THE STATE OF NITROGEN METABOLISM UNDER THE INFLUENCE OF EXOGENOUS NO IN EXPERIMENTAL THERMAL TRAUMA

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Abstract. The effect of therapeutic technologies used in the treatment of burns on the parameters of nitrogen metabolism has not been sufficiently studied. In this regard, the assessment of the level of nitrites and nitrates of blood and tissues in combined thermal trauma (CTT) under the influence of NO is of scientific and practical interest. The aim of the study was to determine the concentration of nitric oxide metabolites in the blood and organs under the influence of NO during CTT. The experiment was carried out on white male rats of the Wistar line. CTT (contact burn on the area of 20% of the body surface and thermal inhalation exposure for 20–30 seconds) was applied under anesthesia. Animals with CTT were treated daily with intraperitoneal injections of a 10% dinitrosyl iron complexes (DNIC) solution or were inhaled with NO (20ppm). The concentration of nitrites and nitrates was determined in the blood plasma and homogenate of the liver, heart, kidneys, and lungs. CTT was accompanied by hyperproduction of NO in blood plasma on the 1st, 7th and 10th days after injury with a maximum growth on the 1st day. The largest decrease in NO_2- and NO_3- levels during CTT was observed in the kidneys > liver > lungs > heart. The use of DNIC in CTT led to an increase in NO_2- and NO_3- in the organs. Inhalations of NO during CTT increased the concentration of NO_2- and NO_3- in the homogenate of the lungs > kidneys > heart > liver.

Keywords: combined thermal injury, nitric oxide, dinitrosyl iron complexes, nitrites, nitrates, blood, tissues.

List of Abbreviations

AOS – antioxidant system
CTT – combined thermal trauma
DNIC – dinitrosyl iron complexes
NO – nitric oxide
iNO – NO inhalation
NOS – NO-synthase
iNOS – inducible NO-synthase
eNOS – endothelial NO-synthase
nNOS – neuronal NO-synthase
mNOS – macrophage NO-synthase
Hb – hemoglobin
HPV – hypoxic pulmonary vasoconstriction
ATP – adenosine triphosphate
PARP – poly(ADP-ribose) polymerase

Introduction

The study of molecular mechanisms of disease development is one of the most dynamically developing areas of molecular medicine. Thermal trauma is a common type of domestic injury, which is becoming more frequent in the conditions of aggravation of the military situation. Free radical processes occur most intensively during a burn. At the same time, the protective mechanisms aimed at binding toxic metabolites are reduced (Porter *et al*., 2016). Separate results of studying the state of lipid peroxidation processes, the antioxidant system (AOS) and the intensity of formation of nitric oxide (NO) in the body during burns are given in the literature (Pozhilova & Novikov, 2015; Jacob *et al*., 2017). However, the authors have no consensus on the role and relationship of AOS as a combination of pro- and antioxidant processes and the NO system in the pathogenesis of thermal trauma. The effect of therapeutic technologies used in the treatment of burns on the parameters of nitrogen metabolism has not been sufficiently studied. In this regard, the assessment of the level of nitrites and nitrates of blood and tissues in normal and thermal trauma under the influence of active forms of nitrogen is of scientific and practical interest.

NO is an important target for pharmacological effects in many pathological conditions, including hypoxia and ischemia (Alimoradi *et al.*, 2019; Liang *et al*., 2021; Lopez-Lorenzo *et al*.,

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2022) . The issues of increasing the body's resistance to the development of hypoxia and ischemia are very relevant in biomedical research, since these conditions accompany the course of many diseases, including burn disease (Mokline *et al*., 2017). However, despite numerous studies, the importance of NO in the systemic regulation of cell and tissue homeostasis still causes scientific discussions.

The aim of the study was to determine the concentration of nitric oxide metabolites in the blood and organs under the influence of nitric oxide during experimental combined thermal trauma (CTT).

Materials and Methods

The experiment was carried out on 60 white male rats of the Wistar line in accordance with the requirements of bioethics and the rules of laboratory practice (GPL), the Geneva Convention for the Protection of Animals «International Guiding Principles for Biomedical Research Involving Animals» (Geneva, 1990), Order of the Ministry of Health of the Russian Federation № 267 of 19.06.2003 "On approval of the rules of laboratory practice". The study was approved by the Local Ethics Committee of the Privolzhsky Research Medical University of the Ministry of Health of the Russian Federation (protocol № 5), in accordance with the provisions of the Helsinki Declaration of 1975, revised in 2008. The rats were received from the «Stolbovaya» branch (Moscow). All animals were kept in standard vivarium conditions in cages with free access to food and water on a diet, according to GOST standards «Maintenance of experimental animals in the nurseries of the Research Institute» (Peretyagin *et al*., 2011).

The animals were separated by stratified randomization with stratification by body weight and age. Rats weighing 200-250 g at the age of 5-7 months were included in the study. After a 14 – day adaptation to the conditions of the local vivarium and quarantine, 6 groups of animals of equal numbers were formed from 60 rats: 1 – intact rats (n = 10) – animals without manipulation; 2 (n = 10), 3 (n = 10), 4 (n = 10) – control groups (animals with CTT on the 1st, 7th and 10th days after the CTT, which corresponds to the periods of burn disease); 5 – experimental group (n = 10) – animals with CTT who received NO inhalation (iNO); 6 – experimental group (n = 10) – animals with CTT who received DNIC.

CTT (contact burn on the area of 20% of the body surface and thermal inhalation exposure to hot air and combustion products in the conditions of the inhalation chamber) was applied under anesthesia («Zoletil-100» («VirbacSante-Animale», France) at a dose of 60 mg/kg and «Xylavet» («Interchemie», Netherlands) at a dose of 6 mg/kg) (Martusevich *et al*., 2014). The animals were removed from the experiment for 1, 7 and 10 days after CTT by decapitation with preliminary ligation of the carotid artery under anesthesia (Zoletil-100 + Xylavet).

The concentration of nitrites and nitrates was determined in the blood plasma and homogenate of the liver, heart, kidneys, and lungs. The blood was stabilized with a solution of sodium citrate (3.8%) in a ratio of 9:1. Organ homogenates were obtained by differential centrifugation (Egorova & Afanasev, 2011). We used a spectrophotometric method for determining NO metabolites (NO_2^- and NO_3^-) in biological media based on the Griss reaction (Metelskay & Gumanova, 2005).

Inhalation-external exposure to NO (concentration 20ppm) on animals with CTT was carried out in a desiccator daily for 10 days for 10 minutes. The feed rate of the gas mixture is 2 l/min. The synthesis of the gas mixture was carried out using an experimental apparatus for generating NO, developed at the Russian Federal Nuclear Center (Sarov).

Animals with CTT received daily treatment in the form of intraperitoneal injections of a 10% solution of DNIC in saline (1:9 by volume) (1 ml; 0.3 micromol/l) for 10 days. DNIC with glutathione was obtained by the method of A.F. Vanin (2017), mixing 300 millimol $NaNO_2$, 200 millimol reduced glutathione and a solution of $FeSO_4$. The concentration of DNIC was determined by the spectrophotometric method on a spectrophotometer Power Wave XS (Bio-Tek, USA) in the wavelength range of 310–700 nm.

Statistical processing of the results was carried out using a computer program «Statistica 6.0». The Shapiro-Wilk criterion was used to test the hypothesis about the nature of the distribution. Descriptive statistics are given by the mean and mean square deviation ($M \pm \sigma$). The comparison of two independent groups was performed using the Student's t-test, depending on the fulfillment of the applicability conditions. When calculating the Student's t-test, we used the Bonferroni correction, which allows us to eliminate the first-kind error that occurs when comparing more than two samples by this method. The differences between the compared groups were considered statistically significant at $p < 0.05$.

Results

Since NO, on the one hand, is a bioregulator, and on the other, can play a negative role (Martusevich *et al*., 2014; Park *et al*., 2021), mainly realized through the formation of peroxynitrite and the possibility of the development of nitrosative stress in the body, the question of assessing the level of NO in biosystems has acquired fundamental importance (Kawakami *et al*., 2021). In addition, with NO therapy, there was a need for quantitative analysis of NO production (Hall & Garthwaite, 2009). Direct quantitative analysis of NO is not possible because it is a short-lived compound. The only stable end product of NO autooxidation in an aqueous medium is nitrites. When NO reacts with oxyhemoglobin or a superoxide radical, nitrates are formed. Therefore, to measure the concentration of NO, the determination of the total level of stable final metabolites of NO in blood plasma and subcellular fractions of organs was used (NO_x), that is, nitrites and nitrates (NO_2^-и NO_3^-, respectively) (Metelskay & Gumanova, 2005; Metelskay & Gumanova, 2005). The method accurately reflects the degree of NOS activity. The results showed that CTT was accompanied by an increase in the concentration of the final metabolites of NO in the blood compared with the indicators of healthy rats (Fig. 1). So, on the 1st day after CTT, the NO_x level in the blood plasma, the content of nitrites and nitrates increased by 188.99% ($p = 0.000$), 11.75% ($p = 0.012$) и 236.45% ($p = 0.000$), on the 7th day after CTT – on 55.70% ($p = 0.004$), 29.41% ($p = 0.011$), 62.74% ($p = 0.001$), on the 10th day – on 105.61% ($p = 0.000$), 19.50% ($p = 0.023$), 128.67% ($p = 0.000$) compared to intact animals.

However, a decrease in the concentration of NO end metabolites in the organs of rats with CTT was revealed compared with the indicators of intact rats. In the homogenate of the liver, kidneys, heart, lungs, on the 1st, 7th, 10th day after CTT, there was a statistically significant decrease in the level of NO_x, nitrites, nitrates (Fig. 2, 3, 4, 5). The maximum decrease in NO_x, nitrites and nitrates in kidney homogenate was noted on day 10, in liver, heart and lung homogenate – on day 1 after CTT.

In the homogenate of the heart, the concentration of NO_x, $NO_2^-$and NO_3^- decreased on 1 day after CTT by 28.29% ($p = 0.019$), 45.23% ($p = 0.007$) и 23.53% ($p = 0,017$) respectively compared to intact animals. In the homogenate of the heart, the concentration of NO_x, $NO_2^-$and NO_3^- remained reduced on the 7th, 10th day after CTT (Fig. 4).

The decrease of NO_x, $NO_2^-$and NO_3^- in lung homogenate on day 1 after CTT occurred by 37.92% ($p = 0.007$), 46.92% ($p = 0.000$), 34.18% ($p = 0.005$) compared to intact animals.

Inhalation of 20 ppm NO with CTT led to a decrease in concentration NO_x (30.56% ($p = 0.028$)) and nitrates (35.86% ($p = 0.012$)) in blood plasma compared with the corresponding indicators in CTT (Fig. 1). Under the influence of DNIC in the blood plasma of rats with CTT, the level of NO_x, nitrites and nitrates decreased by 11.86% ($p = 0.031$), 5.34% ($p = 0.883$), 12.77% ($p = 0.025$) accordingly, compared with the indicators of animals with CTT without treatment.

Inhalation of 20 ppm NO, injection of DNIC in CTT led to a statistically significant increase in the concentration of NO_x, nitrites and nitrates in the liver homogenate compared with the indicators in CTT, which contributed to the normalization of the nitrate content (Fig. 2).

The use of DNIC in CTT led to an increase in the level of NO_x, $NO_2^-$and NO_3^- compared

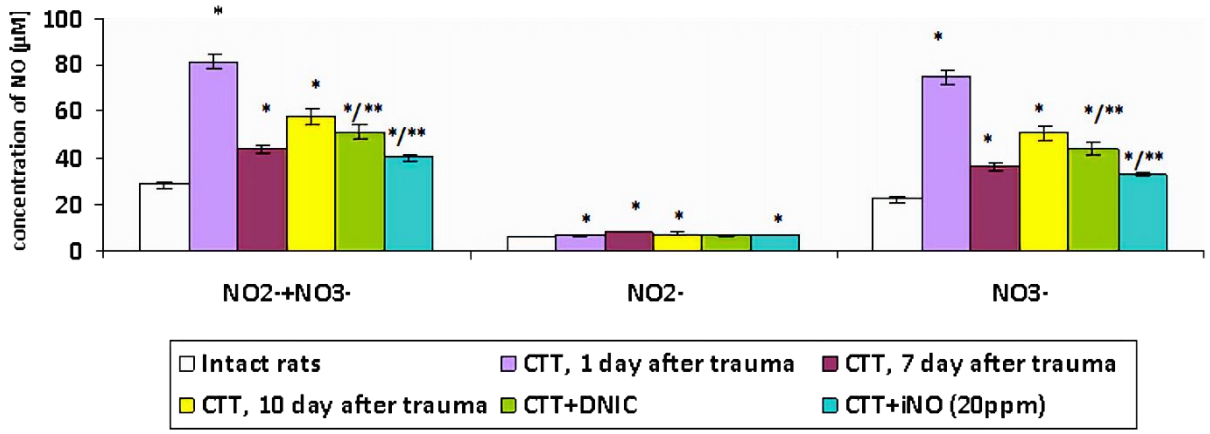

Fig. 1. The content of nitrogen monoxide metabolites (μM) in rat blood plasma
Note: * – differences are statistically significant compared to intact rats ($p < 0.05$); ** – differences are statistically significant compared to CTT on the corresponding day ($p < 0.05$)

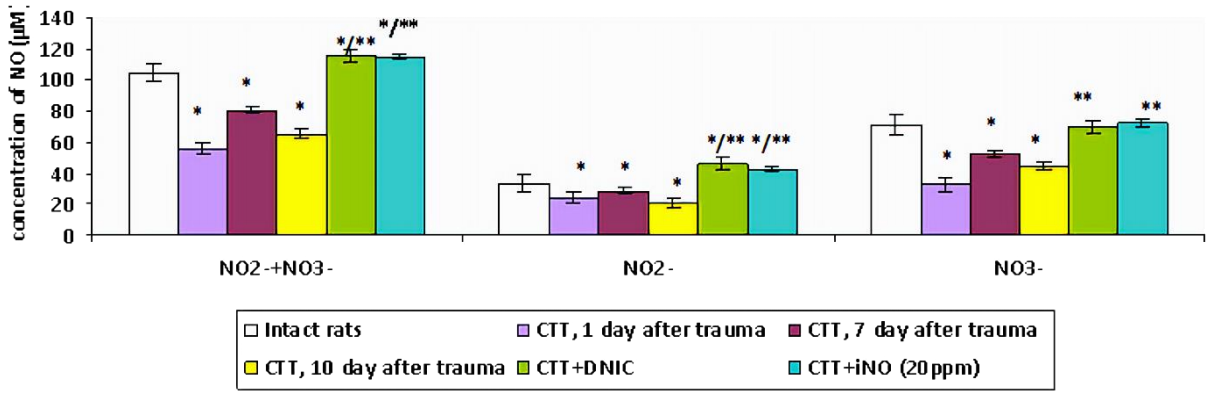

Fig. 2. The content of nitrogen monoxide metabolites (μM) in rat liver homogenate
Note: * – differences are statistically significant compared to intact rats ($p < 0.05$); ** – differences are statistically significant compared to CTT on the corresponding day ($p < 0.05$)

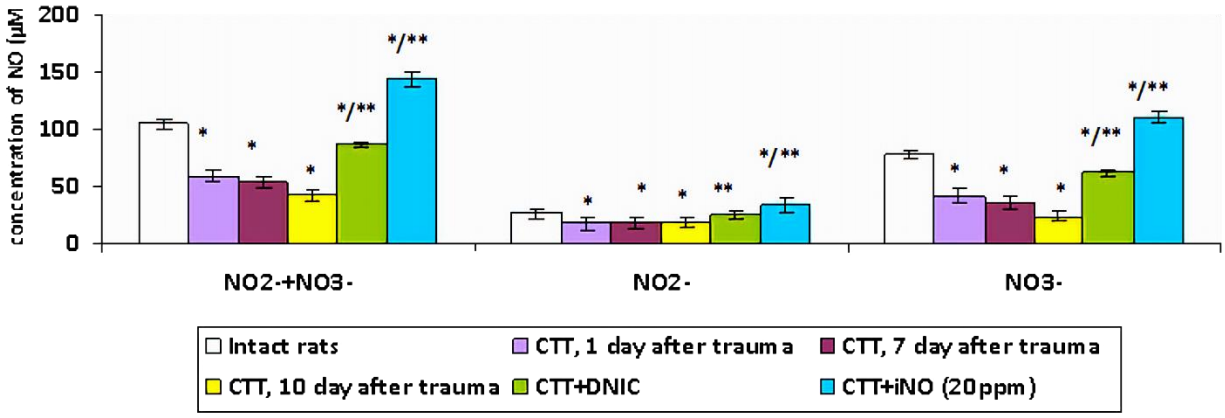

Fig. 3. The content of nitrogen monoxide metabolites (μM) in rat kidney homogenate
Note: * – differences are statistically significant compared to intact rats ($p < 0.05$); ** – differences are statistically significant compared to CTT on the corresponding day ($p < 0.05$)

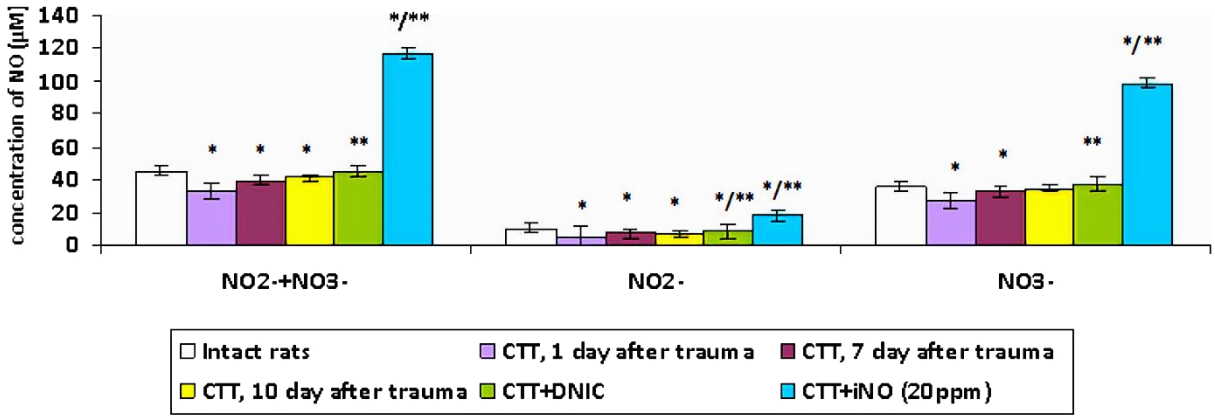

Fig. 4. The content of nitrogen monoxide metabolites (μM) in rat heart homogenate
Note: * – differences are statistically significant compared to intact rats ($p < 0.05$); ** – differences are statistically significant compared to CTT on the corresponding day ($p < 0.05$)

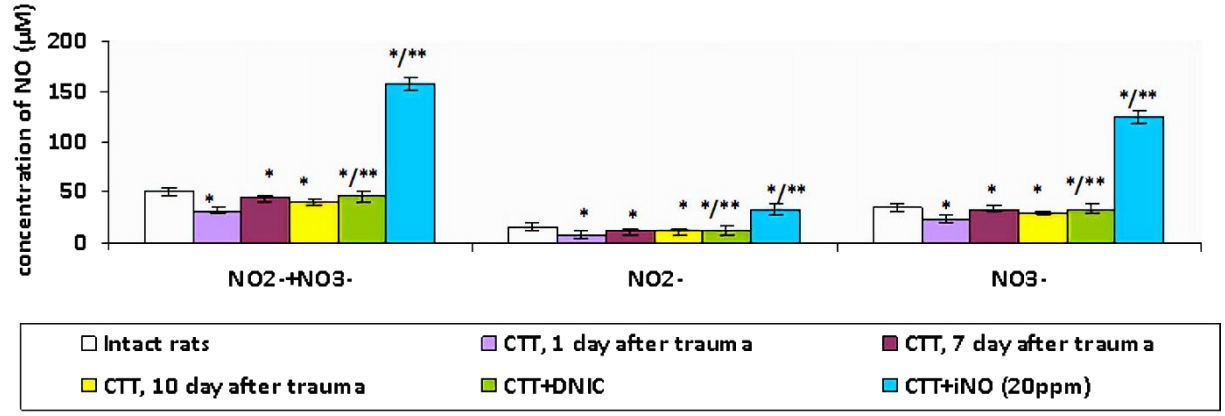

Fig. 5. The content of nitrogen monoxide metabolites (μM) in the homogenate of rat lungs
Note: * – differences are statistically significant compared to intact rats ($p < 0.05$); ** – differences are statistically significant compared to CTT on the corresponding day ($p < 0.05$)

with the indicators of animals with CTT without treatment in the homogenate of kidneys, heart, lungs (Fig. 3–5). DNIC therapy in CTT caused normalization of NO_x and nitrates in the homogenate of the heart. Inhalations of 20ppm NO at CTT caused a statistically significant increase in the concentration of NO_x, nitrites, nitrates in the homogenate of the kidneys, heart, lungs compared with those of rats with burns, exceeding the content of NO_x, nitrites and nitrates of intact animals.

Discussion

Thus, in healthy animals, the maximum NO_x content was found in the liver > kidneys > lungs > heart. The results obtained confirm the literature data (Gunes *et al*., 2017). This distribution of nitric oxide metabolites in the organs of healthy rats is due to the activity of NOS and the presence of enzyme isoforms in the organs. In normoxia, NO activates various signaling pathways depending on the threshold concentration (Shannon *et al*., 2022). In tissues, NO interacts with O_2, forming nitrites and nitrates, which give NO^-_2 or NO^-_3 in the liquid environment of the body (Malahov *et al*., 2009).

NO in the liver can have both a protective effect associated with the regulation of blood flow and cell interaction, and damaging effects on liver homeostasis (Blot, 2021). All isoforms of NOS are expressed in the liver, the main ones for which are *i*NOS and *e*NOS (Saley *et al.*,

2009). In hepatocytes (in vitro), *i*NOS expression is carried out only in the presence of Kupffer cells stimulated by lipopolysaccharide, γ-interferon, tumor necrosis factor, pro-inflammatory interleukin-1, interleukin-6, the synthesis and secretion of which increases with CTT (Menshikova *et al*., 2000).

NO causes inhibition of gluconeogenesis, protein synthesis, mitochondrial respiration, activity of glyceraldehyde-3-phosphate dehydrogenase, aconitase and cytochrome P450 in hepatocytes, as well as activation of soluble guanylate cyclase (Osipov *et al*., 2007). Inhibition of NOS in the body leads to a violation of the prooxidant-antioxidant balance during ischemia and liver reperfusion. The facts of hepatotoxic effects of NO in endotoxemia have been established. In experimental sepsis, a decrease in the level of NO production in the liver protects hepatocytes from necrosis, and large amounts of NO produced by *i*NOS prevent apoptosis (Saley *et al*., 2009). *i*NOS-regulated release of NO causes the expression of protective proteins – Hsp70 protein and cyclooxygenase.

In the kidneys, NO regulates renal blood flow, water-salt metabolism, filtration processes, reabsorption, secretion, incretion, has a natriuretic and diuretic effect (Ahmad *et al*., 2018). NO inhibits Na^+ and water transport in the walls of the tubules of the nephron (Bilalov, 2015). NO is constantly synthesized in the kidneys, in various departments of which all 3 isoforms of NOS are represented: *n*NOS, *i*NOS and *e*NOS (Herrera *et al*., 2006).

The lungs also contain 3 isoforms of NOS, which are products of gene expression NOS_1, NOS_2 и NOS_3. *e*NOS is found in the endothelium of bronchial vessels and epithelial cells, *n*NOS – in cholinergic and noncholinergic/nonadrenergic nerves of the bronchi, epithelial cells. *m*NOS is detected in cells in response to cytokines, endotoxins and oxidants. The participation of NOS gene polymorphism in the formation of respiratory pathology is shown (Uryasev & Shahanov, 2017). Violation of the production and/or destruction of NO is important in the occurrence of hyperreactivity of the respiratory tract in the pathophysiology of the respiratory organs, which is important in case of thermal inhalation injury.

Many regulatory functions of NO are found in the heart, where NO synthesis is carried out in neurons and vascular endothelium and myocytes (Champion *et al*., 2003). NO regulates myocardial function directly and through its effect on blood vessels (Reutov *et al*., 2007). *n*NOS, *i*NOS and *e*NOS are present in the myocardium (Champion *et al*., 2003). In pathology, immunoreactivity to *i*NOS and *e*NOS increases, induction of *i*NOS in cardiomyocytes is caused by proinflammatory cytokines (Reutov *et al*., 2007), an increase in the level of which accompanies CTT. Violation of subcellular distribution of *n*NOS and *e*NOS in cardiomyocytes is associated with the development of various pathological conditions of the myocardium (Rus *et al*., 2011).

NO actively influences the process of opening mitochondrial pores, which is a key link in the development of reperfusion disorders of heart function. This effect of NO is dose-dependent (Piantadosi *et al*., 2002). The blockade of the pore under the action of NO is explained by the inhibition of cytochrome oxidase. High concentrations of NO (more than 20 micromole) cause inhibition of ATP synthesis and nitrosylation of SH-groups of mitochondrial proteins, which leads to the opening of mitochondrial pores. NO, interacting with superoxide, forms peroxynitrite, which through the oxidation of thiol groups of proteins also leads to the opening of mitochondrial pores. On the other hand, NO is an inhibitor of mitochondrial pores, showing activity in concentrations provided by endothelial cells and cardiomyocytes.

The work shows that CTT was accompanied by hyperproduction of NO and accumulation of NO degradation products in blood plasma. Nitrites in the blood interact with Hb, oxidizing divalent iron, resulting in the formation of methemoglobin, which is unable to carry out reversible binding of O_2. As a result, hypoxia develops, lactic acid, cholesterol accumulates and the amount of protein drops sharply, which is observed with CTT (Kovalenko, 2015; Jacob *et al*., 2017). On the other hand, the hyperproduction of NO can be considered as a compensa-

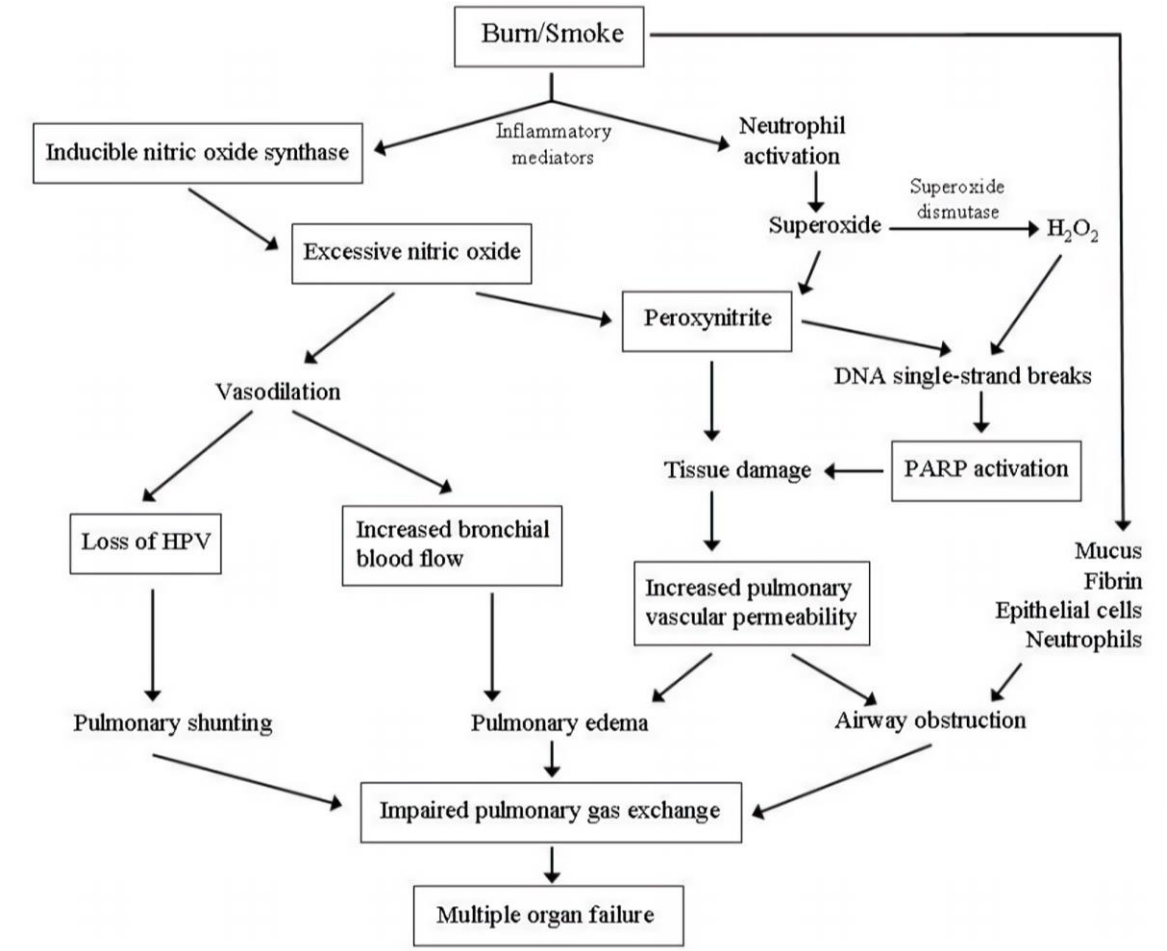

Fig. 6. Possible role of nitric oxide in the pathophysiology of burn and inhalation injury (Lange *et al.*, 2009). Excessive nitric oxide production by the inducible nitric oxide synthase causes pulmonary vasodilation, leading to the loss of hypoxic pulmonary vasoconstriction (HPV) and increased bronchial blood flow which, in turn, result in pulmonary shunting and pulmonary edema. Both nitric oxide-induced peroxynitrite formation and poly(ADP-ribose) polymerase (PARP) activation cause increased pulmonary vascular permeability. In combination with mucus secretion, fibrin clotting, as well as congregation of neutrophils and epithelial cell debris, airway obstruction occurs. Pulmonary shunting, pulmonaryedema and airway obstruction result in impaired pulmonary gas exchange and ultimately multiple organ failure

tory-adaptive reaction under conditions of hypoxia developing during thermal trauma, aimed at increasing blood flow, since NO is a powerful vasodilator. There is a hypothesis that the stress response and long-term adaptation lead (or are a consequence) to a decrease or increase in NO production (Malyshev & Manuhina, 2000). The mechanisms of NO synthesis are determined by the degree of blood oxygenation and can be activated in hypoxia and ischemia (Reutov *et al*., 2007). The Figure 6 shows the systemic effect of NO and its metabolites in burn injury.

In case of burn stress, hypoxia, there is hyperproduction of reactive oxygen species and their interaction with NO with the formation of peroxynitrite. Consequently, NO is one of the key factors in the pathophysiology of oxidative stress, and the peroxynitrite anion is involved in the implementation of oxidative stress, inducing DNA damage and mutations. Excess production of NO is an important link in the pathogenesis of acute circulatory insufficiency in shock. Activation of *i*NOS increases the level of NO in the blood of patients with severe combined trauma (Pozhilova & Novikov, 2015).

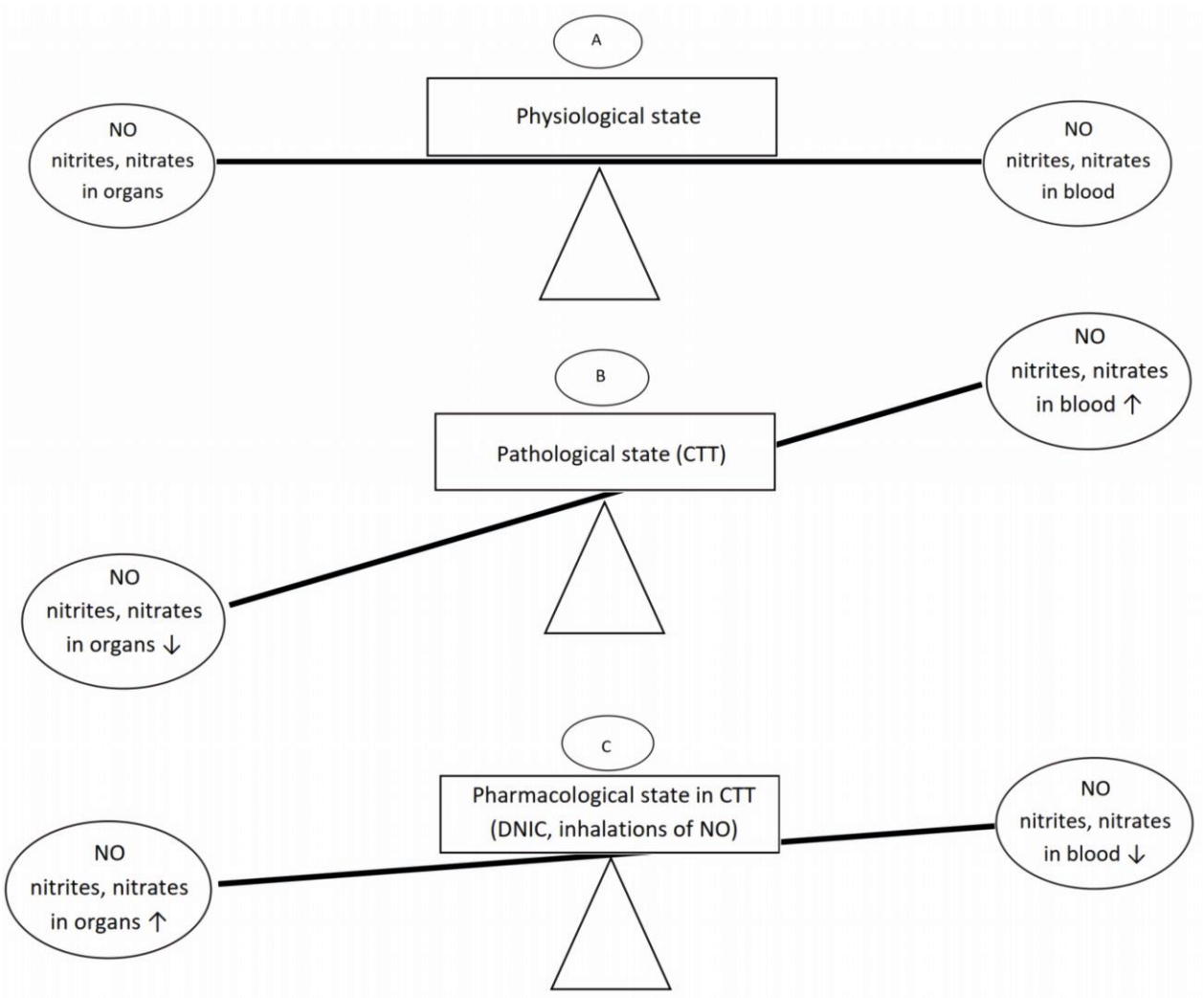

Fig. 7. Different levels of NO (nitrites and nitrates) depicting different states of the body. (A) shows normal levels of NO in normal physiological conditions. (B) represents that levels of NO increased in blood while that of NO is reduced in tissue in pathological states like CTT. (C) indicates that pharmacological treatment by NO donors increases the NO in organs and reduced the levels of NO in blood

Under stress, hypoxia, the activity of *e*NOS increases and, as a consequence, the concentration of intracellular NO (Changjian, 2012).

However, this study revealed a decrease in the concentration of stable NO end metabolites in subcellular fractions of rat organs during CTT, which may be due to a decrease in the amount of L-arginine, as well as a violation of the nitrite-reductase mechanisms of synthesis of the NO molecule. The increase in O_2^--activity observed during thermal trauma causes a decrease in NO content and leads to a mismatch between oxidative and antioxidant mechanisms in tissues, participating in many pathophysiological processes in the body (Kopkan & Cervenka, 2009).

A decrease in the formation of NO can occur under the action of low-density lipoproteins, high glucose concentrations, hypoxia, ischemia due to inhibition of NOS and a decrease in their expression. A low level of NO in tissues leads to an increase in vascular tone, blood clotting, a decrease in immunity and adaptive capabilities of the body, changes in metabolism (Malahov *et al*., 2009). The greatest drop in NO_x, $NO_2^-$and NO_3^- levels during CTT was observed in the kidneys > liver > lungs > heart. The main route of nitrate excretion is the kidneys, which are

significantly affected by endotoxins formed during a burn, accompanied by impaired renal function, negative shifts in the structural and functional parameters of the nephron, modulation of the activity of some humoral systems regulating kidney function and vascular tone. The maximum decrease in the total end products of NO degradation was observed on the 1st day after CTT and then on the 10th day after the burn.

The most pronounced activating effect of iNO in CTT is probably due to the method of exposure to NO, in which, first of all, the respiratory organs are exposed. iNO eliminates pulmonary vasoconstriction caused by hypoxia, heart defects and respiratory distress syndrome. iNO does not give a systemic effect and improves arterial oxygenation (Malahov *et al*., 2009; Radak *et al*., 2013).

The state of nitrogen metabolism under the influence of exogenous no in experimental thermal trauma is explained in Figure 7.

Maintaining NO homeostasis is very important for protecting the tissues of the myocardium, kidneys, liver and lungs. An increase in NO production in the liver, lungs, heart and kidneys under the influence of exogenous NO in CTT may be due to stimulation of NOS isoforms. The results obtained confirmed the literature data on the increase of NO adducts in the heart and kidneys under the influence of DNIC (Timoshin *et al*., 2008).

Conclusions

1. CTT was accompanied by hyperproduction of NO in blood plasma on the 1st, 7th and 10th days after injury with a maximum growth on the 1st day. A decrease in the end metabolites of NO in the organs of rats with CTT was revealed. The largest decrease in NO_x, $NO_2^-$and NO_3^- levels during CTT was observed in the kidneys > liver> lungs > heart. The maximum decrease in the total end products of NO degradation in organs was noted on 1 day after CTT.

2. The use of DNIC in CTT led to an increase in NO_x, NO_2^- and NO_3^- in the organs: kidneys > liver > lungs > heart. Inhalations of NO during CTT increased the concentration of NO_x, NO_2^- and NO_3^- in the homogenate of the lungs > kidneys > heart > liver.

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