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# Effect of dinitrosyl iron complexes with glutathione on hemorrhagic shock followed by saline treatment

Marina I. Remizova [a], Nikolai I. Kochetygov [a], Konstantin A. Gerbout [a], Vladimir L. Lakomkin [b], Alexander A. Timoshin [b], Evgenia N. Burgova [c], Anatoly F. Vanin [c,*]

[a] Russian Research Institute of Hematology and Transfusiology, Russian Ministry of Public Health, Saint-Petersburg, Russia
[b] Russian Cardiological Research-and-Production Complex, Moscow, Russia
[c] Semenov Institute of Chemical Physics, Russian Academy of Sciences, Moscow, Russia

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ABSTRACT

It has been found that dinitrosyl iron complexes with glutathione (DNIC-GS) injected into the blood flow of rats at a dose of 0.05 μmoles/kg prior to hemorrhage significantly improve cardiac function under conditions of hemorrhagic shock manifested in increased stroke volume, left ventricular work and cardiac output to a level exceeding control values 1.5-fold. Enhanced myocardial contractile activity leads to a situation where mean arterial pressure does not decrease further despite the significant decrease of total peripheral resistance. The decrease of total peripheral vascular resistance of the vascular system under vasodilating effects of DNIC-GS used as nitric oxide donors improves microcirculation in experimental rats judging from increased rates of blood flow and low degree of erythrocyte aggregation. Pretreatment of rats with the complexes significantly increases survival (by 21%) under conditions of hemorrhagic shock. It is suggested that beneficial effects of DNIC-GS on systemic circulation parameters under conditions of hemorrhagic shock are determined by their antioxidant activity and the ability to induce S-nitrosylation of proteins.



## 1. Introduction

EPR-active protein-bound and low-molecular weight mononuclear paramagnetic dinitrosyl iron complexes (DNICs) with thiolate ($RS^-$) ligands (formula $\{(RS^-)_2Fe^+(NO^+)_2\}^+$) are generated in living systems endowed with the ability to synthesize nitric oxide (NO) from endogenous or exogenous sources (Vanin, 2009). Their physiological role consists of accumulation, stabilization and transfer of nitric monoxide (NO) molecules into intra- and extracellular spaces. Incorporation of NO into DNICs ensures effective targeted transport of NO into cells able to release this universal metabolic regulator (Ignarro, 2000) (autocrine effect) and its subsequent delivery to other cells and tissues (paracrine effect). This process is similar to incorporation of NO into S-nitrosothiols, which represents an alternative pathway of NO accumulation and transfer in various body cells and tissues. The ability of DNICs with thiol-containing ligands to act as NO donors is determined by the chemical equilibrium between the complexes and their constituent components (Scheme 1):

$$\{(RS^-)_2Fe^+(NO^+)_2\}^+ \Longleftrightarrow Fe^{2+} + NO + \underbrace{(NO^+ + RS^-)}_{\downarrow\ RS\text{-}NO} + RS^-$$

**Scheme 1.**

According to EPR data, DNICs with thiol-containing ligands are represented predominantly by protein-bound complexes in living cells and tissues (Vanin, 2009). By virtue of low mobility of proteins at ambient temperature, the asymmetric shape of their EPR signal, which is determined by the anisotropy of the g-factor ($g_\perp = 2.04$, $g_{||} = 2.014$ $g_{aver.} = 2.03$), does not change over the temperature range from 77K to ambient (Timoshin et al., 2007). Protein-bound DNICs play the role of NO depots and cannot function as NO carriers due to fairly low translational mobility of proteins. This transfer is effected by low-molecular weight DNICs whose existence in various body cells and tissues is determined by the presence, in the latter, of low-molecular thiols, such as cysteine and glutathione, able to compete with protein-bound DNICs for $Fe(NO)_2$ groups, although in rather a weak manner

* Corresponding author at: Laboratory of Physical Chemistry of Biopolymers, Kosygin Str. 4, Moscow 119991, Russia. Tel.: +7 495 939 75 35; fax: +7 495 651 21 91.
*E-mail address:* vanin@polymer.chph.ras.ru (A.F. Vanin).

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(Vanin, 1998). Therefore, low-molecular weight DNICs with thiol-containing ligands have every reason to be regarded as signaling agents responsible for regulation of an immense variety of biochemical and physiological processes occurring in living organisms (Vanin, 1998, 2009).

Low-molecular weight DNICs with thiolate ligands can easily be synthesized by the chemical route and produce miscellaneous physiological effects when injected into animals. Thus, it was found that bolus administration of synthetic low-molecular weight DNIC-GS or cysteine (DNIC-CYS) (2–3 μmoles/kg of body weight) induces long-lasting hypotension (Lakomkin et al., 2007) in experimental rats. In addition, DNICs initiate effective relaxation of isolated blood vessels (Flitney et al., 1992; Vanin et al., 2007; Vedernikov et al., 1992), suppress platelet aggregation (Mordvintcev et al., 1986), reduce the myocardial infarction zone in isolated hearts and in animals with experimental myocardial infarction (Pisarenko et al., 2008), accelerate skin wound healing (Shekhter et al., 2007) and provoke penis erection (Veliev et al., 2008) in experimental animals. Besides, they possess pro- and anti-apoptotic activities (Kim et al., 2000; Kleschyov et al., 2006) and modulate the function of certain genes (Ding and Demple, 2000; Vasil'eva et al., 2001).

Our previous studies on rats demonstrated that DNIC-GS (3.2 μmoles/kg of body weight) enhance myocardial contractile activity manifested as increased cardiac output, stroke volume and left ventricular work (Remizova et al., 2008). The beneficial effect of DNIC-GS on the energy state of the myocardium under ischemia–reperfusion is documented in the literature (Pisarenko et al., 2008). Based on these findings, we set out to examine the feasibility of improving cardiac function by treating rats with DNIC-GS under conditions of hemorrhagic shock followed by saline infusion.

Considering that hemorrhagic shock is concomitant with a drop in arterial pressure (Kelly et al., 1997; Kochetygov et al., 2003; Kochetygov and Kulikov, 1982), we hypothesized that treatment of rats with hypotensive and higher (≥2 μmoles/kg) doses of DNIC-GS (Lakomkin et al., 2007) can accelerate death from blood loss. It seemed, therefore, expedient to examine the beneficial effect of DNIC-GS on animals with hemorrhagic shock by using low (non-hypotensive) doses of the aforementioned complexes. The results obtained corroborated the efficacy of this approach to the study of DNIC-GS treatment for hemorrhagic shock. Thus, treatment of rats with DNIC-GS (0.05 μmoles/kg) had a beneficial effect on systemic circulation parameters and increased survival of experimental animals.

## 2. Materials and methods

### 2.1. Materials

Reduced glutathione and bathophenanthroline disulfonate were purchased from Sigma, USA. Ferrous sulfate was from Fluka (Switzerland).

### 2.2. Synthesis of DNIC-GS and S-nitrosoglutathione (GSNO) and experiment protocols

DNICs with glutathione were synthesized based on the ability of S-nitrosothiols, viz., S-nitrosoglutathione (GSNO), to form DNICs in the presence of iron (II) and thiols (Vanin et al., 1997).

#### 2.2.1. Protocol — synthesis of GSNO

Glutathione (180 mg) was dissolved in 1 ml of distilled water. Because of strongly acidic properties of glutathione, pH of the solution was decreased to 2.5 allowing the formation of GSNO with the addition of 3.5 mg of sodium nitrite in 0.1 ml of distilled water. Immediately after sodium nitrite addition, the solution acquired a pink color indicative of GSNO formation. Then, 4 mL of 15 mM HEPES buffer (pH 7.4) was added to the solution, and the pH was adjusted to 7.4 by dropwise addition of a saturated solution of NaOH.

#### 2.2.2. Protocol — synthesis of DNIC-GS

A mixture of ferrous sulfate (83 mg) and sodium citrate (400 mg) was dissolved in 1 ml of distilled water after which 0.1 ml of the ferrous sulfate citrate complex was added to 5 ml of the GSNO solution as described above. After mixing the citrate iron complex with GSNO, the color of the solution turned orange–green due to the formation of DNIC-GS. The concentration of DNIC-GS in this solution was 5 mM. To obtain stable complexes, the solution was dried in the presence of a water-soluble polymer as described in the Patent of the Russian Federation No. 2 291 880. The resulting preparation was stored in dry air for at least 1 year without any degradation (Vanin et al., 2005).

### 2.3. Animals

The experiments with hemorrhagic shock were performed on female Wistar albino rats weighing 230 to 240 g in full conformity with the Geneva Convention "International Guiding Principles for Biomedical Research Involving Animals" (Geneva, 1990). The hypotensive effect of DNIC-GS and the electron paramagnetic resonance (EPR) of blood samples were assayed in a female Wister albino rat weighing 550 g.

### 2.4. Animal studies

#### 2.4.1. Protocol — experiments with hemorrhagic shock

The animals anesthetized with a single dose of sodium thiopental (35–40 mg/kg) dissolved in distilled water were fixed in a frame. A catheter was inserted into the carotid artery for measuring mean arterial pressure and blood sampling. The stroke volume was determined using the tetrapolar rheography method (Karpinsky et al., 1986). Heart rate was estimated on the basis of the ECG data. Cardiac output (ml/min · 100 g of body weight) was calculated as (cardiac output) • (heart rate); total peripheral resistance (dyne $s\,cm^{-5} \cdot 10^4$/kg of body weight) was determined as (mean arterial pressure)/(cardiac output) · 1332 · 60 · $10^{-4}$/kg; left ventricular work (kGm/kg · min) was estimated as (mean arterial pressure) · (cardiac output) · 0.0135. Microcirculation in the serous membrane of the small intestine was studied using vital contact microscopy in reflected light. Quantitative estimation of the experimental results was performed using a scale developed by E. Bloch (Bloch, 1954) and T. Ditsel (Ditsel, 1959) and modified in our laboratory (Kochetygov and Kulikov, 1982). The microcirculation was estimated in the following way. The initial (normal) state of the blood flow in rat mesentery capillaries in the absence of erythrocyte aggregates was conventionally taken for 0. The decrease in the rate of the capillary blood flow where erythrocytes were still clearly visualized in the circulating blood was estimated as −1 score. The plasma clearance in the intererythrocyte space with further retardation of the blood flow was estimated as −2 scores. The pendular movement of erythrocytes interrupted by periodic arrests was estimated as −3 scores. Complete arrest of erythrocyte movement was estimated as −4 scores. The appearance of single erythrocyte aggregates in blood capillaries was estimated as +1 score. The presence, in the capillaries, of multiple consecutive platelet aggregates was estimated as +2 scores, while the occlusion of the capillaries with platelet aggregates, as +3 scores. The gas content and the acid–base balance of arterial blood were measured with the help of a gas analyzer ABL-500 ("Radiometer").

Hemorrhagic shock was induced by fractional bleeding from the carotid artery for 30–40 min, until the mean arterial pressure dropped down from the initial level (141–142 mm Hg) to 50–60 mm Hg. The common volume of the bleeding consisted of 2.8 ± 0.3 ml/100 g of

body mass. The animal studies were performed in two series. In the first series (control), prior to hemorrhage the animals (n = 13) were treated with 1.0 ml of an isotonic 0.9% solution of NaCl for 20 min after which saline infusion was resumed to the volume of the escaped blood. In the second series, prior to hemorrhagic shock the animals (n = 8) were injected intra-arterially with 0.05 μmoles/kg of DNIC-GS dissolved in 1.0 ml of isotonic saline for 20 min. After cessation of hemorrhage, the isotonic solution was infused to animals in the same volume as in the first series.

Blood samples (0.02 ml) were collected from animals in the initial state, during hemorrhagic shock (prior to saline infusion) and 10 and 60 min after termination thereof. The volume of collected blood was taken into consideration during estimation of the total amount of lost blood. The extent of hemorrhagic shock was estimated as severe since it resulted in the death of all experimental animals not receiving infusion therapy. In the control group (saline), 50% of rats died within 24 h.

Systemic circulation and microcirculation parameters were estimated before and after hemorrhage 10 and 60 min after saline infusion. Blood sampling for measuring gas content and estimation of the acid–base balance in rat blood were performed in the same period.

*2.4.2. Protocol — estimation of the hypotensive effect of DNIC-GS and the electron paramagnetic resonance (EPR) assay of blood samples*

Arterial blood samples of 0.3 ml volume were taken every 3 min. following intravenous injection of DNIC-GS (0.05, 0.5, 3.0 and 10.0 μM/kg of body weight) for EPR assays. Heparin was added to each sample prior to rapid freezing in liquid nitrogen and storage. In parallel experiments, the drop of mean arterial pressure was measured after 3-min treatment of animals with the indicated dose of DNIC-GS.

### 2.5. EPR assays

EPR spectra were recorded at ambient temperature using a Varian E-109E EPR spectrometer (USA) (modulation frequency — 100 kHz; modulation amplitude — 0.2 mT; microwave power — 10 mW).

### 2.6. Statistical analysis

Statistical analysis was performed using Statistica 7.0 software. Statistic group differences were calculated using the Mann–Whitney's *U*-test. All the values were expressed as mean ± S.E.M. of *n* observations, where *n* is the number of animals. $P \leq 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Hypotensive activity of DNIC-GS

Earlier studies of DNIC-CYS (Lakomkin et al., 2007) demonstrated that a dramatic (up to 50%) drop of mean arterial pressure induced by intravenous injection of DNIC-CYS was observed even at low (up to 0.1 μM/kg) doses of DNIC-CYS. At 0.05 μM/kg DNIC-CYS, the mean arterial pressure dropped down to 40%. With an increase in the DNIC-CYS dose, the hypotensive effect of the complexes was attenuated. Consequently, after injection of 2.74 μM/kg DNIC-CYS the decrease of mean arterial pressure did not exceed 65% (Lakomkin et al., 2007).

The decrease of mean arterial pressure induced by DNIC-CYS (Lakomkin et al., 2007) was biphasic. The first (short-term) phase did not exceed several minutes and was characterized by the most conspicuous (≤65%) drop of mean arterial pressure due to formation of NO following the decomposition of the greater part of low-molecular weight DNIC-CYS. The remaining DNICs were converted into the protein-bound form with simultaneous formation of more stable protein-bound DNICs as a result of a transfer of $Fe(NO)_2$ groups from low-molecular weight DNIC-CYS to the thiol groups of proteins. It is the appearance of these DNICs that was the reason for the sustained (up to 1 h) drop of mean arterial pressure (10–35% of the initial level depending on the DNIC-CYS dose). Such a long-lasting decrease of mean arterial pressure was induced by NO molecules released from a small fraction of low-molecular weight DNIC-CYS existing at a chemical equilibrium with protein-bound DNICs (see Introduction).

The dose dependence of the hypotensive effect of DNIC-GS was not studied by the above-cited authors (Lakomkin et al., 2007). However, it was found that the first (short-term) phase of the mean arterial pressure drop was much shorter than in the case of DNIC-CYS, which was attributed to the high stability of DNIC-GS (Lakomkin et al., 2007). It may therefore be assumed that low (0.05–0.1 μM/kg) doses of DNIC-GS might induce only a weak hypotensive effect without causing rapid death of animals with hemorrhagic shock concomitant with a drop of mean arterial pressure (Kelly et al., 1997; Kochetygov et al., 2003; Kochetygov and Kulikov, 1982).

Our experiments with a single large rat weighing 550 g corroborated this hypothesis. Consecutive intravenous injections of DNIC-GS induced a dose-dependent drop of mean arterial pressure. At 0.05 μM/kg DNIC-GS, this drop did not exceed 5 mm Hg, while at 0.5, 3.0 and 10.0 μM/kg DNIC-GS the drop of mean arterial pressure was as high as 20, 60 and 70 mm Hg, respectively. Noteworthy, this decrease was short-term lasting no more than 3–4 min. Detection of a subsequent (more long-lasting) phase of the mean arterial pressure decrease induced by 0.5 and 3.0 μM/kg DNIC-GS was beyond the scope of the present study and was observed only after injection of DNIC-GS at a dose of 10.0 μM/kg of body mass. In this case, the rapid decrease of mean arterial pressure was followed by a sustained decrease of mean arterial pressure (Fig. 1) with a gradual slow return to the initial level (130 mm Hg).

The EPR analysis of the blood samples collected 3 min after DNIC-GS injection revealed the presence of protein-bound DNICs as could be judged from the appearance of a relatively broad EPR signal with a half-width of 4.0 mT at ambient temperature. This signal had an asymmetric shape due to the axial symmetry of the g-factor tensor at $g_{\perp} = 2.04$, $g_{||} = 2.014$, $g_{aver.} = 2.03$ (Fig. 2). A similar signal was recorded in earlier experiments on rats of a lower body weight (200–250 g) (Lakomkin et al., 2007). The lack of a narrow symmetric EPR signal characteristic of low-molecular DNICs with a half-width of 0.7 mT and a peak at $g = 2.03$ at ambient temperature (Vanin et al., 1998) suggests that on entering the blood flow the greater part of low-molecular weight DNIC-GS was converted into protein-bound DNICs. Other low-molecular weight DNIC with thiol-containing ligands existing at equilibrium with protein-bound DNICs could not be detected by the EPR method due to their low concentration (NB: the sensitivity of the EPR method for low-molecular weight DNICs is <100 nM) (Vanin, 2009).

The narrow linewidth of the EPR signal of low-molecular weight DNICs at ambient temperature can be attributed to greater mobility inducing the averaging of the g-factor anisotropy (Vanin et al., 1998). No such averaging takes place in the case of protein-bound DNIC due to decreased mobility of the DNIC-containing protein globule (Vanin et al., 1998). It is the anisotropic shape of the EPR signal recorded in the blood at ambient temperature and the lack of a narrow EPR signal characteristic of exogenous low-molecular weight DNICs that unambiguously suggest that DNICs appearing in the circulating blood largely represent protein-bound complexes.

Similar experiments on rabbits established that protein-bound DNICs appearing in animal blood after injection of low-molecular weight DNICs are predominantly localized in the plasma where they exist in the form of albumin-bound DNICs (Timoshin et al., 2007). At ambient temperature, these DNICs generate a broader (compared to that shown in Fig. 2) EPR signal with the following g-factor values: $g_1 = 2.05$, $g_2 = 2.03$, and $g_3 = 2.014$ (Timoshin et al., 2007). The

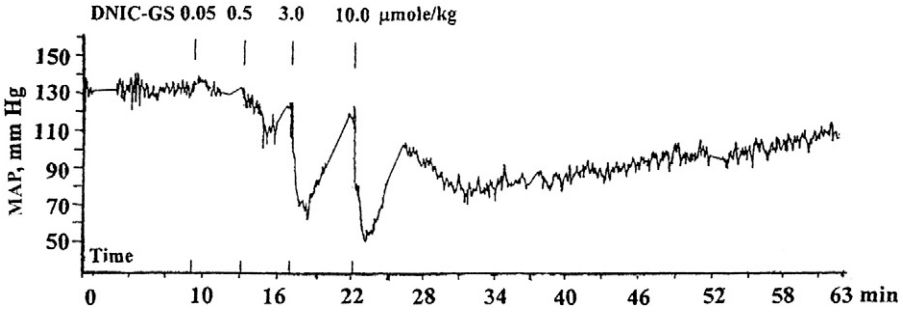


**Fig. 1.** The time-dependent changes (3 min) in the mean arterial pressure (MAP) after consecutive (3 min) intravenous treatment of a rat (550 g) with DNICs with glutathione (0.05, 0.5, 3.0 and 10.0 μmoles/kg). The long-lasting changes in MAP were recorded at 10.0 μmoles/kg DNICs.

difference between the three values of the g-factor tensor for this EPR signal is suggestive of the rhombic symmetry of these DNICs. A less pronounced EPR signal of protein-bound DNICs characterized by an axially symmetric tensor of the g-factor ($g_{\perp}=2.04$, $g_{||}=2.014$) was recorded in the erythrocyte fraction. In-depth analysis revealed that this EPR signal was elicited by hemoglobin-bound DNICs (Timoshin et al., 2007; Vanin et al., 1998). Superposition of the EPR signal of hemoglobin-bound DNICs on the EPR signal of albumin-bound DNICs showed only insignificant distortion of the latter (Timoshin et al., 2007).

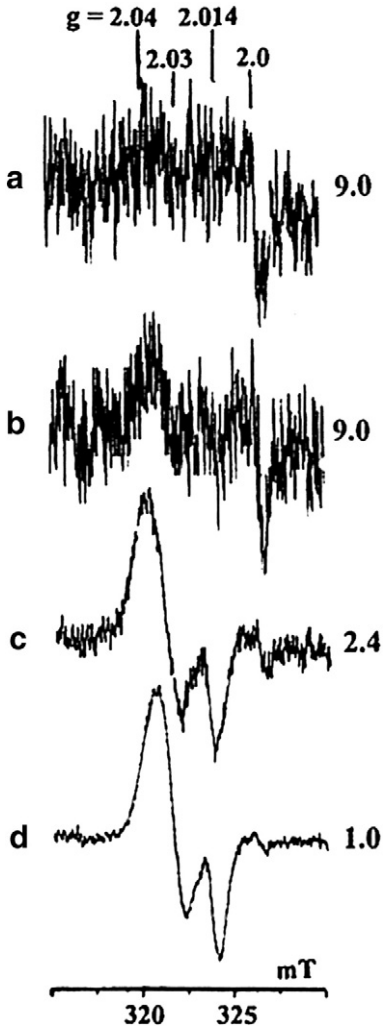


**Fig. 2.** The EPR signal of protein-bound DNICs in arterial blood samples of a rat collected 3 min after injection of DNICs with glutathione (0.05, 0.5, 3.0 and 10.0 μmoles/kg) recorded at ambient temperature. The amplification of the spectrophotometer (in relative units) is shown on the right side of the graph.

Analysis of the EPR signal with the axial symmetry of the g-factor tensor at $g_{\perp}=2.04$ and $g_{||}=2.014$ depicted in Fig. 2 and of an analogous EPR signal recorded in rat blood (Lakomkin et al., 2007) revealed that its characteristics were similar to those of the EPR signal of hemoglobin-bound DNICs (Timoshin et al., 2007; Vanin et al., 1998) suggesting a significant contribution of this signal to the total EPR signal of protein-bound DNICs. In all probability, this contribution depends on the degree of hemolysis of rat erythrocytes, which favors the interaction of low-molecular weight DNICs with hemoglobin resulting in the formation of hemoglobin-bound DNICs.

The EPR signal of protein-bound DNICs was obvious in the blood samples of rats treated with DNIC-GS (≥0.5 μM/kg) (Fig. 2). At lower doses of DNIC-GS, the EPR signal was characterized by a sufficiently high signal-to-noise ratio. In all cases studied, the intensity of the EPR signal of protein-bound DNICs correlated with the magnitude of the hypotensive effect. The decomposition of exogenous DNIC-GS under the action of the water-soluble iron chelator bathophenanthroline disulfonate fully eliminated the hypotensive effect of DNIC (data not shown).

These findings suggest that long-lasting hypotension is a result of formation of stable protein-bound DNICs, which exist in the state of a chemical equilibrium with a small fraction of low-molecular weight DNICs in rat blood. The gradual release of NO from the latter (see above) favors the relaxation of the blood vasculature and a concomitant decrease of MAP.

As mentioned in the foregoing sections, in experiments designed to investigate the effect of DNIC-GS on the physiological status of experimental animals under conditions of hemorrhagic shock, DNIC-GS were used at a low dose (0.05 μmole/kg), which induced only a slight drop of the mean arterial pressure. At higher DNIC-GS doses able to induce a drop of the mean arterial pressure, this decrease and a drastic fall of the mean arterial pressure in rats with hemorrhagic shock might provoke rapid death of experimental animals. However, taking into account other beneficial effects of DNIC-GS and DNIC-CYS, particularly their ability to enhance the synthesis of macroergic compounds in the myocardium and to stimulate its contractile activity (Pisarenko et al., 2008; Remizova et al., 2008), we think it efficacious to investigate DNIC-GS effects on the physiological status of rats with hemorrhagic shock at higher doses of DNIC-GS.

### *3.2. Effect of DNIC-GS (0.05 μM/kg) on the physiological status of rats with hemorrhagic shock*

In the first series of experiments, the blood loss (2.8 ± 0.1 ml/100 g of body weight) was commensurate with that observed in the second series (2.6 ± 0.2 ml/100 g of body weight) and was accompanied by a significant (more than 2-fold) decrease of mean arterial pressure (Table 1). The other systemic circulation parameters measured – cardiac output, stroke volume and left ventricular work – were

**Table 1**
The changes of systemic hemodynamics in hemorrhagic shock and after infusion of saline in the volume of escaped blood. 1st series — injection of 1.0 ml of saline 20 min before bleeding (control, n = 13); 2nd series — injection of DNIC (0.05 μmoles/kg in 1.0 ml of saline) 20 min before bleeding (n = 8).

| Parameters | Series | Initial values | Cessation of hemorrhage | Time after saline treatment, min | |
|---|---|---|---|---|---|
| | | | | 10 | 60 |
| Mean arterial pressure, mm Hg | 1 | 141 ± 3.0 | 59 ± 4.0 | 100 ± 4.0[a] | 100 ± 5.0[a] |
| | 2 | 142 ± 4.3 | 60 ± 8.7 | 108 ± 5.6[a] | 111 ± 7.4[a] |
| Cardiac output, ml/min x 100 g | 1 | 15.5 ± 0.2 | 4.4 ± 0.2 | 12.8 ± 0.9[a] | 11.5 ± 0.8[a] |
| | 2 | 15.2 ± 0.3 | 4.9 ± 0.4 | 24.1 ± 1.2[ab] | 22.1 ± 1.5[ab] |
| Stroke volume, ml/kg | 1 | 0.37 ± 0.01 | 0.13 ± 0.01 | 0.35 ± 0.02[a] | 0.32 ± 0.02[a] |
| | 2 | 0.36 ± 0.01 | 0.15 ± 0.01 | 0.59 ± 0.03[ab] | 0.53 ± 0.03[ab] |
| Total peripheral resistance, dyne s $cm^{-5} \times 10^{-4}$/kg | 1 | 7.3 ± 0.2 | 11.0 ± 0.7 | 6.4 ± 0.3[a] | 7.3 ± 0.5[a] |
| | 2 | 7.5 ± 0.3 | 12.6 ± 1.2 | 3.7 ± 0.4[ab] | 4.2 ± 0.4[ab] |
| Left ventricular work, kGm/kg × min | 1 | 296 ± 6 | 36 ± 3 | 179 ± 18[a] | 162 ± 17[a] |
| | 2 | 291 ± 11 | 41 ± 7 | 349 ± 28[ab] | 330 ± 29[ab] |
| Heart rate, min | 1 | 425 ± 10 | 342 ± 10 | 375 ± 21 | 358 ± 15 |
| | 2 | 428 ± 15 | 323 ± 7 | 413 ± 15 | 413 ± 15 |

Note: Hereinafter, the values represent means ± S.E.M. The significance of differences between the experimental and control values is indicated in comparison with the cessation of hemorrhagic shock (− a) and between Series 1 and 2 — b ($P \leq 0.05$).

decreased more than 3-, 2.5- and 7-fold, respectively. There were substantial changes in the small intestinal wall manifested as significant decreases in the blood flow rates and the erythrocyte aggregation factor (see Table 2).

The $CO_2$ content in the blood was lowered as a result of hemorrhagic shock concomitantly with a pronounced deficit of the basic excess (Table 3). In both experimental series, saline infusion was performed against the background of pronounced disturbances in the parameters of systemic circulation, microcirculation and metabolic acidosis.

Ten minutes after cessation of saline treatment, the mean arterial pressure increased nearly 1.6-fold in both series and remained at this level for 1 h, but was nevertheless lower than the initial level (Table 1). Other systemic circulation parameters were also improved. Thus, in the control group, cardiac output and stroke volume were slightly increased, but still remained below the initial level. In animals treated with 0.05 μmoles/kg DNIC-GS prior to NaCl infusion (2nd series), the increment in the cardiac output and stroke volume was much higher than in the control group, and exceeded the initial level (Table 1). In the second series, the increase in left ventricular work was especially well-pronounced and differed greatly from control values. In contrast, the total peripheral resistance level was notably decreased against the background of a sufficiently high content of mean arterial pressure and cardiac output. As for heart rate, it was increased in both series, but in the second series the increase was more apparent.

**Table 2**
The changes of microcirculation in the serous membrane of the small intestine in hemorrhagic shock and after infusion of saline in the volume of escaped blood. 1st series — injection of 1.0 ml of saline 20 min before bleeding (control, n = 13); 2nd series — injection of DNIC (0.05 μmoles/kg in 1.0 ml of saline) 20 min before bleeding (n = 8).

| Parameters | Series | Initial values | Cessation of hemorrhage | Time after saline treatment, min | |
|---|---|---|---|---|---|
| | | | | 10 | 60 |
| Blood flow rate, scores | 1 | 0 ± 0 | − 3.54 ± 0.08 | − 1.00 ± 0.25[a] | − 1.17 ± 0.18[a] |
| | 2 | 0 ± 0 | − 3.00 ± 0.25 | − 0.50 ± 0.12[a] | − 0.50 ± 0.12[ab] |
| Erythrocyte aggregation, scores | 1 | 0 ± 0 | 2.54 ± 0.08 | 1.15 ± 0.17[a] | 1.33 ± 0.09[a] |
| | 2 | 0 ± 0 | 2.00 ± 0.25 | 0.63 ± 0.12[a] | 0.63 ± 0.12[ab] |

**Table 3**
The gas content and the acid–base balance in arterial blood in hemorrhagic shock and after infusion of saline in the volume of escaped blood. 1st series — injection of 1.0 ml of saline 20 min before bleeding (control, n = 13); 2nd series — injection of DNIC (0.05 μmoles/kg in 1.0 ml of saline) 20 min before bleeding (n = 8).

| Parameters | Series | Initial values | Cessation of hemorrhage | Time after saline treatment, min | |
|---|---|---|---|---|---|
| | | | | 10 | 60 |
| $pO_2$, mm Hg | 1 | 84.6 ± 4.3 | 103.3 ± 6.3 | 94.1 ± 3.0 | 96.0 ± 4.6 |
| | 2 | 84.8 ± 6.5 | 106. ± 6.1 | 96.8 ± 8.1 | 85.3 ± 5.4 |
| $pCO_2$, mm Hg | 1 | 44.6 ± 1.3 | 25.2 ± 1.4 | 31.7 ± 0.9 | 26.2 ± 1.6 |
| | 2 | 42.5 ± 1.5 | 24.7 ± 1.8 | 28.1 ± 1.2 | 26.8 ± 1.7 |
| pH | 1 | 7.38 ± 0.01 | 7.34 ± 0.02 | 7.37 ± 0.02 | 7.37 ± 0.04 |
| | 2 | 7.40 ± 0.01 | 7.39 ± 0.03 | 7.42 ± 0.01[b] | 7.44 ± 0.03 |
| BE, mmol/l | 1 | 1.0 ± 0.6 | − 11.4 ± 0.7 | − 6.5 ± 0.9[a] | − 7.8 ± 1.6 |
| | 2 | 1.7 ± 0.8 | − 8.9 ± 1.6 | − 5.6 ± 0.8 | − 5.5 ± 0.8[a] |

After cessation of saline treatment, the microcirculation parameters in both experimental groups were markedly improved. The blood flow rates in DNIC-GS-treated animals measured 60 min after cessation of saline treatment significantly exceeded the control level, while erythrocyte aggregation was notably decreased (Table 2).

The gas composition of the blood did not change after injection of saline in both series. Basic excess showed a tendency to decrease, while pH increased, in contrast. In the second series, the pH measured 10 min after cessation of saline treatment markedly exceeded that in the first series. However, the increment in pH in this time interval was similar to that recorded after cessation of hemorrhage, viz., 0.03 (Table 3).

In the first series, the survival estimated 24 h after the beginning of measurements was 54%; that in the second series was 75%.

These findings suggest that DNIC-GS used at a dose of 0.05 μmoles/kg prior to bleeding markedly improve systemic circulation parameters in rats with hemorrhagic shock and increase survival without having a hypotensive effect on mean arterial pressure.

## 4. Discussion

The data obtained suggest that, after termination of the hypotension period, DNIC-GS administered to animals at a dose of 0.05 μmole/kg 20 min prior to hemorrhagic shock and saline treatment significantly improved the functional activity of the heart (cardiac output, stroke volume and left ventricular work). The values of these parameters strongly exceeded the initial values. However, used at this dose DNIC-GS failed to induce a notable decrease of mean arterial pressure normally observed at higher concentrations of DNIC-GS (Fig. 1). Ten and sixty minutes after the saline infusion, the mean arterial pressure level in rats treated with 0.05 μmole/kg of DNIGS did not differ greatly from the control level (Table 1).

Interestingly, steady values of mean arterial pressure were recorded at lower values of total peripheral resistance. As a rule, hypotension induced by, e.g. high doses of DNIC-GS is characterized by direct correlation between the decrease of total peripheral resistance and mean arterial pressure. It was assumed that it is the decrease of total peripheral resistance that is responsible for the drop of mean arterial pressure (Lakomkin et al., 2007). The lack of such correlation in rats treated with 0.05 μmole/kg DNIC-GS can be due to significant improvement of the heart function. However, the vasodilator effect of low doses of DNIC-GS on peripheral vasculature should not be ruled out either. This effect can be responsible for the improvement of microcirculation parameters, e.g., increased blood flow rates and low level of erythrocyte aggregation (Table 2).

Such a pronounced improvement of the cardiac function after treatment of rats with 0.05 μmoles/kg DNIC-GS can also be attributed to activation of myocardial contractility. The beneficial effect of DNICs on myocardial contractility (Remizova et al., 2008) is that the

resistance of the animal myocardium to heart rate disturbances increases after ischemia–reperfusion in the presence of DNIC-GS. Notwithstanding, we do not rule out the possibility that the enhancement of the coronary blood flow in response to coronary artery dilatation and improved microcirculation caused by the decrease of total peripheral resistance also plays a role in improved cardiac function. The contributions of enhanced myocardial contractility and relaxation of coronary and peripheral vessels to the improvement of cardiac output, stroke volume and left ventricular work demand further investigation.

Here, it seems appropriate to consider some alternative mechanisms responsible for the beneficial effect of DNIC-GS on the activity of the cardiovascular system in the course of infusion therapy of hemorrhagic shock. Hemorrhage is normally associated with the activation of free radical oxidation processes, which is suppressed under the potent antioxidant effect of DNICs with thiol-containing ligands (Shumaev et al., 2007). Moreover, DNICs enhance oxidative phosphorylation in cardiac muscle mitochondria (Pisarenko et al., 2008). Yet another prerequisite to the beneficial effect of DNICs is their ability to stimulate S-nitrosylation of proteins (Boese et al., 1995). The protective effect of this process against various pathological events was demonstrated in recent studies (Nadtochiy et al., 2007; Prime et al., 2009).

Noteworthy, the survival of rats with hemorrhagic shock increased by 21% in comparison with control animals after treatment with 0.05 μmole/kg DNIC-GS.

The totality of the experimental data strongly suggest that DNIC-GS hold great promise as a means of prophylaxis against hemorrhagic shock, especially in the preoperational period, since low doses of these complexes do not induce a hypotensive effect.

Noteworthy, the beneficial effect of low doses of yet another NO donor, viz., S-nitrosoglutathione, on the cardiovascular system established in experiments on hamsters with hemorrhagic shock was similar to that observed in our experiments on rats treated with DNIC-GS (Cabrales et al., 2009). At first glance, this finding calls into question the specific beneficial effect of DNIC-GS on animals with hemorrhagic shock. However, one should not rule out the possibility that in hamster studies the reaction of S-nitrosoglutathione with free iron and endogenous low-molecular weight thiols would culminate in the conversion of the former into the corresponding DNICs with thiol-containing ligands, which might have a beneficial effect on animals under conditions of hemorrhagic shock. The conversion of S-nitrosothiols into DNICs in chemical systems by this particular reaction is documented in the literature (Constanza et al., 2001; Vanin et al., 1997; Vanin et al., 2004). Therefore, we consider our findings a working hypothesis, which demands further verification and experiment.

## Conflict of interest

There is no conflict of interest.

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## References

Bloch, E., 1954. The bulbar conjunctiva of man as a site for the microscopic study of the circulation. Anal. Rec. 120, 349–361.

Boese, M., Mordvintcev, P.I., Vanin, A.F., Busse, R., Mülsch, A., 1995. S-nitrosation of serum albumin by dinitrosyl-iron complex. J. Biol. Chem. 270, 29244–29249.

Cabrales, P., Tsai, A.G., Intaglietta, M., 2009. Exogenous nitric oxide induces protection during hemorrhagic shock. Resuscitation 80, 707–712.

Constanza, S., Menage, S., Purello, R., Bonomo, R.P., Fontecave, M., 2001. Re-examination of the formation of dinitrosyl-iron complexes during reaction of S- nitrosothiols with Fe(II). Inorg. Chem. Acta 318, 1–7.

Ding, H., Demple, B., 2000. Direct nitric oxide signal transduction via nitrosylation of iron–sulfur centers in the SoxR transcription activator. Proc. Natl. Acad. Sci. USA 97, 5146–5150.

Ditsel, T., 1959. Relationship of blood protein composition to intravascular erythrocyte aggregation. Acta Med. Scand. 164 (suppl. 343), 1–60.

Flitney, F.W., Megson, I.L., Flitney, D.E., Butler, A.R., 1992. Iron–sulfur cluster nitrosyls: a novel class of nitric oxide generator: mechanism of vasodilator action on rat isolated tail artery. Br. J. Pharmacol. 107, 842–848.

Ignarro, L.J., 2000. Nitric Oxide: Biology and Pharmacology. 1st Academic Press, San-Diego.

Karpinsky, V.V., Slovesnov, S.V., Rerikh, R.A., 1986. Measurement of cardiac output in small laboratory animals using tetrapolar rheography. Pathol. Physiol. Exp. Therapy (Rus) 1, 74–77.

Kelly, E., Shah, N.S., Morgan, N.N., Watkins, S.C., Peitzman, A.B., Billiar, T.B., 1997. Physiological and molecular characterization of the role of nitric oxide in hemorrhagic shock: evidence the type of II nitric oxide synthase does not regulate vascular decompensation. Shock 7, 157–163.

Kim, Y.M., Chung, H.T., Simmons, R.L., Billiar, T.R., 2000. Cellular non-heme iron content is a determinant of nitric oxide-mediated apoptosis, necrosis, and caspase inhibition. J. Biol. Chem. 275, 10954–10961.

Kleschyov, A.L., Strand, S., Schmitt, S., Gottfried, D., Skatchkov, M.V., Sjakste, N., Daiber, A., Umansky, V., Munzel, T., 2006. Dinitrosyl-iron triggers apoptosis in Jurkat cells despite overexpression of Bcl-2. Free Rad. Biol. Med. 40, 1340–1348.

Kochetygov, N.I., Kulikov, A.M., 1982. Systemic hemodynamics and microcirculation in the therapy of burn shock using blood transfusion solutions. Probl. Hematol. Blood Transfus. (Rus) 6, 24–30.

Kochetygov, N.I., Remizova, M.I., Gerbut, K.A., Grishina, G.V., 2003. Effect of NO synthesis regulators on hemodynamic parameters in experimental hemorrhagic shock. Med. Acad. J. (Rus) 3, 133–134.

Lakomkin, V.L., Vanin, A.F., Timoshin, A.A., Kapelko, V.I., Chazov, E.I., 2007. Long-lasting hypotensive action of stable preparations of dinitrosyl-iron complexes with thiol-containing ligands in conscious normotensive and hypertensive rats. Nitric Oxide: Biol. Chem. 16, 413–418.

Mordvintcev, P.I., Rudneva, V.G., Vanin, A.F., Shimkevich, L.L., Khodorov, B.I., 1986. The inhibitory effect of low-molecular dinitrosyl iron complexes on platelet aggregation. Biokhimiya (Rus) 51, 1851–1857.

Nadtochiy, S.M., Burwell, L.S., Brooks, P.S., 2007. Cardioprotection and mitochondrial S-nitrosation. Effects of S-nitroso-mercaptopropionyl glycine (SNO-MPG) in cardiac ischemia–reperfusion injury. J. Mol. Cell Cardiol. 42 (8), 12–825.

Pisarenko, O.I., Serebryakova, L.I., Tskitishvili, O.V., Studneva, I.M., Vanin, A.F., Chazov, E.I., 2008. Cardioprotective efficacy of dinitrosyl iron complex with L-cysteine in rats *in vivo*. Izvest. RAS, ser. Biolog. (Rus), 1, pp. 1–5.

Prime, T.A., Blaikie, F.H., Evans, C., Nadtochiy, S.N., James, A.M., Dahm, C.C., Vitturi, D.A., Patel, R.P., Hiley, C.R., Abakumova, I., Requejo, R., Chouchani, E.T., Hurd, T.R., Garvey, J.F., Taylor, C.T., Brooks, P.S., Smith, R.A.J., Murphy, M.P., 2009. A mitochondria-targeted S-nitrosothiol modulates respiration, nitrosates thiols and protects against ischemia–reperfusion injury. Proc. Natl. Acad. Sci. USA 106, 10764–10769.

Remizova, M.I., Kochetygov, N.I., Gerbut, K.A., Vanin, A.F., 2008. Effects of the donor of nitric oxide, dinitrosyl iron complex with glutathione, on blood circulation in healthy animals. Biofizika (Rus) 53, 867–873.

Shekhter, A.B., Rudenko, T.G., Serezhenkov, V.A., Vanin, A.F., 2007. Dinitrosyl-iron complexes with cysteine or glutathione accelerate skin wound healing. Biofizika (Rus) 52, 534–538.

Shumaev, K.B., Gubkin, A.A., Serezhenkov, V.A., Lobysheva, I.I., Kosmachevskaya, O.V., Ruuge, E.K., Lankin, V.Z., Topunov, A.F., Vanin, A.F., 2007. Interaction of reactive oxygen and nitrogen species with albumin- and methemoglobin-bound dinitrosyl-iron complexes. Nitric Oxide: Biol. Chem. 18, 37–46.

Timoshin, A.A., Vanin, A.F., Orlova, T.R., Sanina, N.A., Ruuge, E.K., Aldoshin, S.M., Chazov, E.I., 2007. Protein-bound dinitrosyl-iron complexes in blood of rabbit added with a low-molecular dinitrosyl-iron complex: EPR studies. Nitric Oxide: Biol. Chem. 16, 286–293.

Vanin, A.F., Malenkova, I.V., Serezhenkov, V.A., 1997. Iron catalyzes both decomposition and synthesis of S-nitrosothiols: optical and electron paramagnetic studies. Nitric Oxide: Biol. Chem. 1, 191–203.

Vanin, A.F., Serezhenkov, V.A., Mikoyan, V.D., Genkin, M.V., 1998. The 2.03 signal as an indicator of dinitrosyl-iron complexes with thiol-containing ligands. Nitric Oxide: Biol. Chem. 2, 224–234.

Vanin, A.F., 1998. Dinitrosyl iron complexes and S-nitrosothiols are two possible forms for stabilization and transfer of nitric oxide in biological systems. Biochemistry (Moscow) 67, 782–793.

Vanin, A.F., Papina, A.A., Serezhenkov, V.A., Koppenol, W., 2004. The mechanism of S-nitrosothiol decomposition catalyzed by iron. Nitric Oxide Biol. Chem. 10, 60–73.

Vanin A.F., Lozinsky, V.I., Kapelko, V.I. 2005. Polymeric composition for designing stabilized form of dinitrosyl-iron complex and the method of this complex form synthesis. Russian Patent Application, No 2291880 from 01.12 2005 to 01.12.2025.

Vanin, A.F., Mokh, V.P., Serezhenkov, V.A., Chazov, E.I., 2007. Vasorelaxing activity of stable powder preparations of dinitrosyl iron complexes with cysteine or glutathione. Nitric Oxide: Biol. Chem. 16, 322–330.

Vanin, A.F., 2009. Dinitrosyl iron complexes with thiolate ligands: physico-chemistry, biochemistry and physiology. Nitric Oxide Biol. Chem. 21, 1–13.
Vedernikov, Yu.P., Mordvintcev, P.I., Malenkova, I.V., Vanin, A.F., 1992. Similarity between the vasorelaxing activity of dinitrosyl iron cysteine complexes and endothelium-derived relaxing factor. Eur. J. Pharmacol. 211, 313–317.
Veliev, E.I., Kotov, S.V., Shishlo, V.K., Serezhenkov, V.A., Vanin, A.F., 2008. Effect of dinitrosyl-iron complexes with thiol-containing ligands on penile cavernosum bodies in rats. Biofizika (Rus) 53, 326–335.
Vasilèva, S.V., Stupakova, M.V., Lobysheva, I.I., Mikoyan, V.D., Vanin, A.F., 2001. Activation of *Escherichia coli* SoxRs-regulon by nitric oxide and its physiological donors. Biochemistry (Moscow) 66, 984–988.