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Oxidant Potential of Krunidon *In Vitro* and *In Vivo*

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We studied the effect of Fe^{2+} ions in polymerized hemoglobin (Krunidon blood substitute) and in molecular hemoglobin (Sigma) on $OH^{\bullet}$ radical initiation in the Fenton system. It was found that polymerized hemoglobin, as a component of Krunidon preparation, in contrast to hemoglobin tetramer, did not intensify $OH^{\bullet}$ radical generation. The oxidant potential of Krunidon was evaluated *in vivo* by measuring malondialdehyde level in dog blood plasma after repeated intravenous administration (5 days in a dose of 114 mg/kg) as a biomarker. Administration of the preparation did not significantly increased malondialdehyde content on days 1 and 4 after exposure and did not affect total protein content in blood plasma. Our findings suggest that polymerized hemoglobin in the Krunidon preparation exhibits no pro-oxidant activity and can be used as the basis for the development of non-oxygenic forms of blood substitutes.

Key Words: *blood substitute; polymerized hemoglobin; Krunidon; $OH^{\bullet}$ radicals, malondialdehyde*

Steadily increasing need in blood transfusions, as well as deficiency and limited availability of blood products necessitate the development of blood substitute development, in particular, creation of biocompatible media on the basis of natural or modified human and animal hemoglobin, which implements the idea of modeling natural oxygen transport by red blood cells. Infusion of free hemoglobin solutions revealed serious obstacles to its clinical application; in light of this, various methods for its chemical modification were proposed [6]. Blood safety and availability as well as the interests of military industry limited by the storage conditions and delayed use of donor blood components, prompted the development of blood substitute on the basis of cattle hemoglobin. The American company Biopure has created three generations of the Hemopure drug on the basis of glutaraldehyde-polymerized bovine hemoglobin. Oxyglobin is proposed for use in veterinary practice. Hemopure passed 22 clinical trials in USA, Europe, and South Africa and in 2001 it was registered in South Africa [1].

The Medbiofarm company developed the Krunidon, a lyophilizate for preparing of a solution for infusions containing polymerized purified bovine hemoglobin crosslinked with glutaraldehyde and stabilized with dextrose and ascorbic acid [4].

The most important function of hemoglobin-based blood substitutes is gas transport. Oxygen playing a key role in the energy metabolism is capable of spontaneous non-enzymatic reactions of single-electron reduction leading to the formation of radical and molecular ROS, including highly dangerous hydroxyl radical $OH^{\bullet}$. This radical can oxidize with a high rate almost all organic and inorganic cell compounds, which leads

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to irreversible, often negative consequences [3]. A significant contribution to ROS generation is made by metals of alternating valence, including iron ions [13]. Oxygen is transported to various mammalian tissues by complex iron-containing hemoglobin protein with 4 iron-porphyrin rings, carrying 4 Fe(II) atoms. Hemoglobin iron has a unique property: it reversibly binds oxygen without changing its valence and transports it to various tissues. At the same time, under certain conditions, iron in hemoglobin porphyrin rings can intensify LPO, catalyze peroxide compounds decomposition with formation of hydroxyl radical $OH^{\bullet}$ [12]. It is also known that hemoglobin in erythrocytes is not toxic to the body, while free hemoglobin not bound to erythrocytes is toxic and can impair functioning of many organs and tissues and intensify ROS generation and free radical LPO reactions [10,12].

The aim of this study was to assess the effect of Krunidon preparation on initiation of free radical reactions activated by heme or iron ions in this preparation.

MATERIALS AND METHODS

Krunidon (active ingredient polymerized bovine hemoglobin) was provided by the developer. Krunidon dosage form is a lyophilizate for preparation of a solution for infusions; it represents a lyophilized porous sterile powder of dark red color with a characteristic odor and bitter taste. The prepared solution is sterile, clear, dark red, free of sediment and suspended matter. For the study, a ready-made dosage form of Krunidon with the following composition was provided: polymerized hemoglobin (active substance), 2.0 g; auxiliary substances: dextrose, 1.79 g; sodium chloride, 0.2 g; ascorbic acid, 0.01 g (1 vial).

The effect of Krunidon on activation of free radical LPO reactions was assessed *in vitro* using Fenton reaction system where Fe(II) is oxidized by H_2O_2 to Fe(III) catalyzing H_2O_2 decomposition and formation of hydroxyl radical:

$$Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH^{-}+OH^{\bullet}$$ [5].

Phosphate saline buffer, 2-D-deoxyribose (3 mmol/liter), and H_2O_2 in a concentration of 2 mmol/liter were used as ingredients. Fe(II) in Mohr's salt ($FeSO_4 \times (NH_4)_2SO_4 \times 6H_2O$) and Krunidon, as well as blood hemoglobin (Sigma), were used as catalysts of H_2O_2 decomposition. All preparations containing Fe^{2+} were used in concentration of 27.3 μM (by Fe^{2+}).

The reaction mixture was prepared by adding deoxyribose and H_2O_2 to PBS: 0.14 mol/liter NaCl, 20 mmol/liter phosphate buffer (pH 7.4). The reaction was initiated by adding various iron preparations to the reaction mixture. The samples were incubated for 1 h in a water bath at 37°C; then, 1 ml 3% trichloroacetic acid and 1 ml 1% TBA were added. The mixture was incubated at 100°C for 20 min. Optical density was measured at 532 nm on a UV/Vis 2800 spectrophotometer (UNICO-SIS).

The hydroxyl radical actively interacts with the target deoxyribose molecule and induces its degradation. Among the final products specific for the oxidative deoxyribose degradation, malondialdehyde (MDA) was detected. MDA in the acidic medium binds two TBA molecules with the formation of a stable colored complex. High sensitivity and specificity of the reaction of deoxyribose degradation products with TBA determined wide use of TBA products for evaluation of OH-radical generation. MDA has a maximum absorption at λ=532 nm and a molar extinction coefficient of 156,000 optical units. From the obtained optical density, MDA concentration can be calculated and the level of OH-radical generation proportional to this concentration can be determined.

To assess the oxidant potential of Krunidon *in vivo*, its effect on LPO intensity was investigated in dogs (mongrel male dogs weighing 14.7±2.48 kg). The blood was taken from the saphenous vein. Blood plasma was obtained prior to administration and on days 1 and 4 after completion of 5-day course intravenous administration of Krunidon (114 mg/kg; polymerized hemoglobin). Plasma MDA concentration was determined using $KMnO_4$ and $FeSO_4$ by the method [2]. Protein concentration was measured by the biuret method using a commercial kit (Agat-Med).

The data were processed using parametric and nonparametric statistics. Significance of differences between the groups was assessed using Student's *t* test and Mann—Whitney *U* test.

RESULTS

Heme and iron ions released during hemoglobin oxidation are a potential source of toxicity of hemoglobin-based blood substitutes [10]. Iron ions catalyze chemical reactions with participation of oxygen radical anion (Haber—Weiss reaction) and H_2O_2 (Fenton reaction) and organic peroxides leading to the formation of active $OH^{\bullet}$ hydroxyl radicals, the chemical basis of free radical pathologies [5]. Effect of drugs and bioactive compounds on *in vitro* generation of hydroxyl radicals can serve as an indicator of both prooxidant activity of the compounds under study and their antiradical activity reducing generation of this toxic radical capable of damaging cell membranes, DNA, proteins, and lipids [14].

Fe(II) ions in the Mohr's salt activated generation of $OH^{\bullet}$ radical and increased the content of TBA products (Fig. 1). At the same time, iron present in he-

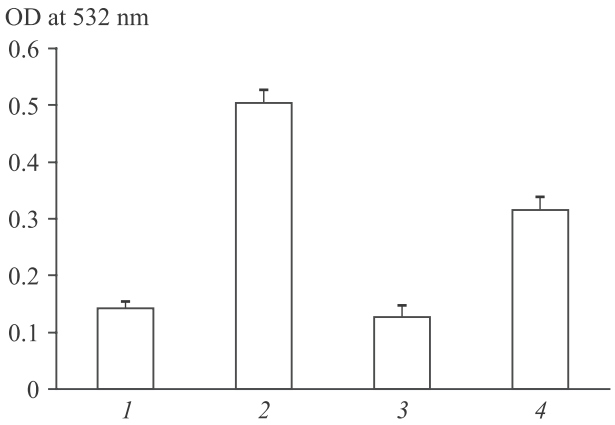

Fig. 1. Effect of Fe^{2+} ions as part of the Mohr's salt (*2*), Krunidon (*3*), and hemoglobin (Sigma) (*4*) on $OH^{\bullet}$ generation. *1*) Baseline (no drugs) level of $OH^{\bullet}$ radical generation.

moglobin of Krunidon preparation did not significantly affected $OH^{\bullet}$ generation. In contrast to Krunidon, hemoglobin (Sigma) enhanced $OH^{\bullet}$ radical generation increasing the content of TBA products. These results indicate that polymerized hemoglobin constituting Krunidon is less toxic than pure hemoglobin (Sigma) and does not intensify $OH^{\bullet}$ hydroxyl radical generation in the Fenton system.

For evaluation of the oxidant potential of Krunidon *in vivo*, its effect on the intensity of LPO was studied in dogs. The increase in concentration of LPO products is considered as universal mechanism cell damage under various pathological conditions, therefore the content of LPO products in the body can be used as a biomarker for evaluation of the effects of drugs and bioactive compounds on the antioxidant protection level. MDA is the most commonly used LPO biomarker [9,14].

Along with phospholipids, albumins play an important role in formation of blood plasma antioxidant and prooxidant properties and can be associated with chronic disorders [11,15]. In this regard, it seems interesting to investigate the effect of intravenous Krunidon administration not only on blood plasma MDA level in dogs, but also on total protein, mainly presented in plasma by albumin.

TABLE 1. Blood Plasma MDA and Protein Level in Dogs at Different Time Points after Intravenous Krunidon Administration (*M*±*m*; *n*=8)

Experimental condition	MDA, μmol/liter	Protein, g/liter
Prior to drug administration	79.26±9.17	76.23±6.11
Day 1 after drug administration	88.10±10.20	69.10±3.00
Day 4 after drug administration	91.10±7.60	71.00±2.90

Krunidon was dissolved in physiological solution and administered intravenously to dogs of the experimental group repeatedly (for 5 days) in a dose of 114 mg/kg, which corresponds to a maximum daily therapeutic dose of 8 g per person. Both plasma MDA and total protein levels were assessed prior to administration and on days 1and 4 after drug administration. Five-fold daily Krunidon administration produced no statistically significant increase in the blood plasma level of MDA (Table 1). Total blood plasma protein level was little altered. These results suggest that Krunidon produces no adverse effect on the body.

It is known that free hemoglobin can intensify ROS generation and free-radical LPO [10,12]. In the study [8], direct quantitative relationship was established between free hemoglobin dose and formation of TBA-reactive products in the complex LPO reaction system *in vitro*. Free hemoglobin is easily subjected to auto-oxidation, as a result of which heme iron (II) is oxidized to iron (III). The proximity of free hemoglobin to endothelial NO sources, as well as insufficiency of natural antioxidant protection give grounds for triggering the cascade of free radical formation reactions, LPO activation and induction of other cytotoxicity and inflammation mechanisms [6]. Other factors, including the presence of erythrocyte stroma fragments, low stability of tetramers that dissociate to low molecular weight dimers within a few hours after the infusion and turn into methemoglobin, and rapid hemoglobin elimination from the bloodstream, also contribute to toxicity of free hemoglobin solutions [6]. Polymerization used in Krunidon manufacturing can be considered as an effective way to eliminate these negative phenomena.

It was established [7] that repeated intravenous administration of low doses (48 and 60 mg/kg) of purified human hemoglobin to Sprague-Dawley rats led to a statistically significant increase in blood plasma MDA and 4-hydroxynonenal concentration. Our results on administration of polymerized endotoxin-free hemoglobin to dogs suggest that the applied therapeutic dose of Krunidon does not initiate free radical LPO reactions based on the blood plasma MDA content data.

Results of *in vitro* and *in vivo* studies suggest that the polymerized hemoglobin in Krunidon preparation exhibit no pro-oxidant activity and can serve as the basis for the development of non-oxygenic forms of blood substitutes.

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