

EFFECTS OF ACUTE TREATMENT OF VITAMIN C ON REDOX AND ANTIOXIDATIVE METABOLISM IN PLASMA AND RED BLOOD CELLS OF RATS

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ABSTRACT. Ascorbic acid (Vit C) is a reducing agent that efficiently quenches potentially damaging free radicals. The aim of this study was to investigate effects of high acute dose of Vit C on redox and antioxidative status in blood of rats. Our study included two groups of *Wistar albino* rats: I – untreated group (control); II – group treated with Vit C (500 mg/kg bw, i.p.). In all cases animals were sacrificed after three days of treatment and blood samples were taken at the day of sacrifice. Concentration of Heinz bodies (HB) was determined in whole blood, while the concentration of reactive oxygen and nitrogen species (ROS and RNS), as well as, parameters of antioxidative defense system were followed in plasma and red blood cells (RBC). Our results showed that Vit C treatment induced higher levels of peroxynitrites and lipid peroxidation in plasma. In RBC, Vit C induced higher level of HB, GSSG/2 GSH ratio and glutathione peroxidase activity, while decreased glutathione reductase activity. In conclusion, acute high doses of Vit C act as prooxidant and induced oxidative stress that resulted in lipid peroxidation in plasma and hemoglobin oxidation in RBC.

Key words: vitamin C, rats, red blood cells, oxidative stress.

INTRODUCTION

The main role of red blood cells (RBC) is transport of hemoglobin which supplies oxygen to all tissues in the body. The combination of several factors, such as active metal protein (hemoglobin), which functions as an oxidase and peroxidase, high pressure oxygen in

the circulation, membrane proteins and unsaturated fatty acids, which can be oxygenated, creates the environment for potentially harmful reactions for RBC (STERN, 1989). Such conditions may induce physiological aging and cell death, but in terms of oxidation stress leads to premature dysfunction and death of cells. Oxidative stress is defined as a seriously disturbed balance between production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) on the one hand, and antioxidant protection (Antioxidative Defense System - AOS) on the other side (HALLIWELL and GUTTERIDGE, 1999). The main source of ROS in cells are mitochondria, peroxisomes, microsomes, cell membranes, membrane bound enzymes (cyclooxygenase and lipooxygenase) (DRÖGE, 2002). Antioxidant defense system has been developed in all aerobic organisms to prevent, limit or repair damage caused by activities of ROS. Antioxidant defense system, present in lesser concentration in relation to the substrates that are oxidized, can prevent or significantly reduce their oxidation (HALLIWELL, 2000).

Humans normally acquire ascorbic acid (vitamin C - Vit C) from a large variety of dietary sources. Ascorbic acid is a cofactor for a number of metabolic enzymes and is an indisputable essential vitamin for humans (REBOUCH, 1991). Under physiological conditions, it functions as a potent reducing agent that efficiently quenches potentially damaging free radicals produced by normal metabolic respiration of the body (ARRIGONI and DE TULLIO, 2002). Vit C is considered to be an important antioxidant in extracellular fluid; it also guards against aqueous radicals in blood and protects plasma lipids from peroxidative damage caused by peroxy radicals (SIES *et al.*, 1992).

The aim of this study was to investigate of *in vivo* effects of acute high doses of Vit C on redox and antioxidative metabolism parameters in plasma and RBC of rats.

MATERIALS AND METHODS

Chemicals

Chemicals for solutions were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Animals and blood collection

In this study RBC of rats (*Wistar albino*, male, 250-350 g body mass) were used. The animals were kept at 21 ± 1 °C and exposed to a 12 h light – 12 h dark cycle. All rats were housed in individual cages and given standard diet and water *ad libitum*. In this study we included two groups of *Wistar albino* rats: I – untreated rats (control); II – vitamin C treated rats (500 mg/kg bw, i.p., in one acute dose). In all cases animals were sacrificed after 3 days of treatment. Blood samples were taken at the day of sacrifice.

Evaluation of ROS and RNS concentrations

The concentrations of ROS and RNS were determined after extraction using the following protocol: ½ vol 3 M perchloroacetic acid and 2 vol 20 mM EDTA were added to 1 vol lysate. After extraction on ice (15 min) and centrifugation for 4 min/15 000 rpm, extracts were neutralized using 2 M K₂CO₃.

The spectrophotometric determination of the superoxide anion (O₂^{•-}) was based on the reduction of Nitroblue Tetrazolium (NBT) in the presence of O₂^{•-} (AUCLAIR and VOISIN 1985). The determination of the hydrogen peroxide (H₂O₂) concentration was based on the oxidation of Phenol Red (PR) in the presence of Horse Radish Peroxidase (HRPO) as a catalyst (PICK and KEISARI 1980).

The spectrophotometric determination of nitrites – NO₂⁻ (indicator of the nitric oxide – NO level) was performed using the Griess method (Green et al. 1982). The concentration of 3-nitrotyrosine (3-NT) as an indicator of the peroxynitrite (ONOO⁻) ion was performed using Riordan and Valle's method (1972).

Heinz body formation level

Heinz body (HB) amount was determined by turbidometric measurement (BATES and WINTERBOURN, 1984). Whole blood (0.1 mL) and Na-PO₄ buffer (3 mL, 5 mM, pH 7.4) were mixed together and incubated for 15 minutes at room temperature in dark. The amount of HB formation can be measured spectrophotometrically at a wavelength of 700 nm.

Evaluation of lipid peroxides level

The level of lipid peroxidation products was determined on the basis of the reaction of lipid peroxidation products (malondialdehydes) using Ohkawa method with TBA (thiobarbituric acid reactive substances - TBARS) (OHKAWA *et al.*, 1974).

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 using BEUTLER method (1975a) and concentration was expressed as nmol/ml plasma (RBC). Concentrations of oxidized glutathione were (GSSG) determined enzymatically by glutathione reductase using BEUTLER method (1975b) after inhibition of GSH oxidation by N-ethylmaleimide. The level of GSSG was expressed as nmol/ml of plasma and RBC.

Evaluation of AOS activities

Superoxide dismutase (SOD) activity was determined after extraction using 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 being diluted with 0.5 ml of distilled water. After centrifugation for 10 min at 1600 g, the pale yellow supernatant was separated from the protein precipitate and was 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 H₂O₂ degradation by the action of CAT contained in the examined samples followed spectrophotometrically at 230 nm in 5 mM EDTA, 1 M Tris-HCl solution, pH 8.0.

Glutathione peroxidase (GSH-Px) activity was assayed following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) with t-butyl-hydroperoxide as a substrate (MARAL *et al.*, 1977). The activity was determined 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 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 mean ± SEM. Statistical evaluation was calculated by one way ANOVA. For all comparisons, p < 0.05 was considered as significant.

RESULTS

ROS and RNS concentrations

The concentration of O₂⁻ and H₂O₂ of the investigated groups of animals in plasma and RBC are shown in Figure 1. Concentration of O₂⁻ in RBC was significantly lower

($p < 0.05$) in Vit C-treated rats (II), compared to control group (I).

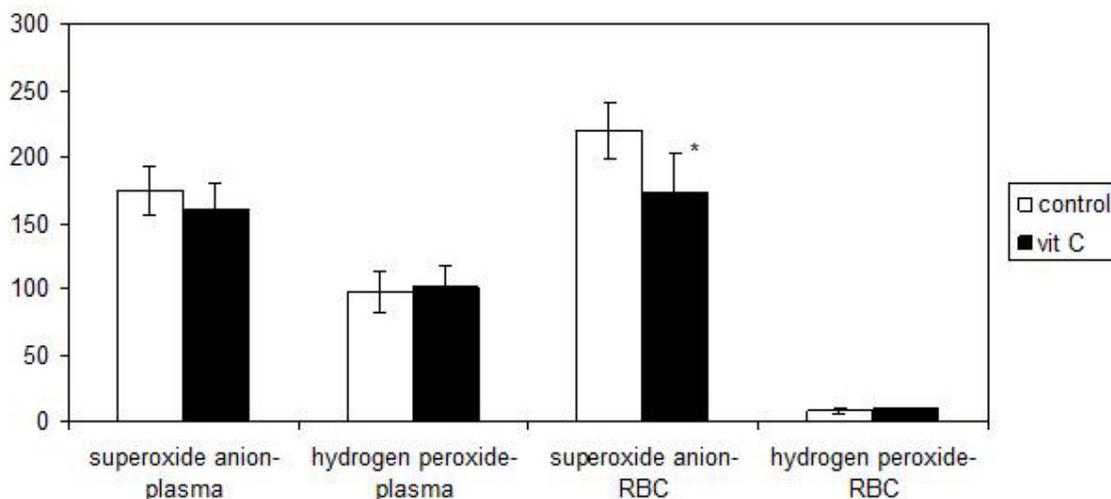


Figure 1. The concentration of ROS in plasma and RBC of control and Vit C-treated rats.

Values represent mean \pm SEM for 5 animals per each group.

Values for O_2^- concentration in RBC are in $nmol/ml \times 10^{-1}$. * $p < 0.05$, Vit C versus control group.

RNS concentrations were showed in Figure 2. Nitrite level was not different in plasma of investigated groups of animals but concentration of nitrite in RBC was significantly lower ($p < 0.05$) in Vit C-treated rats. On the other hand, peroxynitrite (3-NT) level was significantly higher in plasma of Vit C-treated rats, compared to control group.

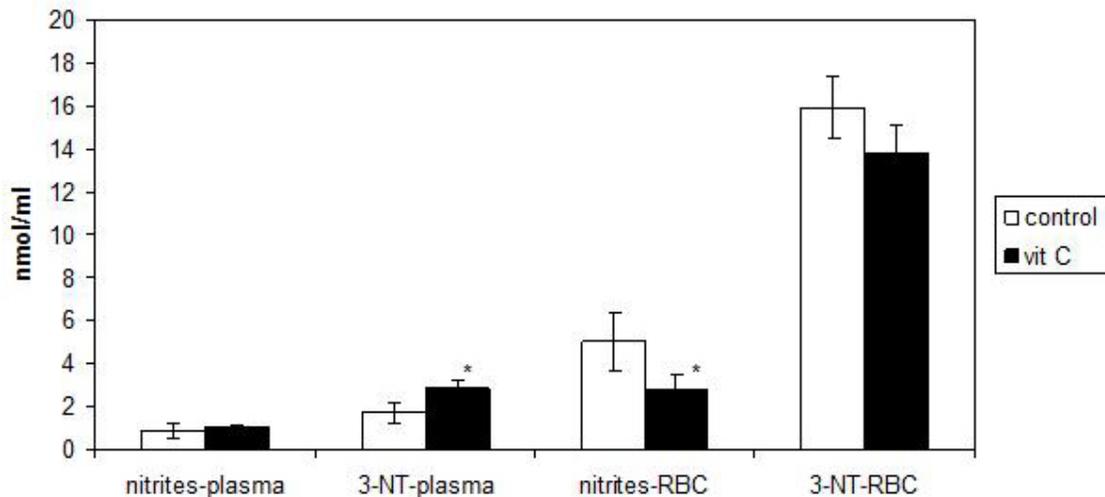


Figure 2. The concentration of RNS in plasma and RBC of control and Vit C-treated rats.

Values represent mean \pm SEM for 5 animals per each group.

Values for nitrites concentration in RBC and 3-NT concentration in plasma and RBC are in $nmol/ml \times 10^{-1}$. * $p < 0.05$, Vit C versus control group.

Oxidative stress induced damage parameters

Heinz's bodies are inclusions denatured globins chains in RBC, and showed an indicator of oxidative damage processes in RBC. HB levels are significantly increased in Vit C-treated rats (Tab. 1).

Table 1. - The concentrations of Heinz's bodies, lipid peroxidation (TBARS), reduced glutathione (GSH) and oxidized glutathione (GSSG) in plasma and RBC in control and Vit C-treated rats. Values represent mean \pm SEM for 5 animals per each group. * $p < 0.05$, Vit C versus control group.

	Plasma		Ercs	
	control	Vit C	control	Vit C
HB			0.055 \pm 0.006	0.094 \pm 0.021*
TBARS nmol/ml	0.88 \pm 0.03	1.54 \pm 0.16*	3.28 \pm 0.20	3.21 \pm 0.25
GSH nmol/ml	52.91 \pm 7.78	50.04 \pm 5.66	5580 \pm 540	3190 \pm 250*
GSSG nmol/ml	283.03 \pm 25.61	347.15 \pm 15.46	1170.23 \pm 96.17	2110 \pm 68.99*
GSSG/2GSH	2.67	3.47	0.10	0.33

TBARS level, as indicator of lipid oxidative damage processes, was significantly higher in plasma of Vit C-treated rats, compared to control group (Tab. 1).

Glutathione status

In plasma of Vit C-treated rats, GSH and GSSG level were not changed (Tab. 1). On the other hand, GSH level was significantly lower, followed by high concentration of GSSG and GSSG/2 GSH ratio in RBC of Vit C-treated rats compared to control group (Tab. 1).

AOS enzymes activities

Figure 3. shows SOD, CAT, GSH-Px, GR and GST activities, five AOS enzymes that are included in ROS metabolism. The SOD, CAT and GST activities were not different in Vit C treated rats. The GSH-Px activity was significantly higher, while GR activity was significantly lower ($p < 0.05$) in RBC of Vit C-treated rats, compared to control group.

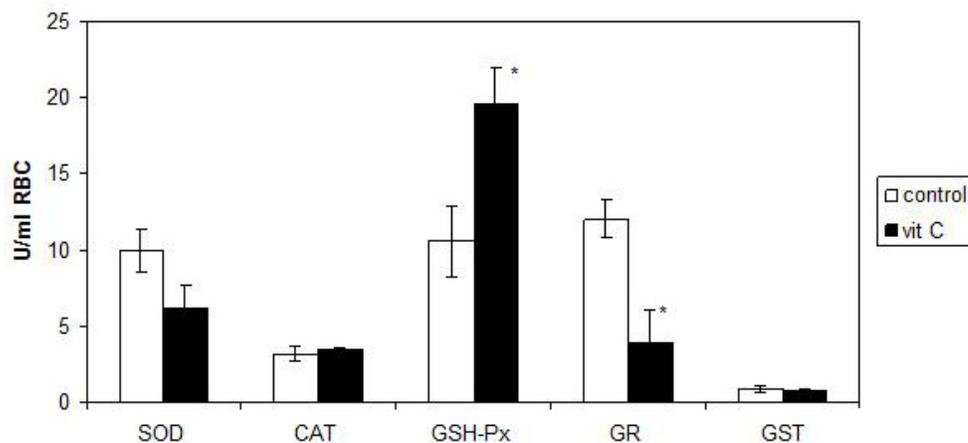


Figure 3. The activities of lysate superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), glutathione-S-transferase (GST) in control and Vit C-treated rats.

Values represent mean \pm SEM for 5 animals per each group.

Values for SOD are in U/ml RBC $\times 10^4$, for CAT are in U/ml RBC $\times 10^6$, for GSH-Px are in U/ml RBC $\times 10^3$, for GR are in U/ml RBC $\times 10^5$, for GST are in U/ml RBC $\times 10^3$. * $p < 0.05$, Vit C versus control group.

DISCUSSION

Traditionally, Vit C is used as antioxidants in co therapy of many diseases. Our study aimed to contribute to effects and possible metabolism of acute Vit C treatment of experimental rats.

Vit C causes a change in the studied parameters, reducing the concentration of ROS and RNS, increasing HB formation in RBC, while activities of antioxidative enzymes SOD and CAT were not changed. Evidently antioxidative capacity of RBC efficiently scavenged ROS that generated in these cells. According to literature data, Vit C acts as breaking antioxidant chain reaction of lipid peroxidation, and as hydrogen donor, participates in the detoxification of various ROS (PADH, 1990). On the other hand, Heinz bodies formation significantly increased in Vit C treated animals, indicating oxidative damage of hemoglobin in RBC. In high doses, and applied alone (without other antioxidants) Vit C can act as pro-oxidant inducing oxidative stress (CARR and FREI, 1999).

Based on our results, acute high dose of Vit C induces accumulation of peroxynitrites and consequently lipid peroxidation in plasma of treated rats. These data show the induction of oxidative stress in plasma which is in accordance to data of previous studies which showed that Vit C can act as pro-oxidant (CARR and FREI, 1999; MACDONALD *et al.*, 2002; BENZIE, 2003; PADAYATTY *et al.*, 2004).

Glutathione redox couple (GSSG/2 GSH) is one of the redox systems of the cell (SCHAFER and BUETTNER, 2001). After Vit C treatment, the GSSG/2 GSH ratio increased in both, plasma and RBC of rats, as consequence of GSSG accumulation, as well as, significantly lower level of GSH in RBC. These data indicate ineffective antioxidative capacity of glutathione in RBC that is followed by hemoglobin oxidation and denaturation. GSSG accumulation may be the consequence of decreased GR activity in RBC of treated rats. The biological function of this enzyme is to maintain glutathione in reduced form and catalyzes reduction of GSSG to GSH with the participation of NADPH as reductive cofactor. Reduced activity of GR is accompanied by accumulation of GSSG and the reduction of available quantity of GSH in RBC of rats acutely treated with Vit C. Regarding changes in glutathione status of RBC, it is obvious that these cells are incorporated by GSH in antioxidant protection against pro-oxidative effects of Vit C. In addition, the higher activity of GSH-Px in the RBC of Vit C treated rats, could contribute to declining level of GSH.

In conclusion, Vit C is widely used as an antioxidant, but when given in high acute doses acts as pro-oxidant in rat's circulatory system. Hence, in order to effective treatment of patients, it is necessary to take care of entered amounts of antioxidants in therapy or supplementary therapy of patients.

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