blood platelets incubated

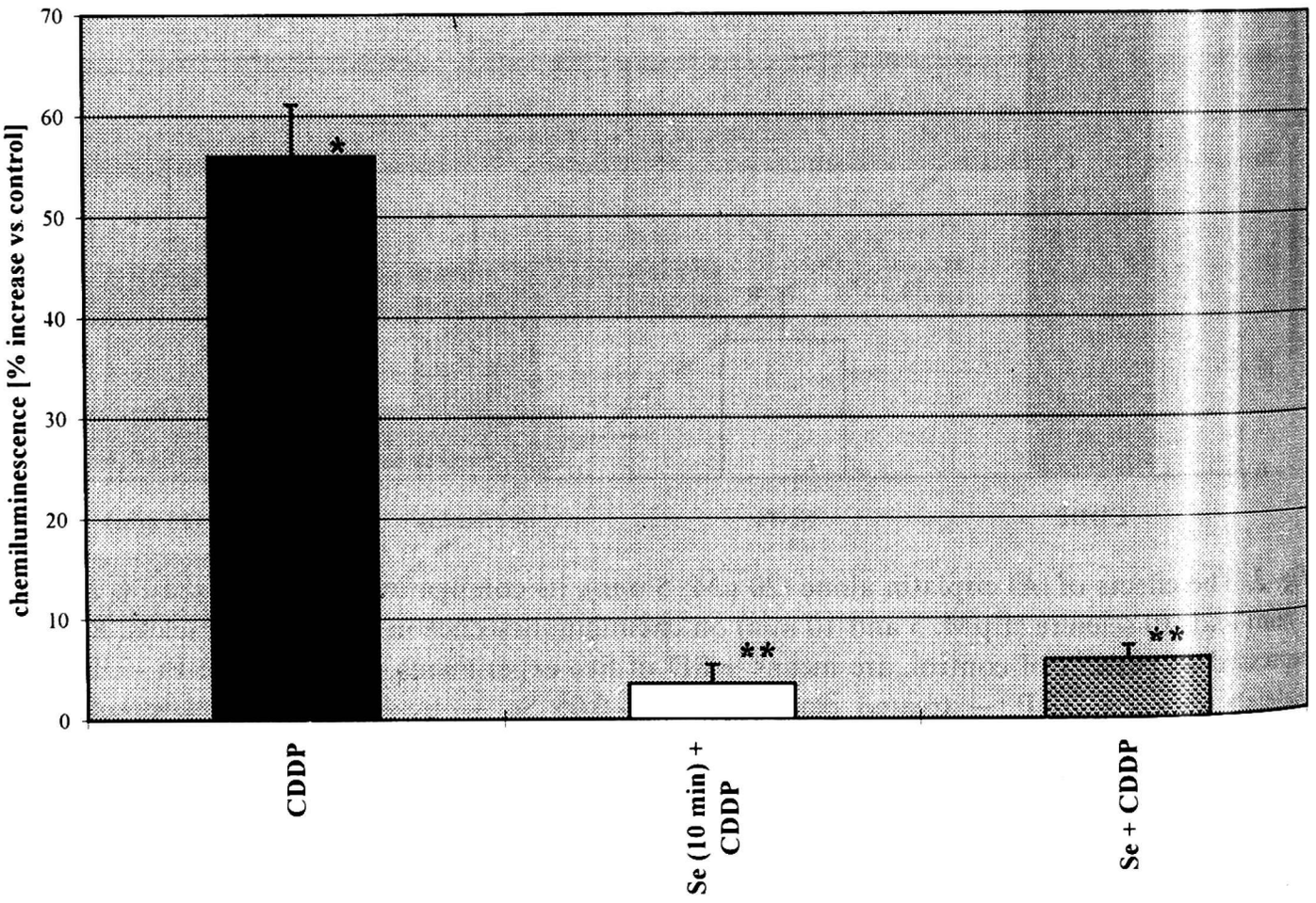

Fig. 3. The effect of cisplatin alone (20 μM; 5 min), cisplatin (20 μM; 5 min) after preincubation of platelets with selenite (1 μM; 10 min) or cisplatin (20 μM) and selenite (1 μM) added to blood platelets together (5 min) on chemiluminescence in pig blood platelets. Results, expressed as percent of control, are means ± SD of five experiments, * $p < 0.05$ CDDP — treated platelets versus control platelets, ** $p < 0.05$ selenite and CDDP — treated platelets versus CDDP — treated platelets.

with CDDP the amount of -SH groups decreased by about 35% (*Fig. 6*). The novel Se-Pt compound at the concentration of 20 μM depleted the level of free thiols in platelet proteins only by about 15% (*Fig. 6*).

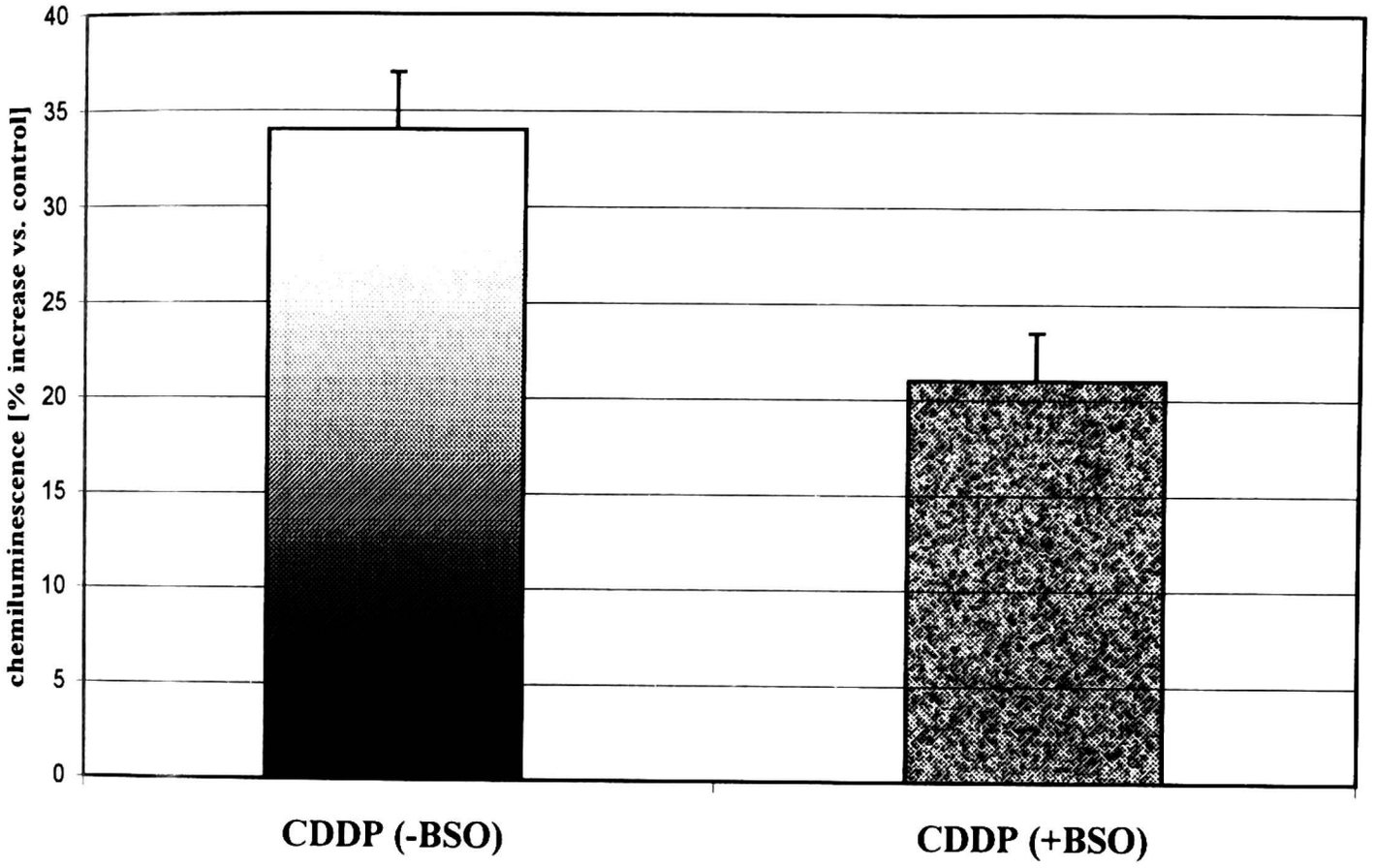

Fig. 4. The effect of BSO pre-treatment (50 μM, 1 h, 37°C) on chemiluminescence of blood platelets induced by CDDP (20 μM, 5 min, 37°C) (n = 5, $p < 0.05$).

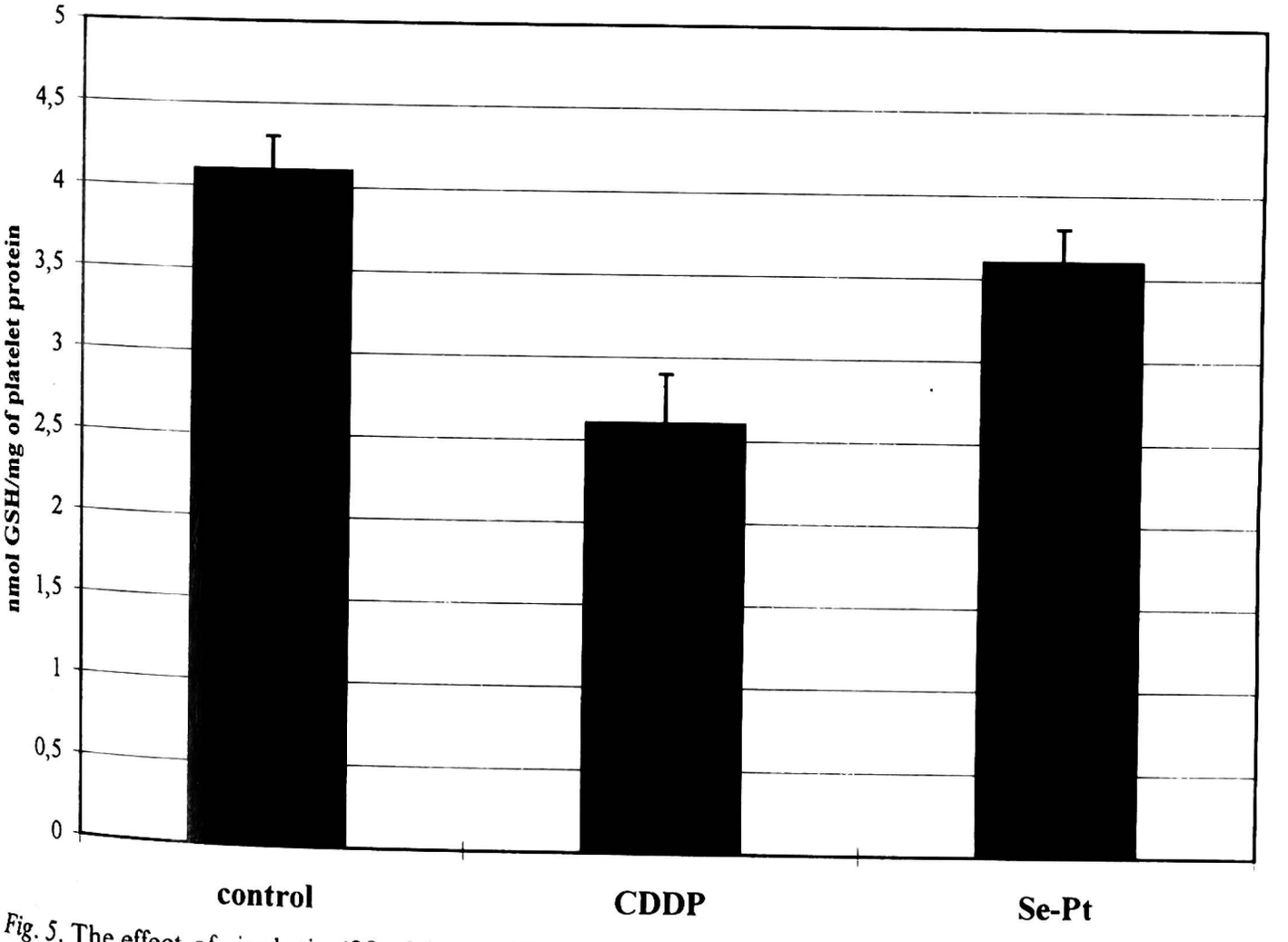

Fig. 5. The effect of cisplatin (20 μM, 1 h, 37°C) and conjugate of selenite with cisplatin (20 μM, 1 h, 37°C) on the level of GSH in blood platelets (n = 8, $p < 0.01$).

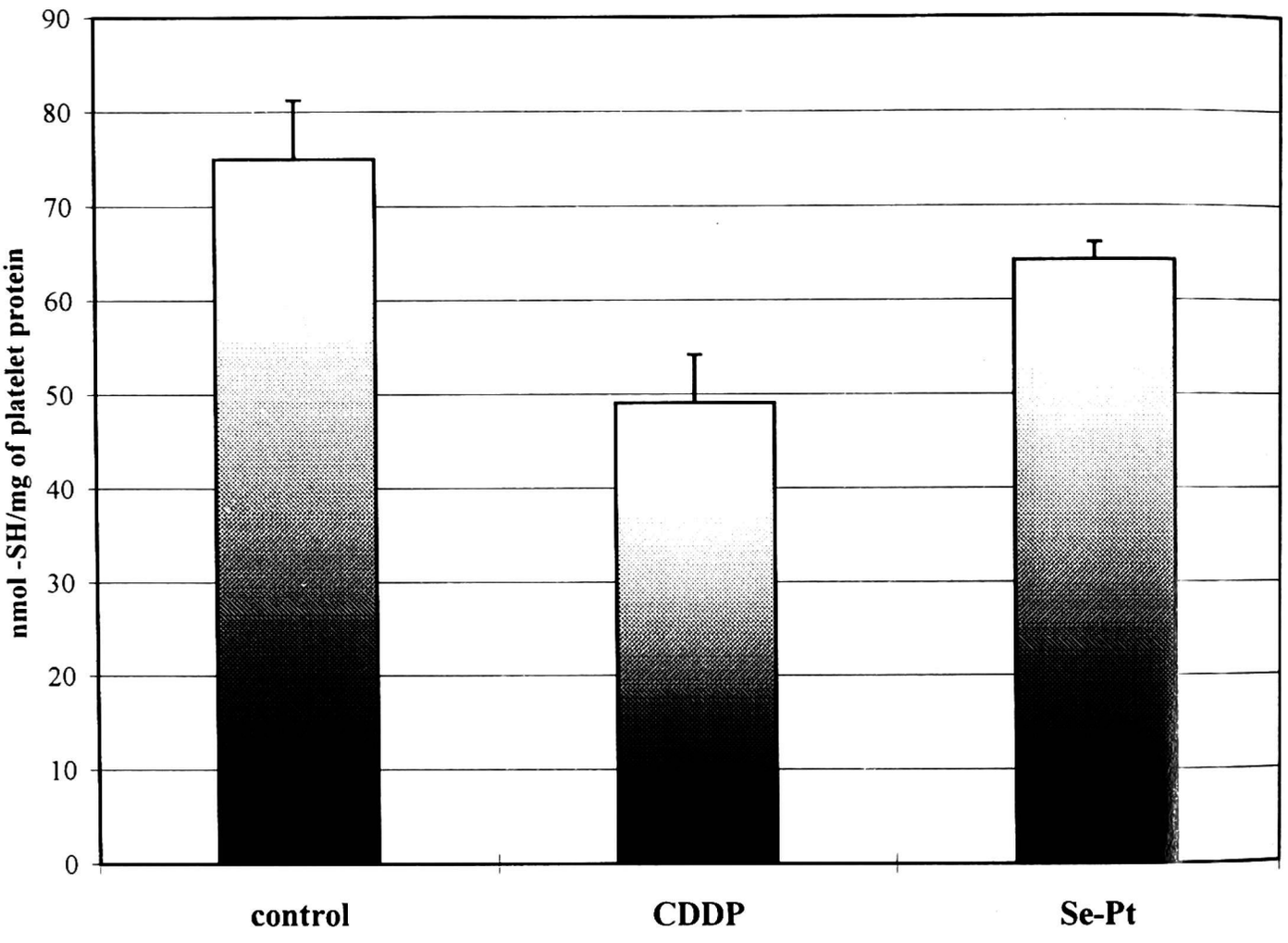

Fig. 6. The effect of cisplatin (20 μM, 1 h, 37°C) and conjugate of selenite with cisplatin (20 μM, 1 h, 37°C) on the level of thiols in proteins platelet fraction ($n = 8$, $p < 0.01$).

DISCUSSION

Several attempts have been made to reduce the side effects — induced by cisplatin. Selenium compounds seem to be significant in preventing the cytotoxic effects of cisplatin. Sodium selenite can reduce these effects without inhibiting the antitumor activity of the drug. The mechanisms of selenite action on the cell and the interaction with CDDP are complex. The exact mechanism underlying the protective action of Se against cisplatin-induced cellular toxicity remains unclear. It is possible that selenium (given as selenite) and cisplatin or its metabolites may interact with each other and that the toxicity of these compounds may be mutually reduced. A novel conjugate of selenite with diammineplatinum [$(NH_3)_2Pt(SeO_3)$] differs from the commercially available platinum complexes. There is a lack of pharmacological information concerning the cytotoxicty of this conjugate Se-Pt. Our earlier results indicate that this compound has no effect on blood platelet activation (5). Selenite does not react directly with cisplatin, but methylselenol (CH_3SeH), a nucleophilic metabolite of selenite is able to form a complex with CDDP *in vitro*. The chemoprotective

activities of Se compounds seem to be mainly attributed to the formation of selenols (18).

We suggest an important role of -SH groups in the generation of free radicals induced by cisplatin and selenite in blood platelets (12). Our preliminary results indicate that cisplatin, like selenite, quickly reacts with thiol groups present in platelets. The results presented in this study demonstrate that in blood platelets cisplatin induces free radicals generation (measured by chemiluminescence method) in a dose — dependent manner concomitant with a decrease of free thiols (*Fig. 1* and *4*). The increase of chemiluminescence correlates with a decrease of free thiols (particularly GSH). This indicates that free thiols present in blood platelets are involved in the generation of free radicals induced by CDDP. From our previous studies it appeared that pre-treatment of platelets with selenium protected these cells against cisplatin — induced lipid peroxidation and $O_2^{\cdot}$ generation (12, 19). The chemiluminescence method used in the present study shows that in blood platelets treated with sodium selenite the level of free radicals — induced by CDDP is diminished (*Fig. 3*). Contrary to CDDP action on platelets, the tested Se-Pt conjugate has an unsignificant effect on free radicals formation in platelets (*Fig. 2*).

In blood platelets incubated with cisplatin, a complex of cisplatin with reduced GSH is formed. We noticed that the reaction of CDDP with GSH *in vitro* resulted in the formation of $O_2^{\cdot}$ (6, 12). This GS-Pt complex seems to be responsible for oxidative stress. Depletion of GSH by L-buthionine sulfoximine leads to decreased amounts of GS-Pt complex and reduces the lipid peroxidation and the formation of $O_2^{\cdot}$ in blood platelets (13). Combining selenium with cisplatin in the form of a novel conjugate could minimise the toxicity and side effects of the chemotherapeutic agent with out effecting antitumor efficacy.

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