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TIME COURSE OF HEMATOLOGICAL PARAMETERS IN BLEEDING-INDUCED ANEMIA

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Abstract — In order to investigate daily changes of hematological parameters in bleeding-induced anemia, we treated Wistar albino male rats by daily bleeding (1.5-2 mL of blood from the tail vein for eight days). Blood samples were taken before (on day zero) and on the first to eighth days of bleeding. The values of hematocrit, hemoglobin, and erythrocyte count decreased significantly after the second, sixth, and second days of bleeding, respectively. The number of leukocytes and platelets, as well as Heinz body levels, increased significantly after the third and second days of treatment. The percentage of reticulocytes increased significantly from the second day and attained the maximum level (32.55 ± 0.96%) on the eighth day.

Key words: Anemia, bleeding, hematological parameters, rats

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INTRODUCTION

In mammals, erythropoiesis is extravascular; if a red cell begins to move into circulation before enucleating, its nucleus is retained in the extravascular space, while the remainder of its cell body passes through the endothelium and is released as a reticulocyte (Wilson and Tavassoli, 1994). The process of erythropoiesis includes maturation of hematopoietic stem cells into mature erythrocytes through increased hemoglobin synthesis and loss of genetic materials and all organelles.

Under normal conditions, reticulocytes are the youngest erythrocytes released from the bone marrow into circulating blood. They mature for one to three days within the bone marrow and circulate for one or two days before becoming mature erythrocytes. The hemoglobin of red cells appears to be synthesized during the early stages of maturation, and its formation is more or less complete by the time the cells leave the bone marrow (Wilson and Tavassoli, 1994). In animal systems, erythropoietic study using blood cells from the peripheral circulation is possible only through the induction of physiological stress, providing the release of a large number of immature cells into peripheral blood.

The most common procedure is the induction of hemolytic anemia by bleeding or by phenylhydrazine-hydrochloride (PHZ) treatment. Excessive bleeding is the most common cause of anemia. When blood is lost, the body quickly pulls water from tissues outside the bloodstream in an attempt to keep the blood vessels filled. As a result, the blood is diluted, and the hematocrit (the percentage of red blood cells in the total blood volume) is reduced. Eventually, increased production of red blood cells may correct the anemia. Over time, bleeding can reduce the amount of iron in the body, so that the bone marrow is not able to increase production of new red blood cells to replace the lost ones. The amount of bleeding-induced reticulocyte generation is a 30-40% increase (Grune et al., 1990; Živković et al., 1990), whereas PHZ-induced reticulocytosis in rats is over 80% (Kostić et al., 1990; Maletić et al., 1999, 2004; Maletić and Kostić, 1999; Marković et al., 2006, 2007).

In order to induce high reticulocytosis, since

reticulocytes are a valuable source of information about many metabolic pathways (especially the oxidative stress response system), we experimentally induced anemia by successive daily bleeding. Anemia is strictly defined as a decrease in red blood cell (RBC) mass. Methods for measuring RBC mass are time-consuming and expensive, and usually require transfusion of radio-labeled erythrocytes. Thus, in practice, anemia is usually discovered and quantified by measurement of the RBC count, hemoglobin (Hb) concentration, and hematocrit (Hct).

In order to evaluate hematological profiles, modification of hematocrit and hemoglobin and changes in the number of erythrocytes (Ercs), leukocytes (Lcs), platelets (Plts), and reticulocytes (Rtcs), as well as the level of Heinz body formation, were monitored in bleeding-treated rats over a period of time.

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, 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*. Reticulocytosis was induced by daily bleeding of rats (1.5-2 mL of blood from tail vein) for eight days. Blood samples were taken before (at zero days) and on the first to eighth days of bleeding.

Hematocrit values (Hct), hemoglobin concentration (Hb), and amounts of erythrocytes (Ercs), leukocytes (Lcs), and platelets (Plts), as well the percentage of reticulocytes (Rtcs), were measured in the collected blood samples. Heinz body (HB) formation was also determined in the collected blood samples.

Hematological parameters

Hematocrit values were determined using the full blood taken with standard microhematocritic tubes (75 mm long) and centrifuged for 5 min at 12000 rpm. Values were expressed in liters of RBCs per liter of blood (L/L).

Hemoglobin concentration in blood and lyzate of RBCs was determined by the cyanmethemoglobin method (Drabkin and Austin, 1935). In a reagent solution, the ferrous ions (Fe^{2+}) of hemoglobin are oxidized to the ferric (Fe^{3+}) state by potassium ferricyanide (KCN) to form methemoglobin. Methemoglobin subsequently reacts with the cyanide ions provided by potassium cyanide to form cyanmethemoglobin. The amount of cyanmethemoglobin can be measured spectrophotometrically at a wavelength of 546 nm and expressed in mmoles/L of blood.

Amounts of erythrocytes, leukocytes, and platelets were counted microscopically and expressed in 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.

Supravital dying technique was used to measure the amount of reticulocytes, since under a microscope with immersion glass, *substantia reticulofilamentosa* (which is an artificial residue and a supravital phenomenon after dying) could be seen. The amount was expressed in % of reticulocytes (number of reticulocytes per total number of RBC x 100%).

Heinz body formation level

The amount of Heinz bodies was determined by turbidometric measurement (Bates and Winterbourn, 1984). Cell suspension (0.1 mL) and Na-PO4 buffer (3 mL, 5 mM, pH 7.4) were mixed together and incubated for 15 min at room temperature in the dark. The amount of HB formation can be measured spectrophotometrically at a wavelength of 700 nm.

Statistical analysis

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

RESULTS

In order to produce a high level of reticulocytes and obtain data about time-dependent changes in hematological parameters during induction, experimental anemia was induced by the method of successive daily bleeding. Blood samples were collected at various times and hematological profiles were evaluated.

The results showed that the values of Hct (Fig. 1), Hb (Fig. 2), and Ercs (Fig. 3) were decreased

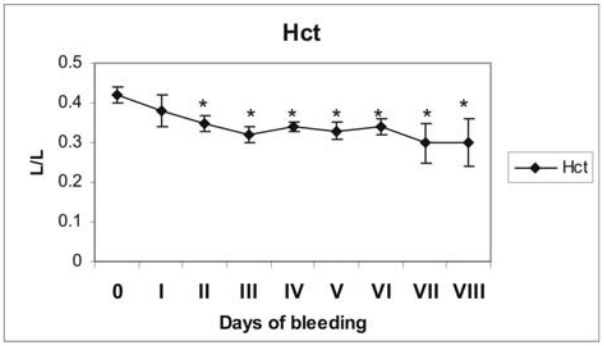

Fig. 1. Time course of Hct values during bleeding treatment of rats. Values represent means ± SEM for five animals. *p < 0.05, compared with control day (0).

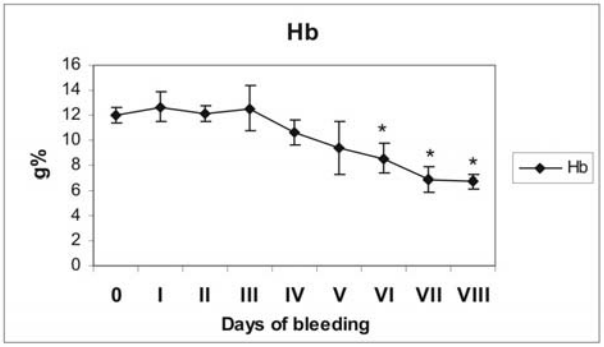

Fig. 2. Time course of Hb concentration during bleeding treatment of rats. Values represent means ± SEM for five animals. *p < 0.05, compared with control day (0).

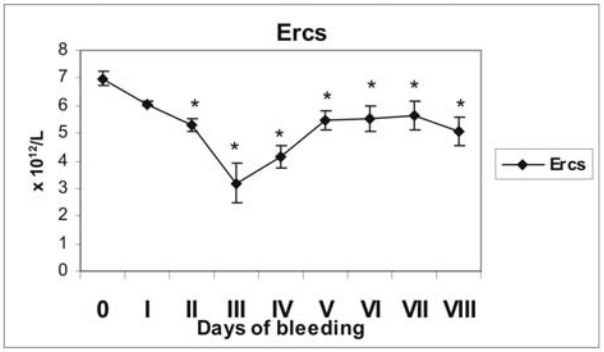

Fig. 3. Time course of Ercs number during bleeding treatment of rats. Values represent means ± SEM for five animals. *p < 0.05, compared with control day (0).

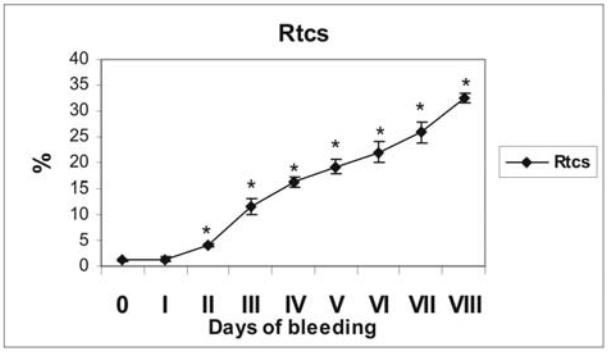

Fig. 4. Time course of Rtcs percentage during bleeding treatment of rats. Values represent means ± SEM for five animals. *p < 0.05, compared with control day (0).

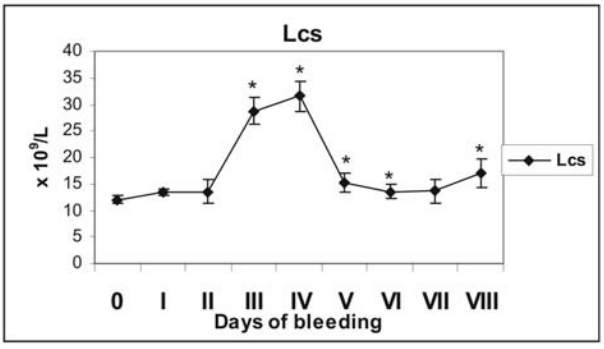

Fig. 5. Time course of Lcs number during bleeding treatment of rats. Values represent means ± SEM for five animals. *p < 0.05, compared with control day (0).

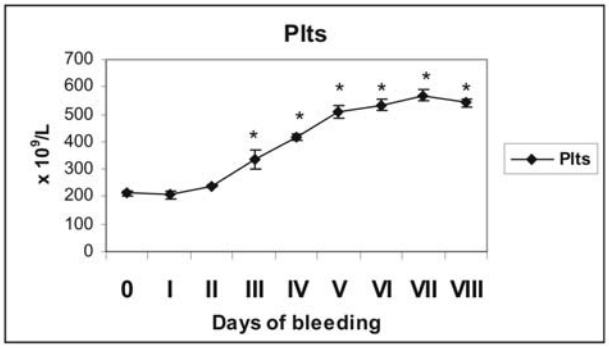

Fig. 6. Time course of Plts number during bleeding treatment of rats. Values represent means ± SEM for five animals. *p < 0.05, compared with control day (0).

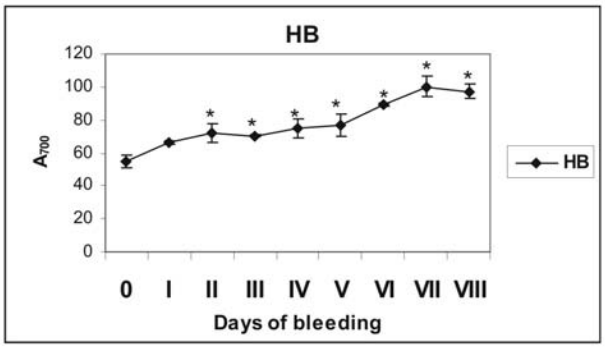

Fig. 7. Time course of Heinz body level during bleeding treatment of rats. Values represent means ± SEM for five animals. *p < 0.05, compared with control day (0).

significantly after the second, sixth, and second days of bleeding, respectively. A tendency toward recovery was evident only in Ercs number after the fifth day, but the control level was not achieved even on the eighth day (Fig. 3). Reticulocyte counts were increased from the second day, and their highest values occurred on the eighth day, when they amounted to 32.55 ± 0.96% (Fig. 4).

The number of both Lcs (Fig. 5) and Plts (Fig. 6) increased significantly after the third day of treatment. The number of Lcs recovered after the fifth day, but the control level was not achieved even on the eighth day (Fig. 5). The number of Plts increased in a time-dependent manner (Fig. 6).

Heinz body counts increased dramatically from the second day ($p < 0.05$) and peaked on the seventh day of bleeding (Fig. 7), indicating oxidative stress in RBCs of bleeding rats.

DISCUSSION

Anemia is usually induced by bleeding or PHZ treatment under experimental conditions (Rapoport, 1986). Successive bleeding of animals or humans caused stimulation of erythropoiesis and resulted in an increased number of reticulocytes in peripheral blood.

The results obtained by following the time dependence of hematological parameters in bleeding rats showed that the values of Hct, Hb, and Erc decreased statistically significantly after the second, sixth, and second days of treatment, while only erythrocytes showed a tendency toward recovery after the fifth day of bleeding. Daily loss of a certain blood volume (1.5-2 mL) induced a decreased capacity for oxygen transportation, i. e., the cell supply with oxygen was insufficient, which caused hypoxic conditions in the organism. The circulating blood of bleeding animals was hypoxic, which induced erythropoetic renal factor formation in the kidneys and consequent intensive erythropoietin production in the liver. The synthesized erythropoietin significantly stimulated erythopoesis in the bone marrow of treated animals (Wilson and Tavassoli, 1994). As a result of stimulated erythropoesis, a high percentage of immature erythrocytes, namely reticulocytes (the last but one stage in the differentiation of erythroid cells), should appear in the blood. This was confirmed by our results – the percentage of reticulocytes in peripheral blood increased depending on time after the second day, with the highest value achieved on the eighth day of bleeding (32.55 ± 0.96%). Due to the increased number of reticulocytes, a tendency to return to control values was expressed after the fifth day of treatment. In the literature, there are data on experimental induction of anemia in rabbits by successive bleeding (Grune et al., 1990; Živković et al., 1990). The intensity of reticulocytosis in those animals was about 30% and was similar to the level in rats shown by our results.

The obtained results indicate that Lcs and Plts counts significantly increased after the third day of bleeding. The number of leukocytes showed the ability to recover, but control values were not achieved even on the eighth day (17.07 ± 2.71). The increase of leukocytes points to the possibility that infections might appear in animals during bleeding from the tail vein. On the other hand, the increase of blood volume due to successive daily bleeding, i. e., successive damaging of blood vessels, results in the physiological reaction of megacariocytopoesis stimulation. All these changes lead to an increased number of platelets and their activation, then to formation of clots and coagulation of blood (Farndale, 2006).

Increase of the Heinz body level in the blood of treated animals was an interesting result of our experiment. Formation of Heinz bodies was more

intensive after the second day, reaching the maximum on the seventh day of bleeding (100.25 ± 6.14). These data indicate the induction of oxidative stress in the blood of animals treated by successive bleeding, since Heinz bodies are inclusions of denaturated globin chains in RBCs (Stern, 1989). Elevation of leukocyte number and consequent high production of reactive oxygen and nitrogen species from these cells (Dröge, 2002) may be the cause of oxidative stress in RBCs.

We have already mentioned that anemia is usually induced by bleeding or PHZ treatment under experimental conditions (Rapoport, 1986). According to previous results, the reticulocytes obtained in the described ways in experimental conditions mature normally and turn into the final stage of erythrocytes (Gronowicz et al., 1984; Kostić et al., 1988; Kostić et al., 1990; Redondo et al., 1995). In the course of induction of anemia by the bleeding method, time-dependent hematological values decrease (hematocrit and hemoglobin level, number of erythrocytes), while leukocytes, platelets, reticulocytes, and Heinz bodies increase. Our results show that none of these parameters attained control values during bleeding. On the other hand, time-dependent changes of hematological parameters during the anemia induced by PHZ are different (Kostić et al., 1988). In the course of treatment with PHZ, hematological values (hematocrit and hemoglobin level, number of erythrocytes) decrease after the third day, but they return to the control level on the seventh and eighth day after the beginning of treatment, when the percentage of reticulocytes is highest (increase of reticulocytosis is time-dependent).

In conclusion, experimental anemia induced by bleeding treatment in rats is characterized by decrease of hematological parameters and subsequent stimulated erythropoiesis and reticulocytosis. Leukocyte and platelet numbers increased, and high production of reactive oxygen and nitrogen species from these cells is a partial cause of increased Heinz body formation and appearance of oxidative stress in RBCs of bleeding rats.

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ВРЕМЕНСКИ ЗАВИСАН ТОК ЕКСПЕРИМЕНТАЛНЕ АНЕМИЈЕ У ПАЦОВА ИЗАЗВАНЕ ДНЕВНИМ КРВАРЕЊЕМ

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У циљу испитивања дневних промена хематолошких параметара у току анемије индуковане дневним крварењем, третирали смо Wistar албино пацове дневним крварењем (1.5-2 ml крви из репне вене у току 8 дана). Узорци крви узимани су пре (0 дан) и 1-8. дана крварења. Вредности за хематокрит, хемоглобин и број еритроцита значајно опадају након другог, шестог и другог дана крварења. Број леукоцита и тромбоцита, као и ниво формирања Heinz-ових телашаца расте значајно након трећег и другог дана третмана. Проценат ретикулоцита значајно расте од другог дана и достиже максимални ниво (32.55 ± 0.96 %) осмог дана тремана.