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The study of the complexes of nitromedicine with cytochrome *c* and NO-containing aqueous dosage form in the wound treatment of rats

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ABSTRACT

The interaction of cytochrome *c* with nitromedicines, such as 5-nitrofural, 5-nitroxoline, metronidazole and sodium nitrite which enables the generation of nitric oxide or nitrosyl complexes in the presence of ascorbic acid or sodium ascorbate in acid medium has been investigated. The pharmaceutical compositions containing cytochrome *c* and nitromedicine complexes as active substances were studied in the experiments by using rats. It has been shown that positive local and systemic effects were estimated when NO-containing gel was used at burn treatment. These positive effects at the local level are due to a sufficient microcirculation index which indicates intensification of the blood flow in the microvessels in the injured area. These effects at the systemic level provide maintenance of the general heart rhythm and gradual recovery of the vegetative balance which is not observed in the animals of the control group.

1. Introduction

At present, nitric oxide (NO) is considered to be one of the most important process control agents *in vivo*, such as endothelial relaxation called "endothelium-derived relaxing factor (EDRF)", with nerve signal transmission being the second messenger in the cellular signaling pathways, gene transcription and translation [1]. For the last thirty years it was proved that the biological variable synthesis of the nitric oxide under the catalysis by NO-synthases, including their isoforms, is related to cardiovascular and immune system performance and ensures antibacterial, cytotoxic, anti-inflammatory and antioxidant actions in *vivo* [2,3].

It has been estimated that some antibacterial nitromedicines are able to release NO *in vivo* and *in vitro* under oxidation, reduction or hydrolytic decomposition [4]. For example, the transformation of 5-nitrofurans to nitric oxide may be presented as in scheme 1 [5]:

Furan ring under reducing conditions may be able to undergo disaromatization with the formation of radical or anion radical species which at least partly will lead to compounds capable of releasing nitric oxide. The behavior of reducing conditions in a number of drugs nitrofuran series such as 5-nitrofural, furagin, furazolidone have been studied by using potassium ferrocyanide–ascorbic acid system [5]. The evidence that the anion radicals formed in the reduction of 5-nitrofurans are able to undergo nitro → nitrite rearrangement was investigated in another paper [6] (scheme 2).

It has been proved that many effects associated with NO due to peroxynitrite being the product of interaction of NO with superoxide anion were generated under the reduction of 5-nitrofurans. Peroxynitrite can inhibit the electron transport chain in mitochondria, disturbing the process of cellular respiration of microorganisms resulting in antibacterial activity [4].

Nitric oxide in the intracellular fluids, including bloodstream, can interact with a variety of substances – thiols, metal ions, sugars – forming different kinds of nitrosyl complexes, among them iron-nitrosyl complexes, which are very important [7]. The numerous examples of "depot" formation of nitric oxide from nitrosyl iron-containing complexes, where the ligands were diethyldithiocarbamic, thiolate, etc., having improved pharmacologic properties in comparison with nitric oxide, are known [8,9]. The great contribution to the development of iron nitrosyl complexes with functional sulfur-containing ligands as the nitric oxide "depot" was made by the Russian scientists S.M. Aldoshin, N.A. Sanina and some others [7,10]. The authors were the first who systematically investigated the coordination of iron (II) by S-functional aza-heterocyclic thiols using triazole, tetrazole, pyrimidin, pyridine, imidazole and its benzo-derivatives and also by natural aliphatic thioamines, many of which are the pharmaceutical substances, the complexes themselves having cardioprotective and antineoplastic activity.

In spite of the success achieved in the nitrosyl complexes invention domain, as potential pharmaceutical substances, there are

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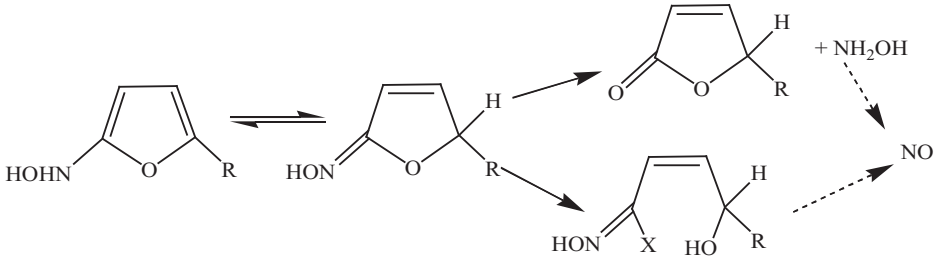


Scheme 1. Decomposition of 5-nitrofurans and NO releasing.

difficulties related to bioavailability, selection of the pharmaceutical substance dose, and also solubility of the nitrosyl complexes in aqueous medium.

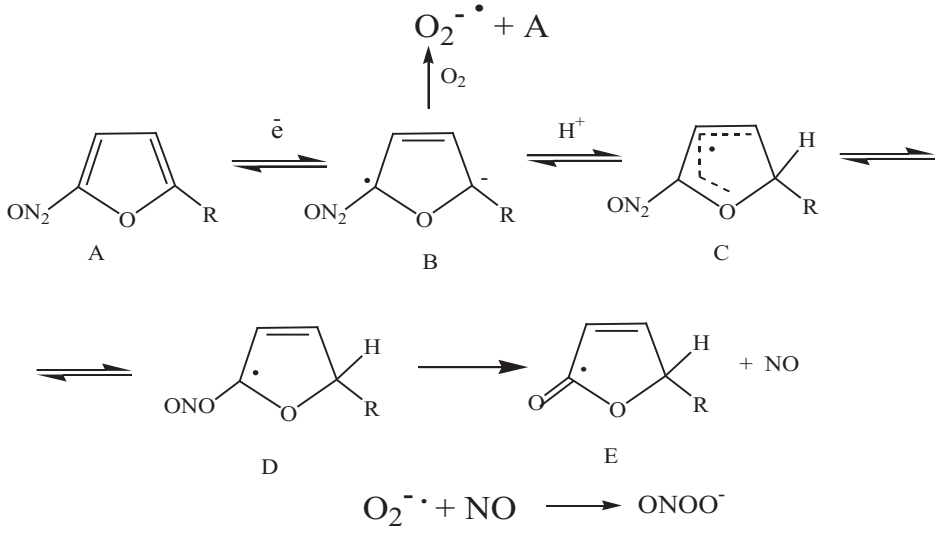
The NO interaction with heme proteins, including the interaction with cytochrome *c*, is of special interest because of the nitrosyl complexes that are formed [11–15].

M.A. Sharpe and C.E. Cooper estimated the aerobic reactions of nitric oxide with mitochondrial cytochrome *c* [13]. According to these authors [13], NO reacts with ferrocytochrome *c* at a rate of 200 $M^{-1}\,s^{-1}$ to form ferricytochrome *c* (visible spectroscopy) and nitroxyl anion (by trapping with metmyoglobin to form the EPR-detectable Mb-nitrosyl complex, and by the formation of dimers in yeast ferrocytochrome *c* via cross-linking of the free cysteine residue), which reacted with oxygen to form peroxynitrite. NO binds to ferricytochrome *c* to form the ferricytochrome *c*-NO complex. The on-rate and the off-rate for ferricytochrome *c*-NO complexation were equaled $1.3 \pm 0.4 \times 10^3\ M^{-1}s^{-1}$ and $0.087 \pm 0.054\ s^{-1}$, consequently. It has been shown that the dissociation constant (*K*d) of the complex is 22 ± 7 μM. These reactions of NO with cytochrome *c* are likely to be relevant to mitochondrial metabolism of NO. Ferricytochrome *c* can act as a reversible sink for excess NO in the mitochondria. The reduction of NO to NO^- by ferrocytochrome *c* may play a role in the irreversible inhibition of mitochondrial oxygen consumption by peroxynitrite.

Nitrosyl complexes can modify the peroxidase activity of cytochrome *c* and control the apoptosis, relatively [15]. Nitrosylation may also be an allosteric regulator of other cytochrome *c* functions [14]. However, studies of work by other authors [14] have shown that cytochrome *c* that is endogenously nitrosylated during apoptosis is unexpected because cytochrome *c* has a 6-coordinate heme that is significantly less reactive with NO than the 5-coordinate heme of guanylate cyclase and cytochrome oxidase. One possible explanation for this result is that cytochrome *c* undergoes a subtle conformational change during apoptosis that increases the reactivity of the heme iron with NO. A number of investigators have demonstrated that cytochrome *c* undergoes a conformational change when bound to anionic phospholipid vesicles that model interactions of cytochrome *c* with the phospholipid-rich mitochondrial membranes [16]. The conformational change involves an opening of the heme crevice, resulting in part from the loss of the iron-methionine 80 ligation. A similar conformational change in cytochrome *c* has been detected in cells early during apoptosis [14]. The data raise the possibility that alterations in mitochondrial membrane lipids during apoptosis may induce a conformational change in cytochrome *c*, resulting in a 5-coordinate heme. Such a conformational alteration may facilitate stable heme nitrosylation of cytochrome *c* during apoptosis.

Usually cytochrome *c* is used as the antioxidant and the antyhypoxant that is caused by its unique enzymatic functions such as electron transmitter at photosynthesis, when breathing, at oxidative phosphorylation and other redox processes [17]. Taking the above mentioned into consideration, the increase in pharmacological action of cytochrome *c* is expected since its biological activity ensures the ability of metalloprotein iron-porphyrin heme to form complexes with nitric oxide and, respectively, to inhibit apoptosis.

The general goal in this paper was to create a new topical reparant dosage form for burn wound treatment on the base antibacterial nitromedicines able to generate NO or nitrosyl complexes in the



Scheme 2. The reduction of 5-nitrofurans by potassium ferrocyanide-ascorbic acid system.

presence of cytochrome *c*. For this purpose we studied the following problems:

- the estimation nitrosyl complexes of cytochrome *c* with nitromedicine and the role of sodium ascorbate in the formation of nitric oxide "depot" from complexes by UV-visible spectra;
- the study of burn wound regeneration in the experiments *in vivo* by rats;
- the detection of the increase of total [NO_x] concentration in blood plasma *in vivo* under treatment of nitromedicine-cytochrome *c*-complexes;
- the estimation of useful side pharmacological effects such as vasodilatation.

2. Experimental section

Materials and reagents. Cytochrome *c* (from heart bovine) (≥95%, lot STBB7839V, Fluka (USA), Sigma-Aldrich), tris(hidroxymethyl)aminomethane (>99.2%, lot 108387, Merck), solvents (analytical grade), 5-nitrofural (>99.8%), metronidazole (>99.8%), sodium nitrite (>99.9%), 5-nitroxolin (>99.8%), ascorbic acid (>99.9%), phosphoric, succinic acid were purchased from Sigma-Aldrich and used without further purification.

NO were prepared by the addition of 2 M H_2SO_4 to solid $NaNO_2$ in a Kipps apparatus. The NO gas was passed through four NaOH (20%) traps (to remove NO_2), and then through a solid CO_2 trap. The gas was collected in a buffer solution that had undergone four vacuum/N_2 deoxygenation cycles. The concentration of the NO solution varied from 1.2 to 2 mM. The NO_2^- concentration was generally approximately 300 μM.

Nitrite was assayed by the Griess reaction [18]. One hundred microliter probes or standards were added to 500 μl of 1% (w/v) sulfanilamide in 5% (v/v) H_3PO_4 and 500 μl 0.15 napthylenediamine hydrochloride. The optical density (A) was measured at 540 nm, and the assay was calibrated using nitrite standards. Determination of nitrite and nitrate using the Griess reaction was carried out according to methods by using Aspergillus nitrate reductase.

Absorption spectra of aqueous solutions (nitromedicine, cytochrome *c*, organic acids, phosphoric acid and their mixtures) were recorded by Bio line Specord S-100 (Analytik Jena), with thickness 10 mm quartz cuvette. Spectrophotometer error is 0.002 units transmittance at a concentration of cytochrome *c* $4.5 \cdot 10^{-5}$ mol/L^{-1} in solution, and the relative standard deviation (RSD%) was 0.9%.

Formulation of pharmaceutical composition. Composition of gel (w, %): sodium hyaluronate – 1.0; hydroxyethylcellulose – 0.1; sodium ascorbate – 0.1; cytochrome *c* – 0.05; methylparaben – 0.15; water up to 100. Sodium nitrite (0.1–1%) was added into gel before application. Composition of powder (w, %): cytochrome *c* –0.05; sodium ascorbate – 0.1; 5-nitrofural (metronidazole, nitroxolin) – 5%; methylparaben – 0.15; starch up to 100. Pharmaceutical composition (gel plus sodium nitrite) should be prepared just before use.

Biomedical research. The experiment with nitromedicine containing dosage form was carried out by using twenty Wistar male rats and ten rats were used as the control group. Two groups of animals were separated, that were exposed to contact thermal burn under anesthesia (the burn area is 20% of the body surface) [19]. After thermal injury modeling a pharmaceutical composition which was a complex of cytochrome *c* and sodium nitrite was applied to the wound surface of the animals in the main group (n = 20) for 10 days. The animals in the control group (n = 10) were treated with 5-nitrofural-containing ointment.

The microcirculation was assessed quantitatively using the LAKK-02 (LASMA, Russia). This device transmits continuous wave laser light (30 mW, 890 nm) and white light (20 W, 500–900 nm) to skin tissue near the wound, where it is scattered and collected on the skin surface with fibers of the probe. The movement of erythrocytes causes a Doppler shift, which in turn is detected by the laser light and analyzed by the LAKK-02. This is then computed and displayed as the blood flow velocity. The detected laser signal correlates with the number of moving erythrocytes in tissue, blood flow velocity for calculation microcirculation parameters, using such arbitrary (relative) units as perfusion units (perf. un.). The rate of microcirculation (the microcirculation level), the regulatory activity of its components and the degree of shunt paths participation with an allowance for the frequency range intervals of the blood flow oscillations in the rats' microvessels were investigated [20–22].

Heart rate variability (HRV) was studied with the help of the "Neurosoft" (Ivanovo, Russia) system with their own adaptation of ECG registration and results management [23]. Time parameters included parameters such as M_o, which is the most frequent mode of RR-cardiointerval values (RR intervals were registered for 5 minutes within 0.001 s in the morning hours in rats at rest), AM_o amplitude of modal value of RR interval set, Standard Deviation of Normal-to-Normal (SDNN) RR intervals, tension index (TI).

Statistical treatment of the results was performed with the Statistica 6.0 program. All heart rate variability data were given with at least two significant digits, except p-values (one significant digits) and were presented as mean relative standard deviation (RSD, %). Two-tailed tests were used. The lower level of statistical significance was set to p = 0.05. All means were accompanied by their ±95% confidence intervals.

3. Results and discussion

3.1. UV-visible study of reaction mixture with nitromedicine generated NO-containing complexes

Sodium ascorbate was used for a spectrum control of cyt *c* reducing form by using absorption of α- (550 nm), β- (520 nm) and γ- (415 nm) bands. In this case at pH 7.1, the initial oxidizing form of cytochrome *c* (cyt c^{3+}), which bands at λ_{max} 410 nm converts to reducing form of cyt c^{2+} in full (Fig. 1a). Fig. 1b shows the changes of UV-visible spectrum of aqueous solution of cyt *c* following the addition of gaseous NO during 15 minutes. The shift of γ-band at 410 nm to 415 nm characterized reducing form was observed. Moreover, new intensive band at 358 nm, two weak bands – 520 nm (β) and 550 nm (α) typical for cyt c^{2+} –appeared.

The same spectral effect was estimated when cyt *c* was interacted with nitrite at pH 3 in the work [24]. At this pH the UV as well as visible spectrum of cyt *c* was changed by nitrite, even in the presence of hydrogen peroxide, probably via the formation of heme iron-nitric oxide complex. This spectrum argument of a heme nitric oxide complex of cyt *c* had been reported previously by Orii and Shimada (1978) and Butt and Keilin (1962). Due to this change, the peroxidase activity of cyt *c* was lost.

The similar change of the heme structure of cyt *c* was shown by absorption spectra in the UV visible range in alternative system, containing a mixture of cyt *c*, sodium nitrite and sodium ascorbate in acid medium, able to generate nitric oxide (Fig. 2a). The UV-Vis spectra of the reaction mixtures with succinic acid are represented in Fig. 2a. We can see the shift of γ-band from 410 nm to 415 nm, the appearance of new intensive band at 358 nm, weak α- and β-bands such as in the spectrum of cyt c^{3+} under gaseous NO action (Figs. 1,2). The significant differences of cyt *c* spectra of reaction mixtures, containing sodium ascorbate and nitrite at pH 5.0, had the reversible shift of γ-band from 415 nm to 410 nm in a time – 30 minutes (Fig. 2b). At the same time, a new band at 358 nm was changed in a slight variation of time, but α- and β-bands disappeared.

The type and character of spectra depended on pH medium and biogenic acid nature. Analysis of spectra data of the reaction

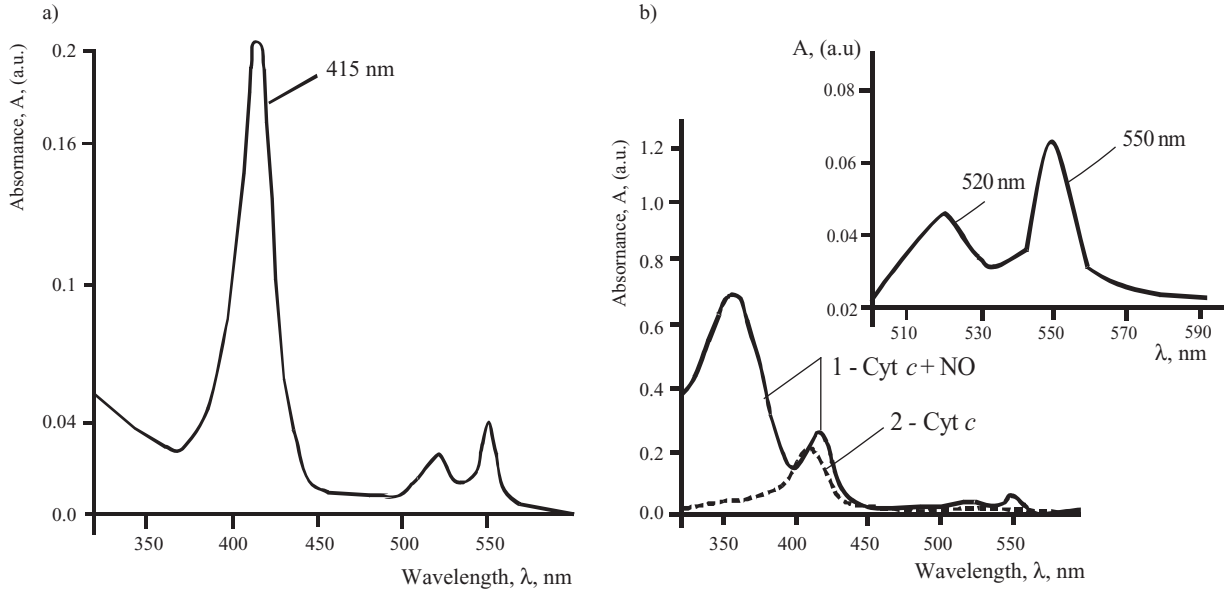


Fig. 1. a, b. UV–visible spectra of 2.4 μM cyt *c* solution after addition: (a) $5{\cdot}10^{-4}$ M sodium ascorbate (reducing form); (b) nitric oxide: *curve* 1 – reaction mixture; *curve* 2 – cyt *c* in water (oxidizing form). Insert shows visible part of spectrum of cyt *c* after NO treatment.

mixtures was performed by comparing with the solutions without sodium nitrite to establish the role of NO-donated component (Table 1).

We suggest that the UV-visible spectra are not only indicated on the changes of heme from cyt c^{3+} to cyt c^{2+}, but also the formation of different nitrosyl cyt *c* complexes with nitric oxide, nitrite (NO_2^-), nitroso- (NO^-) or nitroxyl (NO^+) ions according to the scheme are described in the paper [1].

$$\text{cyt } c^{3+} + \text{NO} \leftrightarrow \text{cyt } c^{3+} - \text{NO} \quad (1)$$

$$\text{cyt } c^{3+} - \text{NO} \leftrightarrow \text{cyt } c^{2+} - \text{NO}^+ \quad (2)$$

$$\text{cyt } c^{2+} - \text{NO}^+ + 2\text{OH}^- \leftrightarrow \text{cyt } c^{2+} + \text{NO}_2^- + \text{H}_2\text{O} \quad (3)$$

$$\text{cyt } c^{2+} + \text{NO} \rightarrow \text{cyt } c^{3+} + \text{NO}^- \quad (4)$$

The interaction cyt *c* with nitrite-ascorbate system in acid medium, probably, lead to two types complexes – NO-cyt c^{2+} (λ_{max} 415 nm) and nitrosyl cyt c^{2+}-NO^+ (λ_{max} 385 nm), but NO-cyt c^{2+}-complex is not stable and converts to NO-cyt c^{3+} complex (λ_{max} 410 nm).

The antibacterial nitromedicines (5-nitrofural, 5-nitroxoline, metronidazole) also formed complexes with cyt *c* in the presence of sodium ascorbate at pH 7.

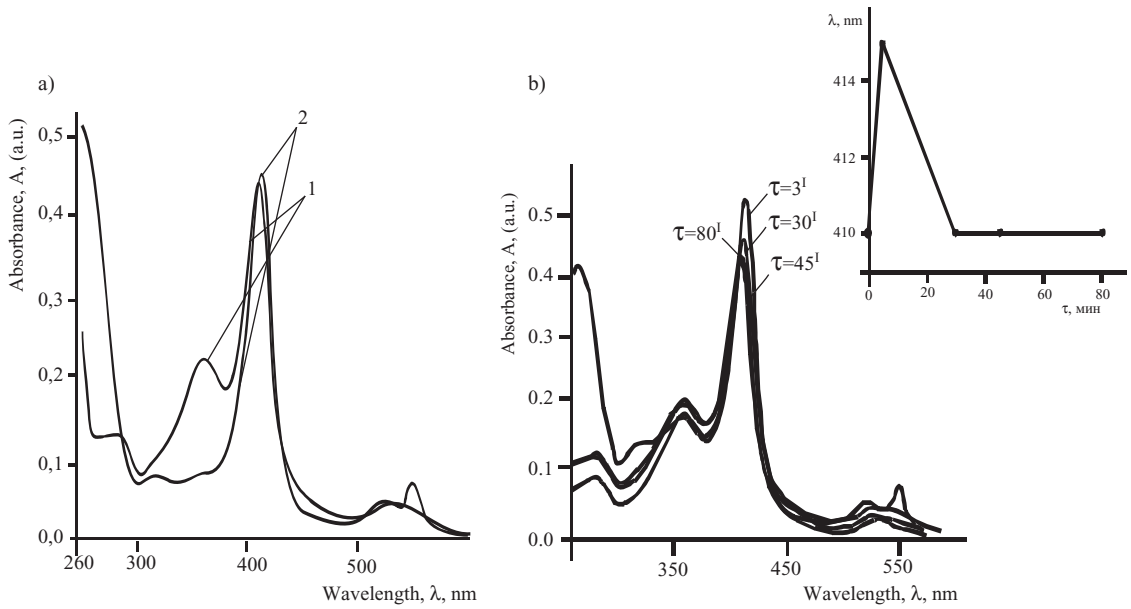
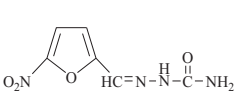


Fig. 2. a, b. UV-Vis spectra of $1{\cdot}10^{-5}$ M cytochrome *c* solution, $1.45{\cdot}10^{-2}$ M sodium nitrite, $5{\cdot}10^{-4}$ sodium ascorbate, $8.5{\cdot}10^{-3}$ M succinic acid (*curve 1*) and in the absence of sodium nitrite (*curve 2*); the spectra of reactions mixture in time (panel b). The insert shows dependence $\lambda = f(\tau)$.

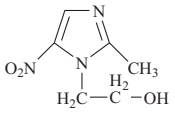
Table 1
UV-Vis data of the aqueous reaction mixtures of $1{\cdot}10^{-5}$ M cytochrome *c*, $5{\cdot}10^{-4}$ M sodium ascorbate, $6.6{\cdot}10^{-5}$ M biogenic acid at different pH in 40 minutes.

Acid	$NaNO_2$, $1.45{\cdot}10^{-2}$ M	pH	410–415 nm		350–360 nm	
			λ_{max}	A	λ_{max}, nm	A
no band	-	7.1	415	0.60 ± 0.02	no band	no band
	+		415	0.60 ± 0.02	358	0.21 ± 0.02
Succinic	-	4.5	410	0.40 ± 0.02	no band	no band
	+		413	0.45 ± 0.02	358	0.22 ± 0.02
Oxalic	-	4.3	410	0.43 ± 0.03	no band	no band
	+		412	0.46 ± 0.03	359	0.20 ± 0.02
Tartaric	-	4.4	411	0.42 ± 0.03	no band	no band
	+		411	0.41 ± 0.03	359	0.22 ± 0.02
Citric	-	4.3	413	0.44 ± 0.03	no band	no band
	+		410	0.40 ± 0.03	359	0.21 ± 0.02

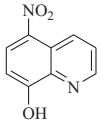
no band, In the experiment in the absence of biogenic acid and sodium ascorbate λ_{max} = 410 nm, A = 0.40 ± 0.02 (pH 6.2).



5-nitrofural



Metronidazole



5-nitroxoline

It has been shown by UV-visible spectra that the absorbance of nitromedicine in the range of 270 nm (C = O of ascorbate) was strongly decreased and in the range of absorption of γ-band of cyt c^{3+} was changed from 410 nm up to 415 nm for all nitromedicines, and the absorbance intensity was increased linearly when the nitromedicine concentration was increased too. In Fig. 3a,b these data demonstrated the formation of NO-cyt c^{2+}-complex from cytochrome *c* and nitromedicines, with 5-nitrofural as an example.

The complexation of cyt c^{2+} with nitromedicine and later on with NO in reaction mixture during the initial ten minutes was suggested by the determination of total $[NO_X]$-concentration which equaled 15 ± 5.4 μM at $C_{5\text{-nitrofural}}$ = 30 μM and 22 ± 5.8 μM at $C_{5\text{-nitrofural}}$ = 60 μM, respectively.

It is necessary to note that NO-cyt c^{2+} complexes formed by nitromedicine are very stable: the absorbance of reaction solution didn't change during one mouth.

These results allowed us to propose two types of the dosage form on base nitromedicine, including sodium nitrite, cytochrome *c* and sodium ascorbate as pharmaceutical substance: powder, combined dosage form gel consisting of gel and powder of sodium nitrite. The pharmaceutical composition was made directly before the study by adding the sodium nitrite into the gel while mixing thoroughly.

3.2. The biological activity of the pharmaceutical compositions in the experiment on rats

3.2.1. Regeneration effects of combined dosage form (cyt c-containing gel plus sodium nitrite)

$[NO_2^-]$ concentration in "NO"-gel didn't change during 60 minutes, but $[NO_x]$ didn't change significantly for one day, if $NaNO_2$ powder was added right after into gel-base with cyt *c* and sodium ascorbate. "NO"-gel was used only freshly prepared in all days of the experiment.

Rat skin surface concentration of $[NO_x]$ of combined gel was equal to 1.81 ± 0.21 μM/cm², taking note that the average mean of surface area of wound equaled 40 cm² and 0.5 mL gel that treated the rat wound . Under "NO"-gel treatment of burn wound during 30 minutes $[NO_x]$ concentration in plasma was changed from 4.82 ± 0.34 μM as soon as the onset of injury to 5.91 ± 0.25 μM of approximating control (Table 2).

The area of skin wounds in the experimental group decreased under "NO"-gel treatment much faster than in the control group (Fig. 4). While the average time of complete wound healing in the

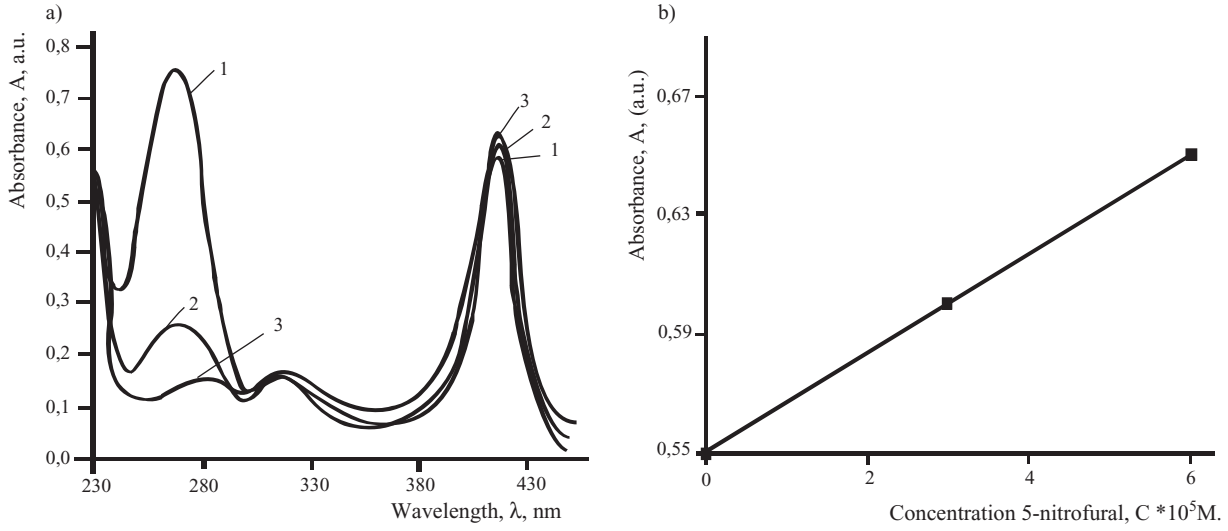


Fig. 3. UV-Vis spectra of aqueous solutions of $1{\cdot}10^{-5}$ M cyt *c* and nitrofural. (a). Concentration of 5-nitrofural (mol/L) 1 – C = 0; 2 – C = $3.0{\cdot}10^{-5}$; 3 – C = $6.0\ 10^{-5}$; C(cyt *c*) = const; (b). Dependence of A (in the region of γ-band of cyt *c*) on C (nitrofural). C (Na-Asc) = $5{\cdot}10^{-4}$M.

Table 2
Total [NO_x] concentration in blood plasma of rats during "NO"-gel treatment.

	[NO_x], μM
Control	6.81 ± 0.22
"NO"-gel after injury (τ = 2 min)	4.82 ± 0.34
"NO"-gel treatment (τ = 30 min)	5.91 ± 0.25
"NO"-gel treatment (10 days)	6.51 ± 0.23

control group was equal to 29.15 ± 1.71 days, such term of the "NO"-gel treatment wound decreased to 21.17 ± 1.32 days.

On the third day after thermal injury under "NO"-gel treatment the skin wound of the rats got better. There were great differences in the skin wounds at tenth and third days under "NO"-gel treatment (Fig. 4B,D).

The same result of burn wound healing was achieved when pharmaceutical composition with other nitromedicines (5-nitrofural, 5-nitroxoline, metronidazole) was used.

3.2.2. Vasodilatation effect of "NO"-gel

With the local microcirculation assessment, the application of the tested pharmaceutical composition proved maintenance of an increased microcirculation index in the area around the wound on both the third and tenth post-burn days (Fig. 5).

At the same time the animals in the control group showed marked depression of microcirculation ($p < 0.05$ relative to the level of the intact animals and the members of the main group) on the 3rd day, with partial recovery by the 10th day after the thermal injury. At the same time it is in the early period when the most effective stimulation of regenerative processes is provided which is triggered through NO-dependent mechanisms [3,22,23].

It should be mentioned that the level of the microcirculation index of the rats in the control group practically returned to the values characteristic of the intact animals, whereas it remained elevated ($p < 0.05$) in the main group, optimizing conditions for skin regeneration.

The analysis of the parameters of heart rhythm variability allowed us to determine that on the 3rd day after the thermal injury, the animals in the control group gave a stress response, whereas the rats treated with NO-containing composition showed less marked vegetative changes with parasympathetic stimulation emphasis (Fig. 6).

Thus, the members of the control group showed change with the shift to the sympathetic side on the third post-burn day, but when using a NO-containing composition, the ratio between the tension index reflecting the myocardium sympathetic stimulation and the mode amplitude visualizing vagotonic effects, corresponded, on the whole, to the initial level. On the 10th day heart rhythm structure optimization in the rats of both groups was observed.

The most marked tendencies are seen with respect to the spectral parameters of heart rhythm variability, the ratio of spectrum power in low- and high-frequency range, in particular (Fig. 7).

It was established that on the 3rd and 10th days of the postburn period, marked hypersympathicotonia was found in the animals in the control group (up to 0.2 relative units) while it was significantly lower in the rats of the main group, a tendency to the normalization of the specified index being registered on the 10th day after injury.

We think that the positive effects in the wound healing are due to not only to the action of NO or nitrosyl containing cytochrome *c*-complexes, but the excess of antioxidant-sodium ascorbate in pharmaceutical composition because metabolism of NO and related N-oxides in the systemic circulation depends on concentration of blood antioxidants. When human plasma is exposed to NO or NO_2, a rapid loss of antioxidants, such as ascorbic acid, uric acid and most importantly protein thiols, occurs [25].

4. Conclusion

In this paper we suggested new combined dosage forms of cytochrome *c* nitrosyl complexes which consist of hydrophilic gel of cytochrome *c* and powder of antibacterial nitromedicine, such as

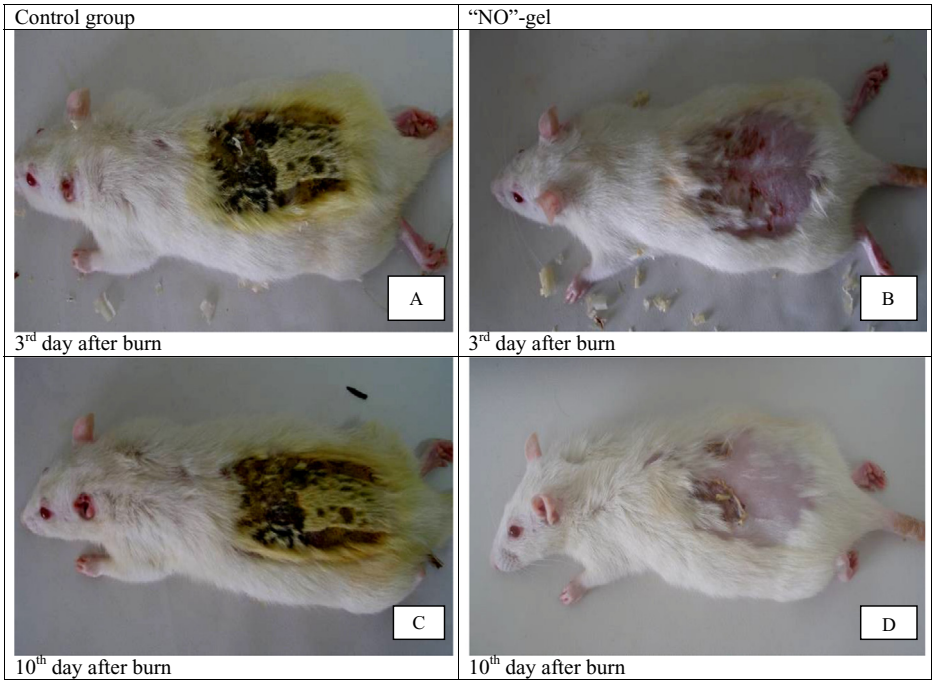
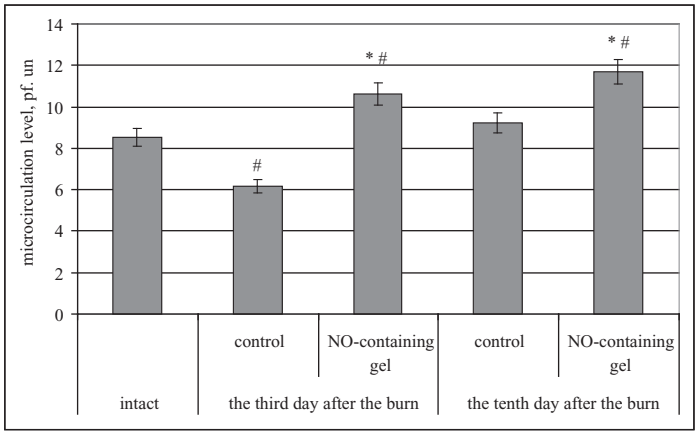
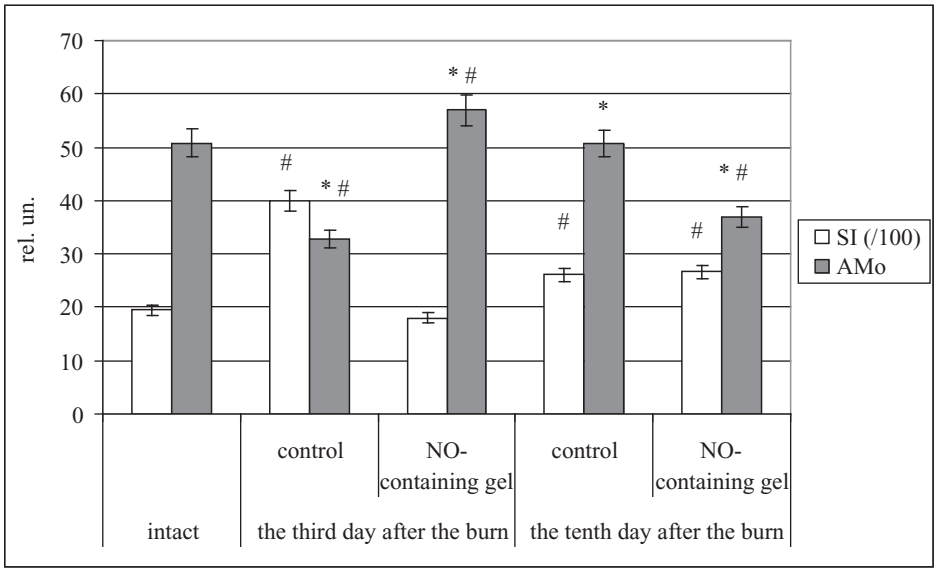


Fig. 4. Healing of the burn wounds: (A) 3rd day after burn in control group; (B) under "NO"-gel treatment during third day; (C) 10th day after burn in control group; (D) under "NO"-gel treatment during tenth day.



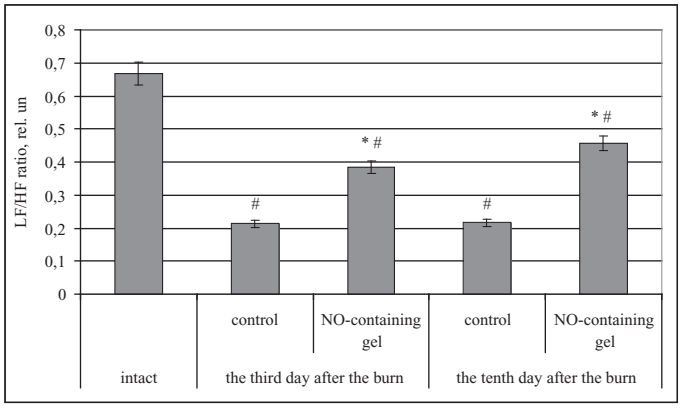
	Day	Level of microcirculation, PM, perf. un.	RSD, %
Control	3	6,18±0,02	0,25
"NO"-gel	3	10,65±0,02	0,19
Control	10	9,25±0,05	0,38
"NO"-gel	10	11,90±0,02	1,30

Fig. 5. The influence of topical treatment by "NO"-gel on level microcirculation (LM, perf. un.) in periwound area of animals. Level of microcirculation was estimated by laser Doppler flowmetry in third and tenth days after burn. The level microcirculation is the microcirculatory rate presented in perfusion units (perf. un.); *, the statistical differences of control and main (with "NO"-gel) groups, $p < 0.05$; #, the statistical differences of control and intact groups, $p < 0.05$.



	Day	TI, stand. units	AM_o, stand. units
Control	3	40,0±2,5	32,8±2,3
"NO"-gel	3	18,1±1,8	57,0±4,0
Control	10	26,1±1,3	50,7±4,2
"NO"-gel	10	26,6±1,4	36,9±2,5

Fig. 6. The dependence of heart rate variability (HRV) parameters on the topical treatment of rats with thermal injury (SI, stress index, AM_o, amplitude of modal value of RR interval set). Heart rate variability was estimated by special Neurosoft ECG system in third and tenth days after burn. The rate of stress index and amplitude of modal value was demonstrated in relative units (rel. un.); *, the statistical differences of control and main (with "NO"-gel) groups, $p < 0.05$; #, the statistical differences of control and intact groups, $p < 0.05$.



	Day	Ratio LF/HF	RSD, %
Control	3	0,21±0,02	0,31
"NO"-gel	3	0,38±0,03	0,11
Control	10	0,22±0,01	0,57
"NO"-gel	10	0,46±0,03	0,82

Fig. 7. The dependence of ratio LF/HF on the topical treatment of the animals with thermal injury; LF (ms^2) and HF (ms^2) mean power of heart rate in low (LF)- and high-frequency (HF) range. The ratio LF/HF was shown in relative units (rel. un.); *, the statistical differences of control and main (with "NO"-gel) groups, $p < 0.05$; #, the statistical differences of control and intact groups, $p < 0.05$.

5-nitrofural, metronidazole, 5-nitroxolin, and sodium nitrite, for burn wounds treatment.

The formation of cytochrome *c* nitrosyl complexes in the reactions with nitromedicine was proved in comparison with reaction mixtures of cytochrome *c* and gaseous nitric oxide, in the presence of sodium nitrite as the nitric oxide "depot" by using UV-visible spectra. Ascorbic acid may promote the complexation of ferrocytochrome *c* with nitromedicines in aqueous solutions too. It has been shown that absorption of γ-band (415 nm) typical for ferricytochrome *c* linearly increased with medicine concentration, but ascorbic acid absorption band extremely decreased in the region of 270 nm and new bands of nitrosyl content compounds in the region of 350–390 nm appeared.

With the use of rats in the experiments, the vasodilatation and regeneratting effects were shown when the wounds were treated with a combined dosage form of cytochrome c and nitromedicine on the base complexes, thus being able to generate nitric oxide. These positive effects at the local level are shown by a sufficient microcirculation index which indicates intensification of the blood flow in the microvessels in the injured area [26]. The effects at the systemic level provide maintenance of the general heart rhythm and gradual recovery of the vegetative balance, which is not observed in the animals of the control group.

The developed combined dosage form has the advantages over reparant ointments that usually are used because they show positive local and systemic effects. We suppose that pharmaceutical composition containing cytochrome *c*, sodium ascorbate and nitromedicine may be used as transdermal dosage forms for cardiological disease treatment in the future.

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