ALTERATION OF OXIDATIVE STRESS PARAMETERS IN RED BLOOD CELLS OF RATS AFTER CHRONIC *IN VIVO* TREATMENT WITH CISPLATIN AND SELENIUM

SNEŽANA D. MARKOVIĆ*, JOVANA B. ŽIŽIĆ, DRAGANA S. DJAČIĆ, ANA D. OBRADOVIĆ,
MILENA G. ĆURČIĆ, DANIJELA M. CVETKOVIĆ, NATAŠA Z. ĐORĐEVIĆ,
BRANKA I. OGNJANOVIĆ and A. Š. ŠTAJN

Department for Biology and Ecology, Faculty of Science, University of Kragujevac, 34000 Kragujevac, Serbia

Abstract - In this study we evaluated the possible protective effects of selenium (Se) on hematological and oxidative stress parameters in rats chronically treated with cisplatin (cisPt). Four groups of Wistar albino rats were examined: a control, untreated rats (I), rats treated with Se (II), rats treated with cisPt (III), and rats treated with Se and cisPt (IV). All animals were treated for 5 days successively and killed 24 h after the last treatment. Hematological and oxidative stress parameters were followed in whole blood and red blood cells (RBC). Results showed that the chronic application of Se was followed by a higher number of reticulocytes and platelets, increased lipid peroxidation and GSH content in the RBC. Cisplatin treatment induced depletion of RBC and platelet numbers and an elevation of the superoxide anion, nitrites and glutathione levels. Se and cisPt co-treatment was followed by an elevation of the hematological parameters and the recovery of the glutathione status when compared to the control and cisPt-treated rats.

Key words: Antioxidative defense system, cisplatin, hematology, oxidative stress, selenium.

INTRODUCTION

Cisplatin (*cis*-diamminedichloroplatinum II, cisPt) is one of the most potent antitumor agents. Its activity has been demonstrated against a variety of tumors, notably in head and neck, testicular, ovarian, bladder and small-cell lung cancers (Rosenberg, 1985). The clinical success of cisPt for the treatment of cancer is clear, however, it causes severe side effects (nephrotoxic and hepatotoxic) while intrinsic or acquired resistance limits its application in high doses (Yoshida et al., 2000). The therapeutic effects of cisplatin are based on the interaction with DNA in the cell which prevents proliferation (Perez, 1998), as well as on inducestion of apoptosis in tumor cells (Friesen et al., 1999). On the other hand, cisPt is highly mutagenic, inducing chromosome aberrations in pe-

ripheral blood lymphocytes in patients and in rat bone-marrow cells (Osanto et al., 1991; Antunes et al., 1999; Antunes et al., 2000). Cisplatin also induces the production of reactive oxygen species (ROS) in renal epithelial cells mainly by decreasing the activity of antioxidant enzymes and by depleting intercellular concentrations of reduced glutathione (GSH) (Santos et al., 2008). Thiols such as the sulfur in GSH bind to the platinum molecule, replacing one of the chloride ions and preventing binding to other cellular nucleophiles (Berners-Price and Kuchel, 1990). These ROS can also cause extensive tissue damage through reactions with all biological macromolecules, e.g., lipids, proteins and nucleic acids, leading to the formation of oxidized substances such as the membrane lipid peroxidation product malondialdehyde (MDA) (Halliwell and Gutteridge, 1999)

and failure of the antioxidative defense mechanism against free radical-mediated organ damage.

Selenium (Se) is an essential dietary trace element which plays an important role in a number of biological processes. As an integral part of glutathione peroxidases and thioredoxin reductase, Se interacts with nutrients that affect the cellular redox status (i.e., pro-oxidant/antioxidant balance). Selenoenzymes are also known to play roles in carcinogen metabolism, in the control of cell division, oxygen metabolism, detoxification processes, apoptosis induction and the functioning of the immune system (Schrauzer, 2009). Red blood cells (RBC) are the main place of inorganic Se utilization and reduction by GSH (Suzuki and Ogra, 2002; Imai et al., 2009). There is a great deal of evidence indicating that Se supplementation at high levels reduces the incidence of cancer in animals. Many experimental studies in animals have demonstrated the ability of Se to prevent carcinogenesis, and epidemiological studies have suggested that a decreased Se status in humans is associated with an increased risk of cancer (Weijl et al., 1997). Different therapeutic adjuvants have been tested in an attempt to reduce the nephrotoxicity of cisplatin. Burk et al. (1974) showed that heavy metals, among them platinum, disclosed an interaction with Se and formed a complex of metal-Se with reduced toxicity.

In the present study, we evaluated the possible protective effects of Se on hematological and redox status parameters, as well on the activity of antioxidative enzymes in the RBC of rats chronically treated with cisPt.

MATERIALS AND METHODS

Chemicals

Chemicals and enzymes were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Cisplatin was obtained from Acros Organics (Liège Area, Belgium) and Na_2SeO_4 from Bor Copper Mining and Smelting Combine licensed by the Institute for Chemistry, Belgrade, Serbia.

Animals and blood collection and incubation

In this study the RBC of healthy rats (*Wistar* albino, male, 250-350 g body mass) were used. The animals were kept at $21 \pm 1^{\circ}C$ and exposed to a 12 h light/dark cycle. All rats were housed in individual cages and given standard diet and water *ad libitum*. In this experimental study four groups of rats were included: the control group comprised untreated rats (I), rats treated with Se (1.5 mg/kg of body weight of Na_2SeO_4 in 0.9% NaCl, i.p.) (II), rats treated with cisPt (4 mg/kg of body weight of cisPt in 0.9% NaCl, i.p.) (III), and rats treated with Se and cisPt (1.5 mg/kg of body weight of Na_2SeO_4 in 0.9% NaCl, i.p. 1 h before treatment with 4 mg/kg of body weight of cisPt in 0.9% NaCl, i.p.) (IV). All animals were treated for 5 days successively. Rats were anaesthetized by ether and sacrificed 24 h after the last treatment. Blood was collected in tubes containing heparin.

The hematocrit value (Hct), hemoglobin concentration (Hb), numbers of erythrocytes (Ercs), leukocytes (Lcs), platelets (Plts), as well as, the percentage of reticulocytes (Rtcs) were measured in the collected blood samples.

For oxidative stress parameter determination the collected blood was centrifuged for 10 min at 5,000 rpm, plasma was separated, while the RBC were washed three times with 0.9% NaCl. Washed-out erythrocytes were lysed with dH_2O (1:3, v/v) at $0^{\circ}C$ for 30 min. All samples were extracted from lysate. After extraction, samples were stored at $-80^{\circ}C$ before performing the appropriate analytical methods.

Hematological parameters

Hematocrit value determination was performed using full blood taken with standard microhematocritic tubes (75 mm length) and centrifuged for 5 min at 12,000 rpm, expressed in liter of RBCs per liter of blood (L/L). Hemoglobin concentration in the blood and lysate of RBCs was determined by the cyanmethemoglobin method (Drabkin and Austin, 1935). The counts of Ercs, Lcs and Plts were determined microscopically and were expressed as the

number of Ercs x 10^{12}/L of blood, Lcs x 10^9/L of blood and Plts x 10^9/L of blood, respectively. The supravital dying technique was used for measuring the amount of Rtcs.. The amount was expressed in % of reticulocytes (number of reticulocytes per total number of RBC x 100%).

Evaluation of ROS and RNS concentrations

The concentrations of ROS and reactive nitrogen species (RNS) were determined after extraction using the following protocol: ½ vol of 3 M perchloric acid and 2 vol of 20 mM EDTA were added to 1 vol of lysate. After extraction on ice (15 min) and centrifugation for 4 min at 15 000 rpm, extracts were neutralized with 2 M K_2CO_3.

The spectrophotometric determination of the superoxide anion (O_2^-) was based on the reduction of Nitro Blue Tetrazolium (NBT) in the presence of O_2^- (Auclair and Voisin, 1985). Determination of hydrogen peroxide (H_2O_2) concentration was based on the oxidation of Phenol Red (PR) in the presence of horse radish peroxidase (HRPO) (Pick and Keisari, 1980).

The spectrophotometric determination of nitrites – NO_2^- (indicator of the nitric oxide – NO levels) was performed by the Griess method (Green et al., 1982). The level of hydroxylamine as an indicator of the nitroxyl ion (NO^-) was determined by indooxine formation from 8-hydroxyquinoline as described by Arnelle and Stamler (1996).

Evaluation of lipid peroxide level

The level of lipid peroxidation products was determined on the basis of the reaction of lipid peroxidation products (malondialdehydes) by the Ohkawa method with thiobarbituric acid (thiobarbituric acid reactive substances – TBARS) (Ohkawa et al, 1979).

Evaluation of glutathione level

The level of reduced glutathione (GSH) was determined on the basis of GSH oxidation with 5.5-dithio-bis-6.2-nitrobenzoic acid by the Beutler method (1975); concentration was expressed as nmol/ml RBC. Levels of oxidized glutathione (GSSG) were determined using a fluorimetric assay according to the Hissin and Hilf (1976). For the estimation of the GSSG, the supernatant was mixed with N-ethylmaliemide (NEM) (5 mg/ml methanol) and incubated at room temperature for 30 min to inhibit the GSH at alkaline pH 12. Then, ortho-phthaldialdehyde (OPT) (1 mg OPT/ml methanol) was added and the intensity of fluorescence was measured (λ_{exc} = 350 nm; λ_{em} = 420 nm). The level of GSSG was expressed as nmol/ml RBC.

Evaluation of AOS enzymes activities

Antioxidative enzymes activities were determined in the lysate.

Superoxide dismutase (SOD) activity was determined after extraction using the following protocol: to remove the hemoglobin (Tsuchihashi, 1923), 1.0 ml of an ethanol/chloroform (1:1,v/v) mixture was added to an aliquot (0.5 ml) of the lysate cooled on ice. This mixture was stirred constantly for 15 min before dilution with 0.5 ml of distilled water. After centrifugation for 10 min at 1,600 g, the pale yellow supernatant was separated from the protein precipitate and used to assay SOD enzyme activity. SOD activity was determined owing to its ability to inhibit the auto-oxidation of pyrogallol according to the method of Marclund and Marclund (1974).

Catalase (CAT) activity was measured by the method of Beutler (1982). The method is based on the rate of H_2O_2 degradation by the action of CAT contained in the examined samples and was followed spectrophotometrically at 230 nm in 1 M Tris-HCl solution (with 5 mM EDTA, pH 8.0).

Glutathione peroxidase (GSH-Px) activity was assayed by following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) with t-butyl-hydroperoxide as a substrate (Maral et al., 1977). The activity of glutathione reductase (GR) was determined by using method of Glatzle et al. (1974).

The method is based on the capacity of GR to catalyze the reduction of GSSG to GSH using NADPH as a substrate. Glutathione-S-transferase (GST) activity towards 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate was measured according to the method of Habig et al. (1974). The activities of all followed enzymes were expressed in U/ml RBC.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical evaluation was calculated by one way ANOVA. For all comparisons, $p < 0.05$ was considered as significant.

RESULTS

The effects of chronic treatment in the group treated with Se (II), cisPt (III) and combined chronic treatment with Se and cisPt (IV) on the hematological parameters were evaluated in comparison with the control group (I), as well as between the Se and cisPt-treated group (IV), versus the cisPt-treated group (III) of *Wistar* albino rats.

Data for the hematological parameters is shown in Table 1. Results showed that chronic treatment with Se (II) induced a higher percentage of Rtcs and number of Plts compared to the control group (I). Cisplatin treatment (III) was followed by lower numbers of Ercs and Plts, as well as a higher Lcs number compared to the control group (I). The combined effect of Se and cisPt treatment (IV) induced, according to the data, a significant increase in the levels of Hct and Hb, a higher percentage of Rtcs and a lower number of Ercs, in comparison with the control group (I). Our results also showed a significant increase in the values of Hb, a higher percentage of Rtcs, higher number of Ercs and Plts in group of rats treated with Se and cisPt (IV) when compared to group treated with cisPt (III). In the same group of treated rats, the number of Lcs and Plts were recovered to control level (Tab. 1).

The effects of chronic treatments on the redox status and antioxidative enzymes were evaluated in
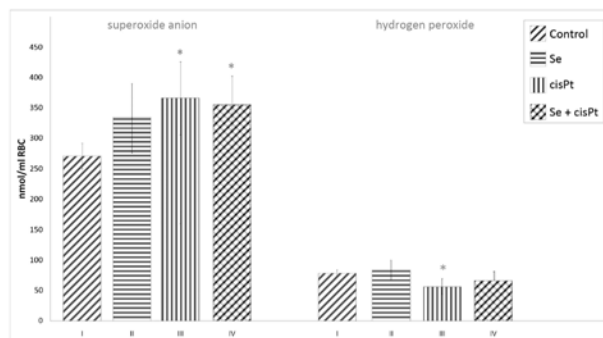

Fig. 1. The concentration of ROS in lysates from control (I) rats and rats exposed to chronic treatment with Se (II), cisPt (III) and Se and cisPt (IV) treated rats. Values represent the mean ± SEM for 5 animals per each group. The values for the superoxide anion radical are expressed in nmol/ml RBC x 10^{-1}. *$p < 0.05$, compared to control group (I). #$p < 0.05$ Se and cisPt-treated group (IV) compared to cisPt-treated group (III).

RBCs in the next part of the study. The ROS and RNS levels in the lysate obtained from the investigated groups of animals are shown in Figs. 1 and 2, respectively. All applied chronic treatments elevated O_2^- concentration (alterations are significant in the presence of cisPt (II and IV groups)), while the H_2O_2 concentration significantly decreased in the presence of cisPt, compared to the control (I) group (Fig. 1). In addition, chronic treatment with cisPt induced the elevation of nitrite and depletion in hydroxylamine (NO^-) level (Fig. 2). These results showed that cisPt induced the generation of reactive oxygen and nitrogen species in the RBC of the treated rats. So, the next step was investigation of RBC antioxidative capacity.

Lipid peroxidation - TBARS level, as an indicator of oxidative damage processes, was significantly higher in the group treated with Se (II), as well as in the group co-treated with Se and cisPt (IV) (Table 2). As a possible answer of RBC to Se-induced lipid peroxidation, the concentration of reduced glutathione significantly increased, consequently elevating the glutathione antioxidative capacity (decreased GSSG/2 GSH ratio) (Table 2). Chronic application of cisPt induced a significant elevation of reduced and oxidized glutathione (Table 2), indicating adaptation of the rats' RBC to cisPt-induced oxidative stress.
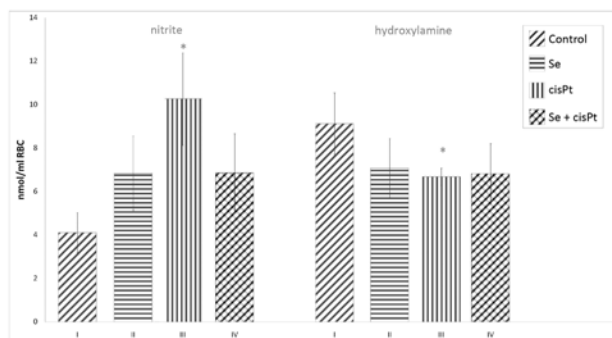
Fig. 2. The concentration of RNS in lysate of control (I) rats and rats exposed to chronic treatment with Se (II), cisPt (III) and Se and cisPt (IV) chronically treated rats. Values represent mean ± SEM for 5 animals per each group. *p < 0.05, compared to control group (I). #p< 0.05 Se and cisPt-treated group (IV) compared to cisPt-treated group (III).
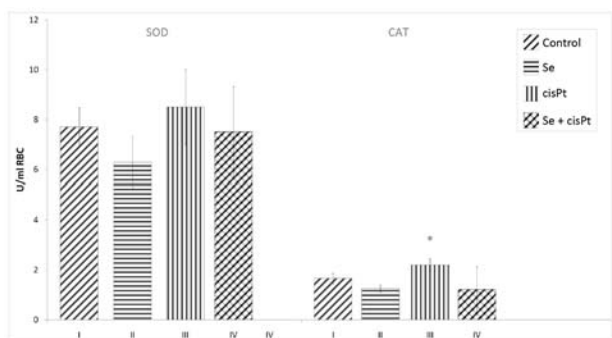

Fig. 3. The activities of lysate superoxide dismutase (SOD) and catalase (CAT) in control (I), Se (II), cisPt (III) and Se and cisPt (IV) treated rats. Values represent mean ± SEM for 5 animals per each group. Values for SOD are in U/ml RBC x 10^4 and for CAT are in U/ml RBC x 10^6. *p < 0.05, compared to control group (I). #p< 0.05 Se and cisPt-treated group (IV) compared to cisPt-treated group (III).

Fig. 4. The activities of lysate glutathione peroxidase (GSH-Px), glutathione reductase (GR) and glutathione-S-transferase (GST) in control (I) rats and rats exposed to chronic treatment with Se (II), cisPt (III) and Se and cisPt (IV). Values represent the mean ± SEM for 5 animals per each group. Values for GSH-Px are in U/ml RBC x 10^4, for GR are in U/ml RBC x 10^5 and for GST are in U/ml RBC x 10^3. *p < 0.05, compared to control group (I). #p< 0.05 Se and cisPt-treated group (IV) compared to cisPt-treated group (III).

DISCUSSION

Cisplatin is a very effective chemotherapeutic agent, used in the treatment of a wide range of malignant diseases. However, it exhibits certain toxic effects on the kidneys and liver which interfere with its therapeutic efficiency (Taguchi et al., 2005). Some antioxidants may prevent these toxic effects (Durak et al., 2002, Naziroglu, 2004). In this study we evaluated oxidative stress effects of chronically applied cisPt and the possible preventive action of Se.

Wood and Hrusheeky (1995) showed that cisPt causes significant effects on hematological parameters only during chronic treatment in humans and rats. Cisplatin-induced anemia is also a well-known side-effect (Von Hoff et al, 1979) which occurs in 9-40% of patients (Philipps and Tannock, 1998). Our previous results showed that high, acute doses of cisPt did not affect the RBC maturation in rats (Marković et al., 2010). The results of our study are in accordance with literature data, and show that chronic application of cisPt induced depletion in RBC number and maturation. Animals also were treated with Se which as an antioxidant can prevent the toxic effects of cisPt. Chronic treatment of rats with Se did not affect the properties of RBC, while

Results showed that the activities of antioxidative enzymes partially changed in the treated groups of rats. The activity of CAT was significantly higher in the cisPt (III) treated rats in comparison with the control group (I) (Fig. 3). Combined chronic treatment with Se and cisPt induced a lower activity of GSH-Px and GR in comparison with the control group (I) (Fig. 4). The activities of lysate SOD and GST were not significantly changed in all the groups of treated rats compared to the control group (I).
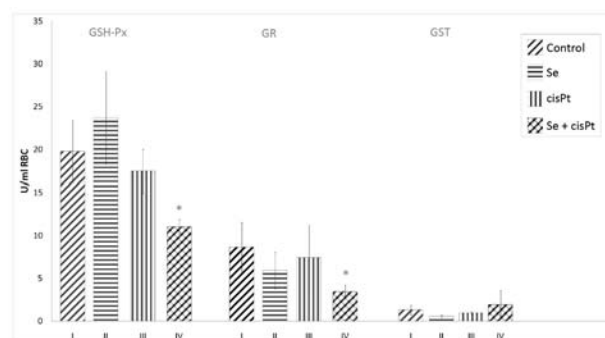
Table 1. Hematological parameters of control (I) rats and rats exposed to chronic treatment with Se (II), cisPt (III) and Se and cisPt (IV). Values represent mean ± SEM for 5 animals per each group. *p < 0.05, compared to control group (I). #p< 0.05 Se and cisPt-treated group (IV) compared to cisPt-treated group (III).

	I	II	III	IV
Hct (L/L)	0.32 ± 0.02	0.33 ± 0.02	0.37 ± 0.01	0.40 ± 0.03*
Hb (g%)	12.03 ± 0.93	13.51 ± 0.91	10.96 ± 1.63	17.70 ± 0.53*#
Ercs (x10^{12}/L)	5.66 ± 0.33	5.61 ± 0.29	2.64 ± 0.20*	4.26 ± 0.95*#
Rtcs (%)	0.30 ± 0.04	0.68 ± 0.12*	0.14 ± 0.01	1.03 ± 0.26*#
Lcs (x10^9/L)	3.5 ± 0.45	3.76 ± 0.39	7.9 ± 1.9*	3.8 ± 0.57 #
Plts (x10^9/L)	185 ± 15.33	248 ± 8.2*	120 ± 20*	188.3 ± 11.66 #

Table 2. TBARS and glutathione status in RBC of control (I) rats and rats exposed to chronic treatment with Se (II), cisPt (III) and Se and cisPt (IV) treated rats. Values represent mean ± SEM for 5 animals per each group. *p < 0.05, compared to control group (I). #p< 0.05 Se and cisPt-treated group (IV) compared to cisPt-treated group (III).

	I	II	III	IV
TBARS (nmol/ml RBC)	3,10 ± 0,23	10,00 ± 2,09*	3,00 ± 0,25	11,44 ± 1,13*#
GSH (nmol/ml RBC)	1164,76 ± 175,20	1612,00 ± 113,71*	1400,66 ± 27,57*	1326,95 ± 128,81
GSSG (nmol/ml RBC)	772,92 ± 49,60	723,33 ± 72,08	1012,66 ± 41,34*	998,89 ± 99,36*
GSSG/2GSH	0,33	0,22	0,36	0,38

the effect of the combined Se and cisPt treatment partially recovered the cisPt-induced depletion in Ercs number and RBC maturation. Heavy metals, among them platinum, interact with Se, forming a metal-Se complex with reduced toxicity (Burk et al. 1974). Brandao and associates (2005) showed that incubation of human venous blood in the presence of Se is followed by hemolysis. This could explain the occurrence of reticulocytosis, an indicator of stimulated erythropoiesis, in our study. The neph-

rotoxic effects of cisPt (Yoshida et al., 2000) could be related to renal failure, leading to the loss of body fluids and hemoconcentration and therefore an increased hematocrit in cisPt-treated animals, shown by our results.

Aside from the reduction in RBC number, chronic application of cisPt induced a reduction in the number of platelets and an increase in the number of leukocytes in the blood of rats. In support of the above, Olas et al. (2005) have shown that cisPt causes oxidative stress in human platelets and lymphocytes, which might reflect on their life expectancy, the induction of apoptosis, and thereby ultimately reduce the number of these cells in the blood. On the other hand, the increase in the leukocyte number could be the consequence of infection and inflammation during cisPt treatment and cisPt metabolism in our experimental rats. Se in the co-treatment with cisPt recovered the number of these blood cells to the control level, indicating that it has preventive and protective effects against the platinum complex.

Oxidative stress and ROS accumulation are one of the main mechanisms of cisPt-induced nephrotoxicity (Santos, 2008). Our study shows that chronic doses of cisPt induced higher superoxide anion and nitrite concentrations, while decreasing the hydrogen peroxide and hydroxylamine (NO$^-$) levels. Literature data suggest that nitrite, the stable end-product of nitric oxide metabolism, reacts with superoxide radicals which finally leads to nitrosative stress (Wink and Mitchell, 1998). Srivastava et al. (1996) reported that treatment of rats with cisplatin resulted in a significant increase in the activity of calcium-independent nitric oxide synthase in the kidney and liver, leading to an enhancement in NO formation. Chronic treatment with cisPt induced a significantly higher activity of CAT in comparison with the control group and consequently a decreased concentration of hydrogen peroxide, as shown in our results.

Chronic doses of Se were associated by increased lipid peroxidation in the RBC. Recent studies have shown the protective effects of Se against Cd-induced lipid peroxidation in rat liver and kidneys (Ognjanović et al., 2008), as well as hemoprotective effects against cyclophosphamide-induced toxicity in rats (Ayhanci, 2009). However, our results show that the antioxidative activity of Se as a constituent of GSH-Px, did not satisfactorily prevent oxidative damage of lipid molecules. In addition, Se acted as a pro-oxidant after chronic treatment. Chronic Se and cisPt co-treatment induced an accumulation of lipid peroxides in the RBC, indicating the prooxidant action of these substances. Literature data also show that the co-administration of Se with mercury was followed by lipid peroxidation in the liver, kidney and brain of mice (Agarwal and Behari, 2007).

As a consequence of the chronic administration of heavy metals (Se and cisPt) and their toxic effects, the RBCs of treated rats were mostly involved in antioxidative mechanisms through the glutathione pathways. In all treated groups of animals, the GSH level was elevated while the GSSG level was the highest in the cisPt-treated group. The pool of GSH was preserved by the antioxidative action of Se (high activity of GSH-Px); the GSSG/2 GSH ratio was decreased in comparison with cisPt single treatment. The protective effects of Se seem to be primarily associated with its presence in the GSH-Px and other selenoenzymes which are known to protect cellular components from oxidative stress damage, and to play roles in carcinogen metabolism, cell division, oxygen metabolism, detoxification processes, apoptosis induction and the functioning of the immune system (Schrauzer, 2009).

The combined chronic treatment with Se and cisPt induced a lower activity of GR compared to the control group (Fig. 4). The reduced GR activity was also followed by the accumulation of GSSG and an increase in the GSSG/2 GSH ratio, indicating the pronounced utilization of GSH and alteration in glutathione metabolism favoring the oxidized form. Literature data showed that many trace elements, as well as arsenic-, platinum- and gold-containing drugs, significantly influence the fate of exogenous Se, whereby they may adversely

affect the availability of this essential element for synthesis of selenoenzymes (Sugawara and Sugawara, 1987; Schrauzer, 2009). While Se remains the key cancer-protective trace element, the interpretation of its mode of action necessitates consideration of the effects of Se antagonists (Schrauzer, 2009).

Based on the results of this study we can conclude that (*i*) chronic Se treatment stimulated erythropoiesis, increased lipid peroxidation and altered glutathione metabolism, favoring itsa reduced state; (*ii*) chronic cisPt treatment induced anemia, the generation of reactive species and an increase of the glutathione status; (*iii*) co-treatment with Se and cisPt and their synergistic effects can in part protect against cisPt-induced toxicity. At present, few interactions between Se and medications are known. ,Based on the presented results it can be argued that it is necessary to take into account the interactions of Se with cisPt in a discussion of its mechanism of antioxidative and anticarcinogenic action.

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