Acta Biologica Hungarica 62(2), pp. 122–132 (2011)
DOI: 10.1556/ABiol.62.2011.2.2

ENERGY PRODUCTION AND REDOX STATUS OF RAT RED BLOOD CELLS AFTER RETICULOCYTOSIS INDUCED BY VARIOUS TREATMENTS

SNEŽANA D. MARKOVIĆ,[1]* JOVANA B. ŽIŽIĆ,[1] ANA D. OBRADOVIĆ,[1] BRANKA I. OGNJANOVIĆ,[1] A. Š. ŠTAJN,[1] ZORICA S. SAIČIĆ[2] and M. B. SPASIĆ[2]

[1] Department for Biology and Ecology, Faculty of Science, University of Kragujevac, 34000 Kragujevac, Serbia
[2] Department of Physiology, Institute for Biological Research "Siniša Stanković", 11060 Belgrade, Serbia

(Received: May 10, 2010; accepted: August 11, 2010)

Stimulated erythropoiesis and reticulocytosis can be induced by daily bleeding, or by phenylhydrazine (PHZ) treatment. We compared the *in vivo* effects of PHZ and bleeding treatment on haematological, energy and redox status parameters in red blood cells (RBC) of rats. The results showed that all followed haematological parameters were significantly lower in bleeding, compared to PHZ-treated rats. PHZ induced even 2.58-fold higher reticulocytosis as compared to bleeding treatment. Although PHZ induced higher reticulocytosis, respiration intensity and energy production was lower than in bleeding-induced reticulocytes. These alterations were the consequence of increased superoxide anion and peroxynitrite concentrations in PHZ-treated rats. Bleeding treatment resulted in increased activity of an antioxidative enzyme, superoxide dismutase. In conclusion, differences in these two experimental models for reticulocytosis may be used as tools for appropriate pharmacological testing of redox-active substances considering energy and redox processes, as well as apoptosis pathways.

Keywords: Bleeding – energy production – phenylhydrazine – redox status – reticulocytes

INTRODUCTION

In mammals, the process of erythropoiesis includes maturation of haematopoietic stem cells through increased haemoglobin synthesis and loosing of genetic material and all organelles in mature erythrocytes. The last but one stadium of this differentiation, the reticulocytes do not possess a full range of metabolic pathways compared to proliferating cells. However, reticulocytes are still equipped with a set of metabolic pathways, due to the presence of mitochondria and ribosomes [21]. Primary consumers of oxygen and primary ATP generators are mitochondria and are a permanent source of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells [4, 8]. In comparative studies, the reticulocytes and erythrocytes were used as simple model systems to differentiate the metabolic processes in these cells. In addition, reticulocytes are adequate model system for investigations of mitochondrial proc-

*Corresponding author; e-mail: smarkovic@kg.ac.rs

esses, considering energy and redox metabolism [12, 14, 15], as well as mechanisms of apoptosis.

In experimental conditions, reticulocytosis can be induced by bleeding or by phenylhydrazine-hydrochloride (PHZ) treatment. The amount of bleeding-induced reticulocyte generation is a 30–40% increase [16, 28], whereas PHZ-induced reticulocytosis in rats is over 80% [10, 12, 14, 15]. Phenylhydrazine and its derivatives are well-known active substances that induce deleterious changes of red blood cell (RBC, including both erythrocytes and reticulocytes) properties and lead to haemolysis and/or phagocytosis. It is generally assumed that all these changes are mediated by formation of superoxide radicals, hydrogen peroxide, phenyl radicals and other reactive species derived from PHZ [18]. In RBCs, PHZ reacts with oxyhaemoglobin to form methaemoglobin, oxidizes to haemichrome, and causes Heinz body formation, inducing membrane alterations due to lipid peroxidation and changes in membrane proteins [26]. Studies *in vitro* showed that PHZ induced significant changes in energy metabolism of RBCs in humans [11] and rabbits [28].

Recent studies showed that PHZ-induced reticulocytes are simple model system of anaemia and apoptosis investigation [5, 20, 24]. In order to clearly define this model system, the question which we tried to resolve in this study is whether PHZ-induced reticulocytosis produce the energy functional cells. We compared *in vivo* effects of PHZ treatment with bleeding regarding respiratory processes and energy production in reticulocytes of rats. In addition, we followed redox status parameters in RBC of treated groups of rats and found positive correlation between PHZ-induced inhibition of energy production and oxidative stress appearance.

MATERIALS AND METHODS

Chemicals

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

Animals, RBCs collection and incubation

In this study, RBCs 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*. Control groups were obtained from untreated (I) and 0.9% NaCl-treated (0.5 ml 0.9% NaCl for three days) rats (II). Reticulocytosis was induced by PHZ treatment (35 mg/kg body weight for 3 days) (III) and by daily bleeding of rats (1.5–2 ml of blood from tail vein) for 8 days (IV). After 7 (I, II and III) and 9 days (IV) rats were anaesthetized by ether and blood was taken by exsanguinations. Blood was collected into tubes containing heparin.

Haematocrit value (Hct), haemoglobin concentration (Hb), amounts of erythrocytes (Ercs), leukocytes (Lcs), platelets (Plts), and percentage of reticulocytes (Rtcs) were measured in the collected blood samples. Formation of Heinz bodies (HB) was also determined in the collected blood samples.

For energy production determination, collected blood was centrifuged 10 min at 5000 rpm, plasma and leukocytes were separated, while RBCs were washed three times with NaCl 0.9%. Washed RBCs were resuspended in incubation buffer containing 50 mmol/l Hepes, 100 mmol/l NaCl, 1 mmol/l $MgCl_2$, 1 mmol/l NaH_2PO_4, 5 mmol/l glucose and 2 mmol/l $CaCl_2$, pH 7.4 at 37 °C [10]. Cell suspensions (final haematocrit value about 0.20) obtained from bleeding and PHZ-treated rats were incubated aerobically for 2 h at 37 °C.

For redox status determination, the collected blood was centrifuged for 10 min at 5000 rpm, plasma and leukocytes were separated, while RBCs were washed three times with NaCl 0.9%. Washed-out RBCs were lysed with dH_2O (1 : 3, v/v) at 0 °C for 30 min. All samples were extracted from lysates. After extraction, the samples were stored at –80 °C until analysis.

Haematological parameters

Haematocrit value determination was preformed using the full blood taken with standard microhaematocritic tubes (75 mm length) and centrifuged for 5 minutes on 12,000 rpm, expressed in litre of RBCs per litre of blood (L/L). Hemoglobin concentration in blood and lysate of RBCs was determined by cyanmethemoglobin method [6]. Amounts of erythrocytes, leukocytes and platelets were counted microscopically and were expressed in number of Ercs $\times 10^{12}$/L of blood, Lcs $\times 10^9$/L of blood and Plts $\times 10^9$/L of blood, respectively. Supravital dying technique was used for measuring the amount of reticulocytes. *Substantia reticulofilamentosa,* which is an artificial residue and a supravital phenomenon after dying (1% Brilliant cresyl blue in 150 mM NaCl), could be seen under the microscope with immersion glass. The amount was expressed in % rtcs (number of reticulocytes per total number of RBC × 100%).

Heinz body formation level

Heinz body amount was determined by turbidometric measurement [2]. Cell suspension (0.1 mL) and Na-PO_4 buffer (3 mL, 5 mM, pH 7.4) were mixed together and incubated for 15 minutes on room temperature in dark. The amount of HB formation was measured spectrophotometrically at a wavelength of 700 nm.

Oxygen consumption measurement and evaluation of energy production

Oxygen consumption (ΔO_2) was measured by the Warburg technique [27]. Coupled ΔO_2 (the part of total ΔO_2 used for ATP production in oxidative phosphorylation – OxP) was calculated as the difference between total and (5 μM) oligomycin-resistant (uncoupled) ΔO_2 [25]. The oxygen consumption intensity was calculated and expressed as μmol/h/ml reticulocytes. Energy production through OxP was calculated on the basis of the estimated coupled ΔO_2 and the estimated P/O ratio of 2.5 (1 M atoms of oxygen consumed equivalent to 2.5 M produced ATP) [25].

Evaluation of ROS 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 (4 min/15,000 rpm), extracts were neutralized using 2 M K_2CO_3.

The spectrophotometric determination of the superoxide anion ($O_2^{\cdot -}$) was based on the reduction of Nitro Blue Tetrazolium (NBT) in the presence of $O_2^{\cdot -}$ [1]. The determination of the hydrogen peroxide (H_2O_2) concentration was based on the oxidation of Phenol Red (PR) in the presence of Horse Radish Peroxidase (HRPO) as a catalyst [19].

The spectrophotometric determination of nitrites – NO_2^- (indicator of the nitric oxide – NO level) was performed using the Griess method [9]. The concentration of 3-nitrotyrosine (3-NT) as an indicator of the peroxynitrite ($ONOO^-$) ion was performed using Riordan and Valle's method [23].

Evaluation of Antioxidative System (AOS) enzymes activities

Superoxide dismutase (SOD) activity was determined after extraction using the following protocol: to remove the haemoglobin, 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 5000 rpm, 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 [13].

Catalase (CAT) activity was measured by the method of Beutler [3]. The method is based on the rate of H_2O_2 degradation by the action of CAT contained in the examined samples followed spectrophotometrically at 230 nm in 1 M Tris-HCl solution, containing 5 mM EDTA, pH 8.0. The activities of SOD and CAT were expressed as 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

Haematological parameters

The haematological parameters of bleeding and PHZ-treated rats are shown in Table 1. Value for "mock-treated" control (II) was not different in comparison to control (I). In PHZ-treated rats (III) concentration of Hb was lower ($p>0.05$), while Plts number, percentage of Rtcs and HB levels were significantly higher, compared to control (I). In bleeding rats (IV), RBC's parameters were lower, while Lcs and Plts number, percentage of Rtcs and HB levels were significantly higher, compared to control (I). When compared PHZ- (III) and bleeding-treated (IV) rats, Hct value, Ercs and Plts number, percentage of Rtcs and HB levels were significantly lower in group IV, indicating that anaemia in bleeding rats was more pronounced than in PHZ-treated rats. Moreover, PHZ treatment induced a 2.58-fold higher reticulocytosis compared with bleeding treatment. In addition, extreme elevation of Heinz bodies' level (12.58-fold higher than in control group), indicated oxidative stress appearance in RBC of PHZ-treated rats.

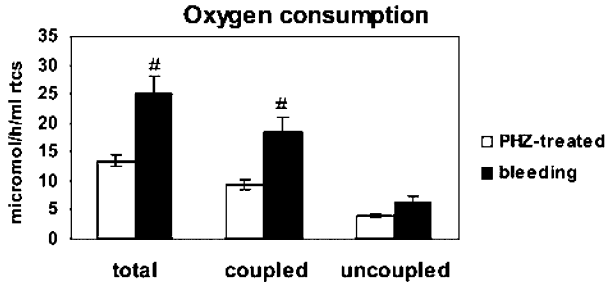
Table 1
The haematological parameters in control (I and II), PHZ-treated (III) and bleeding rats (IV)

	I	II	III	IV
Hct (L/L)	0.43 ± 0.02	0.39 ± 0.02	0.40 ± 0.02	0.30 ± 0.06*#
Hb (g%)	12.02 ± 0.84	11.89 ± 1.09	8.92 ± 1.21	6.71 ± 0.56*
Ercs ($\times 10^{12}$/L)	7.09 ± 0.35	6.84 ± 0.42	7.18 ± 0.30	5.08 ± 0.53*#
Lcs ($\times 10^{9}$/L)	11.90 ± 1.00	12.16 ± 1.09	10.91 ± 0.66	17.07 ± 2.71*#
Plts ($\times 10^{9}$/L)	203.33 ± 15.77	221.60 ± 10.0	645.71 ± 13.40*	545.37 ± 15.26*#
Rtcs (%)	2.45 ± 0.17	3.67 ± 0.39	84.11 ± 4.60*	32.55 ± 0.96*#
HB	51.16 ± 3.46	59.40 ± 7.77	643.71 ± 23.42*	97.25 ± 4.01*#

Values represent mean ± SEM for 7 animals per each group. *$p<0.05$, compared with control group (I), #$p<0.05$, PHZ-treated versus bleeding rats.

Oxygen consumption and energy production

The total ΔO_2 amounted to 13.48 ± 1.08 and 25.04 ± 3.23 μmol/h/ml rtcs in PHZ and bleeding induced reticulocytes, respectively (Fig. 1). Although PHZ-treatment induced a higher reticulocytosis (Table 1), respiration intensity was lower than in



	III	IV
OxP-ATP prod. µmol/ml cells/h	47.50 ± 4.15	93.10 ± 11.50#

Fig. 1. Oxygen consumption level and energy production in reticulocytes obtained from the PHZ-treated (III) and bleeding rats (IV). Values represent mean ± SEM for 7 animals per each group. #$p < 0.05$, PHZ-treated versus bleeding rats

bleeding-induced reticulocytes (Fig. 1). The coupled ΔO_2 was even "two-fold" lower in PHZ-treated rats. On the basis of the coupled ΔO_2, the energy production in OxP was calculated and showed (Fig. 1). The energy production was even "two-fold" lower in PHZ-treated, compared with bleeding rats, indicating PHZ-induced inhibition of energy producing processes.

ROS and RNS concentrations

The concentration of $O_2^{\cdot -}$ and H_2O_2 in RBC of treated animals are shown in Figure 2. Concentration of $O_2^{\cdot -}$ was significantly higher in PHZ-treated rats (III) compared to control, while significantly lower in bleeding rats (IV), compared to control (I) and PHZ-treated groups (III). Level of H_2O_2 was not changed in the investigated groups of animals (Fig. 2).

RNS (nitrite and peroxynitrite) concentrations are shown in Figure 3. Nitrite level was not different in investigated groups of animals. On the other hand, peroxynitrite level was significantly higher in RBC of PHZ-treated rats (III), compared to control (I) and bleeding rats (IV). These results indicate accumulation of superoxide and peroxynitrite in RBC of PHZ-treated rats. Oxidative stress appearance may be cause of oxygen consumption and energy production damage in PHZ-induced reticulocytes. Therefore, the following step was the determination of AOS enzymes activities.

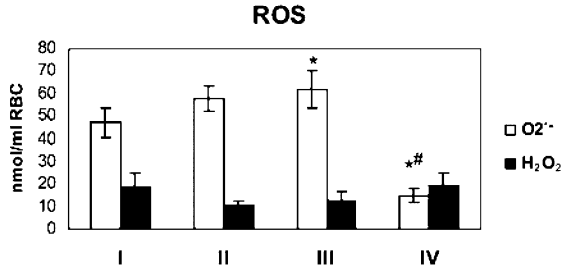


Fig. 2. The lysate ROS concentrations in control (I and II), PHZ-treated (III) and bleeding rats (IV). Values represent mean ± SEM for 5 animals per each group. Values for $O2^{\cdot-}$ are in nmol/ml RBC × 10^{-1}. *p < 0.05, compared with control group (I), #p < 0.05, PHZ-treated versus bleeding rats

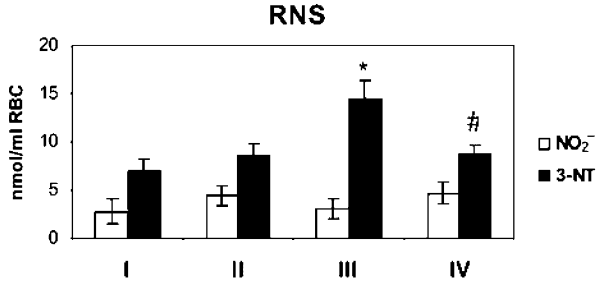


Fig. 3. The lysate RNS concentrations in control (I and II), PHZ-treated (III) and bleeding rats (IV). Values represent mean ± SEM for 5 animals per each group. Values for 3-NT are in nmol/ml RBC × 10^{-1}. *p < 0.05, compared with control group (I), #p < 0.05, PHZ-treated versus bleeding rats

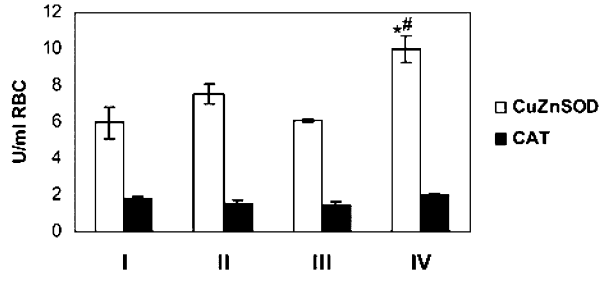


Fig. 4. The activities of lysate Cu, Zn containing superoxide dismutase (CuZnSOD) and catalase (CAT) in control (I and II), PHZ-treated (III) and bleeding rats (IV). Values represent mean ± SEM for 5 animals per each group. Values for CuZnSOD are in U/ml RBC × 10^4, and for CAT are in U/ml RBC × 10^6. *p < 0.05, compared with control group (I), #p < 0.05, PHZ-treated versus bleeding rats

AOS enzyme activities

Figure 4 shows that the CuZnSOD activity was significantly higher in bleeding rats (IV), when compared to both control (I) and PHZ-treated rats (III). The CAT activity was not different in the investigated groups of rats.

DISCUSSION

Anaemia is usually induced by bleeding or PHZ treatment under experimental conditions [21]. Successive bleeding of animals or humans causes stimulation of erythropoiesis, and results in the increase of number of reticulocytes in peripheral blood. PHZ-induced anaemia is the result of rapid haemolysis due to damage and complex interactions between PHZ and RBCs' proteins and lipids [26]. Results of this study showed that PHZ treatment induced even $84.11 \pm 4.60\%$ reticulocytes, which was 2.58-fold higher than in bleeding rats. Our results are in accordance with the published data [10, 12, 14–16, 28]. In addition, according to the presented data, severe anaemia occurred in bleeding, compared with PHZ-treated rats, although Redondo et al. [22] showed similar decrease of Hct and Hb concentration in two of the same experimental groups. Our results showed that PHZ-induced experimental anaemia in rats yielded a greater degree of reticulocytosis with less anaemic problems than bleeding treatment.

Significantly elevated number of leukocytes and platelets in bleeding rats were found in this study. High level of leukocytes may be the consequence of some infection during bleeding procedure, while stimulated megakaryopoiesis followed by high level of platelets may be physiological response of hematopoietic system on bleeding. PHZ treatment also induced higher level of platelets, in comparison to control and bleeding groups of animals. Freedman and Karpatkin [7] showed that intravenous injection of PHZ into intact rabbits resulted in thrombocytosis and megathrombocytosis, as consequence of splenic blockade by RBC haemolysate.

The one of the essential metabolic processes for energy production – OxP was followed in this study. Total and coupled ΔO_2 was almost 2-fold lower in reticulocytes obtained from PHZ-treated than in bleeding rats. Studies *in vitro* showed that PHZ caused inhibition of coupled ΔO_2 in rabbit reticulocytes [28]. Due to the lower coupled ΔO_2 in reticulocytes of PHZ-treated rats, ATP production via OxP was significantly diminished, compared to bleeding rats, indicating PHZ-induced inhibition of energy producing processes.

The presented results showed that concentration of $O_2^{\cdot -}$ and peroxynitrite were significantly higher in PHZ-treated rats, compared to control and bleeding rats. The accumulation of $O_2^{\cdot -}$ and peroxynitrite is the consequence of two facts: (*i*) reticulocytes contain functional mitochondria, which is one of the main source of ROS and RNS in cells [4, 8] and (*ii*) PHZ metabolism in the cells results in the formation of reactive species [18]. Peroxynitrite formation is the result of the reaction between nitric oxide (NO) and $O_2^{\cdot -}$. NO level (determined as its oxidative product – nitrites)

was not different in investigated groups of animals, and perhaps NO was metabolized to peroxynitrite. Taking everything into account, the depletion of energy production during OxP in reticulocytes of PHZ-treated rats was the consequence of irreversible inhibition of enzymatic complexes (I–V) of the respiratory chain by peroxynitrite [4]. In feedback, high production of $O_2^{\cdot -}$ was a consequence of PHZ-induced inhibition of OxP [4].

The high level of $O_2^{\cdot -}$ results in extremely elevated formation of Heinz bodies in RBC of PHZ-treated rats (12.58-fold higher than in control group and 6.62-fold higher than in bleeding group) and indicate oxidative stress appearance. Namely, in RBCs, PHZ reacts with oxyhaemoglobin to form methaemoglobin, oxidises to haemichrome, and causes Heinz body formation [26]. Due to the fact that there was not any change in lipid peroxidation in the investigated groups of animals (data not shown), it seems that proteins (haemoglobin) are the main site of PHZ-induced damage of RBC, which is in accordance with literature data [17]. In addition, 1.90-fold higher levels of Heinz bodies in bleeding rats, compared to control, indicate instability of Hb molecules in these cells as the consequence of oxidative stress.

The activities of CuZnSOD and CAT, two AOS enzymes included in $O_2^{\cdot -}$ and H_2O_2 metabolism were not different in RBC of PHZ-treated rats. On the other hand, the CuZnSOD activity was significantly higher in bleeding rats, compared to control and PHZ-treated rats. The high CuZnSOD activity in bleeding RBCs may be the consequence of erythrocyte maturation and the cause of lower $O_2^{\cdot -}$ concentration in RBC of bleeding rats.

In conclusion, bleeding-induced experimental anaemia in rats was followed by relatively low amount of reticulocytes and low level of haematological parameters in peripheral blood (strong anaemia). The RBCs obtained after bleeding had stable energy production and redox status. On the other hand, anaemia caused by PHZ yielded a greater degree of reticulocytosis with lower haematological anaemic problems. There is inhibition of energy production in PHZ-induced reticulocytes, as the consequence of oxidative stress in these cells. However, the energy processes in PHZ-induced reticulocytes were stable enough, resulting in maturation of these cells into normal erythrocytes [10]. Differences in these two experimental models for reticulocytosis may be used as tools for appropriate pharmacological testing of redox-active substances.

ACKNOWLEDGEMENTS

This work was supported by the Ministry for Science and Technological Development of the Republic Serbia, Grant No 143035B. This work is dedicated in memory to late Prof. dr Milosav M. Kostić.

REFERENCES

1. Auclair, C., Voisin, E. (1985) Nitroblue tetrazolium reduction. In: Greenwald, R. A. (ed.) *Handbook of Methods for Oxygen Radical Research.* CRC Press, Inc., Boca Raton, pp. 123–132.
2. Bates, D. A., Winterbourn, C. C. (1984) Haemoglobin denaturation, lipid peroxidation and haemolysis in phenylhydrazine-induced anemia. *Biochem. Biophys. Acta 798*, 84–87.
3. Beutler, E. (1982) Catalase. In: Beutler, E. (ed.) *Red Cell Metabolism. A Manual of Biochemical Methods*. Grune and Stratton, Inc., New York, pp. 105–106.
4. Brown, G. C., Borutaite, V. (2002) Nitric oxide inhibition of mitochondrial respiration and its role in cell death. *Free Radic. Biol. Med. 33,* 1440–1450.
5. Diwan, A., Koesters, A. G., Capella, D., Geiger, H., Kalfa, T. A., Dorn, G. W. 2nd (2008) Targeting erythroblast-specific apoptosis in experimental anemia. *Apoptosis 13*, 1022–1030.
6. Drabkin, D., Austin, H. (1935) Spectrophotometric studies preparations from washed blood cells. *J. Biol. Chem. 112,* 51–55.
7. Freedman, M.L., Karpatkin, S. (1975) Heterogeneity of rabbit platelets. IV. Thrombocytosis with absolute megathrombocytosis in phenylhydrazine-induced hemolytic anemia in rabbits. *Thromb. Diath. Haemorrh. 33*, 335–340.
8. Gonchar, O., Mankovskaya, I. (2009) Effect of moderate hypoxia/reoxygenation on mitochondrial adaptation to acute severe hypoxia. *Acta Biol. Hung. 60,* 185–194.
9. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., Tannenbaum, S. R. (1982) Analysis of nitrate, nitrite and [15N]nitrate in biological fluids. *Anal. Biochem. 126*, 131–138.
10. Kostić, M. M., Živković, R. V., Rapoport, S. M. (1990) Maturation-dependent changes of the rat reticulocyte energy metabolism and hormonal responsiveness. *Biomed. Biochim. Acta 49*, 178–182.
11. Magnani, M., Rossi, L., Cucchiarini, L., Stocchi, V., Fornanini, G. (1988) Effect of phenylhydrazine on red blood cell metabolism. *Cell Biochem. Funct. 6*, 175–182.
12. Maletić, S. D., Kostić, M. M. (1999) Effects of nitroglycerin on energy metabolism of rat reticulocytes. *J. Physiol. Pharmacol. 50*, 75–87.
13. Marklund, S., Marklund, G. (1974) Involvement of superoxide anion radical in the autoxidation of pyrogallol and convenient assay for superoxide dismutase. *Eur. J. Biochem. 47*, 469–474.
14. Marković, S. D., Ognjanović, B. I., Štajn, A. Š., Žikić, R. V., Saičić, Z. S., Radojičić, R. M., Spasić, M. B. (2006) The effects of nitroglycerine on the redox status of rat erythrocytes and reticulocytes. *Physiol. Res. 55*, 389–396.
15. Marković, S. D., Vukajlović, M. Dj., Ognjanović, B. I., Štajn, A. Š., Žikić, R. V., Saičić, Z. S., Radojičić, R. M., Jones, D. R., Spasić, M. B. (2007) The effects of molsidomine and 3-morpholinosydnonimine on redox status of rat erythrocytes and reticulocytes: a comparative study. *Cell Biochem. Funct. 25*, 251–258.
16. Marković, S. D., Milošević, M. P., Djordjević, N. Z., Ognjanović, B. I., Štajn, A. Š., Saičić, Z. S., Spasić, M. B. (2009) Time course of hematological parameters in bleeding-induced anemia. *Arch. Biol. Sci. 61*, 165–170.
17. McMillan, D. C., Powell, C. L., Bowman, Z. S., Morrow, J. D., Jollow, D. J. (2005) Lipids versus proteins as major targets of pro-oxidant, direct-acting hemolytic agents. *Toxicol. Sci. 88,* 274–283.
18. Nakanishi, A., Kinuta, K., Abe, T., Araki, K., Yoshida, Y., Liang, S., Li, A., Takei, K., Kinuta, M. (2003) Formation of *meso*, *N*-diphenylprotoporphyrin IX by an aerobic reaction of phenylhydrazine with oxyhemoglobins. *Acta Med. Okayama 57*, 249–256.
19. Pick, E., Keisari, Y. (1980) A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *J. Immunol. Meth. 38*, 161–170.
20. Ramot, Y., Koshkaryev, A., Goldfarb, A., Yedgar, S., Barshtein, G. (2008) Phenylhydrazine as a partial model for β-thalassemia red blood cell hemodynamic properties. *Br. J. Haematol. 140*, 692–700.
21. Rapoport, S. M. (1986) *The Reticulocyte*. CRC Press, Inc., Boca Raton, Florida.
22. Redondo, P. A., Alvarez, A. I., Diez, C., Fernandez-Rojo, F., Prieto, J. G. (1995) Physiological response to experimentally induced anemia in rats: a comparative study. *Lab. Anim. Sci. 45*, 578–583.

23. Riordan, J. F., Vallee, B. L. (1972) Nitration with tetranitromethane. In: Hirs, C. H. W., Timasheff, S. N. (eds) *Methods in Enzymology.* Academic Press, New York, pp. 515–521.
24. Savill, N. J., Chadwick, W., Reece, S. E. (2009) Quantitative analysis of mechanisms that govern red blood cell age structure and dynamics during anaemia. *PLOS Comput. Biol. 5*, 1–19.
25. Siems, W., Müller, M., Dumdey, R., Holzhütter, H. G., Rathmann, J., Rapoport, S. M. (1982) Quantification of pathways of glucose utilization and balance of energy metabolism of rabbit reticulocytes. *Eur. J. Biochem. 124,* 567–576.
26. Stern, A. (1989) Drug-induced oxidative denaturation in red blood cells. *Semin. Hematol. 26*, 301–306.
27. Umbreit, W. W., Burris, R. H., Stauffer, F. (1964) *Manometric Techniques*. Burgess Publishing Co., Minneapolis, pp. 1–17.
28. Živković, R. V., Kostić, M. M., Siems, W., Werner, A., Mojsilović, L. P., Gerber, G. (1990) Effects of phenylhydrazine hydrochloride on energy metabolism in rabbit erythrocytes and reticulocytes. *Biomed. Biochim. Acta 49*, 172–177.