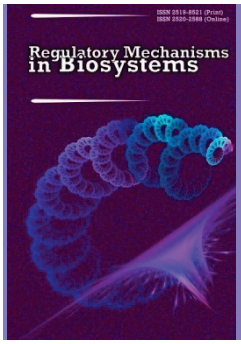

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Parameters of oxidative, nitrasive and anti-oxidative status in men with erectile dysfunction due to combat trauma

O. K. Onufrovych, R. V. Fafula, M. Z. Vorobets, A. S. Besedina, O. V. Melnyk, D. Z. Vorobets, Z. D. Vorobets

Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

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Danylo Halytsky Lviv National Medical University, Lviv, 79010, Ukraine.
Tel.: + 38-032-275-49-66.
E-mail: kaf_medicalbiology@meduniv.lviv.ua

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The development of oxidative and nitrative stress and the processes of free radical oxidation are associated with many pathological processes. Damage of any origin leads to the activation of free radical processes not only in the place of damage, but also in the whole organism. The aim of the study is to assess the state of lipid peroxidation, content of GSH and GSSG and the activity of NO-synthase and arginase in lymphocytes and peripheral blood serum in men with erectile dysfunction due to combat trauma. The research was conducted on peripheral blood lymphocytes of men injured as a result of combat operations (shrapnel and bullet wounds) in the Russian-Ukrainian war, and who were treated at the Military Medical Clinical Center of the Western Region (Lviv, Ukraine). The research group of men with combat injuries was divided into two age groups: men aged 20–39 years and men aged 40–53 years. The MDA content in the blood serum of patients of both age groups was 1.35 times higher than in the control group. In peripheral blood lymphocytes, the MDA content in patients of the young age group was 1.27, and in patients of the middle age group in 1.39 times higher than in the control group. Simultaneously, no significant changes in the concentration of oxidized glutathione in blood serum and blood lymphocytes were found between men with erectile dysfunction due to combat trauma and healthy men. GSH content in blood serum in patients of both age groups was significantly lower than in the control group. The arginase/NOS ratio in blood serum was 9.75 times lower in the young age group and in 20.45 times lower in the middle age group compared to healthy men. It was established that in the blood serum and blood lymphocytes of men with erectile dysfunction due to combat trauma, processes of lipid peroxidation were intensified and the GSH level was reduced. The GSH/GSSG ratio was reduced only in blood serum. It was found that the oxidative stress is associated with development of nitrative stress. The arginase/NOS ratio was shifted towards increased NOS activity. Activation of iNOS was accompanied by significant inhibition of cNOS. Further study of biochemical mechanisms is important to understand the triggers of erectile dysfunction due to combat trauma.

Keywords: trauma; erectile dysfunction; MDA content; glutathione; NO-synthase; arginase.

Introduction

According to modern ideas, the development of pathological processes in the body are associated with disturbances of the mechanisms of antioxidant protection (Cacciatore et al., 2010; Fafula et al., 2017; Vorobets et al., 2022; Averill-Bates, 2023). In physiological conditions, the balance between prooxidant and antioxidant processes is maintained. In a pathological condition, this balance is disturbed in the direction of uncontrolled generation of free radicals. It is known that reactive oxygen species (ROS) are free radical derivatives of oxygen that have high reactivity (Ko et al., 2014; Chaudhary et al., 2023). Damage to any origin leads to the activation of free radical processes not only in the place of damage, but also in the whole organism. Trauma, shrapnel and bullet wounds, as well as nervous disorders can cause oxidative stress. The last arises as a result of an imbalance between the ROS generation and the activity of antioxidant protection (Hosseinzadeh Colagar et al., 2013; Agarwal et al., 2014; Ko et al., 2014; Chaudhary et al., 2023; Fafula et al., 2023).

Over the past decades, the scientific literature has accumulated a lot of information about the close functional interaction between ROS and nitric oxide (NO). In particular, it is known that at low physiological concentrations, ROS and NO are physiological modulators and perform signaling functions. Hyperproduction of ROS and NO causes the development of oxidative and related nitrative stress. The last can cause irreversible damage in all bio(macro)molecules, in particular in DNA and proteins. In high concentrations, NO can act as a cytotoxic agent with pro-oxidant and apoptotic properties. It has been shown that NO inhibits reparative and bioenergetic processes. Also, the damaging effect of NO is mediated by peroxynitrite, which is produced with high generation of superoxide anion and nitric oxide (Akimov et al., 2016).

It is known that NO is generated enzymatically during the oxidation of L-arginine with the participation of NO synthases (NOS). In the human body, NOS exists in three isoforms, such as endothelial, neuronal, and inducible (eNOS, nNOS, iNOS), which are encoded by different genes, are different in localization, and differ in the nature of induction (Wu et al., 2013). The neuronal and endothelial isoforms are Ca^{2+}-dependent, constitutive enzymes (cNOS) that synthesize physiological or basal small amounts of NO. The Ca^{2+}-independent iNOS isoform is not expressed under physiological conditions (in the absence of extreme endo- and exogenous factors). An expression of iNOS can be induced by pro-inflammatory factors, in particular, lipopolysaccharides of bacteria, pro-inflammatory cytokines, activated macrophages. Under pathophysiological conditions iNOS is known to produce NO in concentrations several orders of magnitude higher than cNO. Competing with the NO-synthase pathway is the non-oxidative pathway of L-arginine conversion with the participation of arginase (Pautz et al., 2010).

The development of oxidative and nitrative stress and the processes of free radical oxidation are prevented by a complex multi-component system of antioxidant protection under physiological conditions. It interrupts chain free radical reactions, eliminates ROS to non-toxic products. The antioxidant defense system includes both enzymatic and non-enzymatic

components that neutralize ROS and free radicals and protect against excessive exposure to oxidative stress (Cacciatore et al., 2010; Adeoye et al., 2018; Averill-Bates et al., 2023; Chaudhary et al., 2023). In order to study the biochemical changes that occur in the body under the action of various exogenous factors on the body, the most adequate model can be peripheral blood lymphocytes, which are able to objectively reflect changes in the body's metabolic homeostasis (Henkel et al., 2011).

The aim of the study is to assess the state of lipid peroxidation, content of GSH and GSSG and the activity of NO-synthase and arginase in lymphocytes and peripheral blood serum in men with erectile dysfunction due to combat trauma.

Materials and methods

Patients and bioethics approval. The research was carried out in compliance with the principles of medical ethics and the protection of patients' rights, human dignity and moral and ethical norms, in accordance with the principles of the Helsinki Declaration of Human Rights, the Council of Europe Convention on Human Rights and Biomedicine, the laws of Ukraine; permission of the Bioethics Committee of the Danylo Halytsky Lviv National Medical University.

The research was conducted on peripheral blood lymphocytes of men injured as a result of combat operations (shrapnel and bullet wounds) in the Russian-Ukrainian war, and who were treated at the Military Medical Clinical Center of the Western Region (Lviv, Ukraine). The research was conducted in September-December 2023 and January 2024. The research group of men with combat injuries was divided into two age groups: men aged 20–39 years (group 1) and men aged 40–53 years (group 2). The control group consisted of 48 practically healthy men without complaints of sexual dysfunction or cardiac, neurological or endocrinological pathology. Among the men of the control group 30 men were aged 20–39 years (group 3) and 18 men aged 40–53 years old (group 4). The collection of peripheral blood was carried out after the preliminary completion of their clinical examination, before assigning them a course of treatment.

Biochemical assays. Serum was obtained from blood collected in disposable tubes without anticoagulant. Blood was kept at room temperature for 30 minutes until complete clot formation or placed in a thermostat at 37 °C for 15 min. After that, blood was centrifuged at 1200–2000 g for 15 minutes. Peripheral blood lymphocytes were isolated according to the modified method of Boyum A. The integrity and viability of blood lymphocytes in all experiments was at least 95%. Saponin was added to the suspension to permeabilize blood lymphocyte membranes and reveal latent enzymatic activities.

The state of lipid peroxidation was assessed by determining the concentration of malondialdehyde. The principle of the MDA determination method is that at a high temperature in an acidic environment, it reacts with 2-thiobarbituric acid, creating a colored trimethylene complex with an absorption maximum at $\lambda = 532$ nm (Nabil et al., 2008; Ayala et al., 2014). The content of total glutathione (GSHt) was determined in the cell suspension after complete reduction of glutathione with the help of glutathione reductase ("Sigma", USA) using Ellman's reagent (Monostori et al., 2009). The enzymatic reaction was initiated by adding glutathione reductase (1.9 units, 0.037 mL of 50 U/mL) and the level of formation of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNBA) was recorded spectrophotometrically at 412 nm every 30 s during a 120 s period. Standard solutions were made from reduced glutathione. To determine the content of oxidized glutathione (GSSG), 60 min before the determination, 2-vinylpyridine was added to the incubation mixture to a final concentration of 2%, and the content of reduced glutathione (GSH) was calculated as the difference between the concentrations of total and oxidized glutathione. The redox index (RI) of GSH was calculated as the ratio ([GSHt]-[GSSG])/[GSHt]).

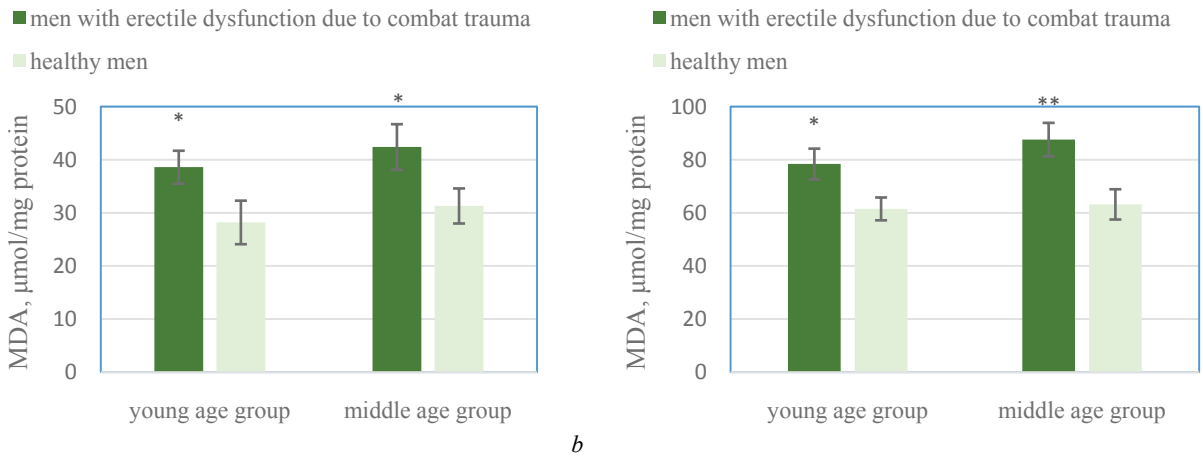
Arginase activity in blood serum and blood lymphocytes was measured by determining levels of urea production. Incubation media of the following composition (mmol/mL): $MnCl_2$ – 2, L-arginine – 100, Tris-HCl – 20 (pH 9.5) was used. The protein concentration was 50–100 mg. The mixture was incubated at 37 °C for 90 min. The reaction was stopped by adding 1 mL 50% trichloroacetic acid. After centrifugation, the urea content was determined in the supernatant spectrophotometrically at 520 nm according to the assay kit "Simko Ltd". Enzyme activity was expressed as nmol urea per min per mg protein.

Determination of NO-synthase activity in blood serum and blood lymphocytes was carried out at 37 °C in an incubation medium (1.5 mL) containing: Tris-HCl – 0.08 M (pH 7.4), $CaCl_2$ – 10 mM, L-arginine – 0.15 mM, NADPH(H^+) – 0.12 mM. Control samples contained bidistilled water instead of NADPH(H^+) and L-arginine. The reaction was initiated by adding an aliquot of protein to the incubation medium (protein content did not exceed 50–70 μg/mL). Absorbance was measured at $\lambda = 340$ nm. The selective iNOS inhibitor aminoguanidine was added to the incubation medium instead of $CaCl_2$ to determine the activity of Ca^{2+}-independent iNOS. cNOS activity was expressed as the difference between total NO synthase activity and iNOS activity. The activity of NO-synthase isoforms was expressed in nanomoles of oxidized NADPH(H^+)/min per 1 mg of protein.

Statistical analysis. The results are presented as the mean ± standard error (x ± SE). Analysis of variance (ANOVA) was used to compare the difference in the means between studied groups. Differences were considered statistically significant at $P < 0.05$.

Results

Indicators of oxidative stress were assessed by the content of MDA and oxidized glutathione in blood serum and blood lymphocytes. The MDA content in the blood serum of patients of both age groups was 1.35 times higher than in the control group ($P < 0.05$, Fig. 1). In peripheral blood lymphocytes, the MDA content in patients of young age group was 1.27, and in patients of the middle age group 1.39 times higher than in the control group ($P < 0.05$, $P < 0.01$). Simultaneously, no significant changes in the concentration of oxidized glutathione in blood serum and blood lymphocytes were found between men with erectile dysfunction due to combat trauma and healthy men (Fig. 2).



a *b*

Fig. 1. The MDA content in blood serum (*a*) and blood lymphocytes (*b*) of men with erectile dysfunction due to combat trauma: x ± SE, n = 48–68; * – $P < 0.05$; ** – $P < 0.01$ changes are statistically significant compared to healthy men

Fig. 2. The GSSG content in blood serum (*a*) and blood lymphocytes (*b*) of men with erectile dysfunction due to combat trauma: x ± SE, n = 48–68

The state of the non-enzymatic component of the antioxidant system was assessed by the content of reduced glutathione (Fig. 3) and GSH/GSSG ratio (Fig. 4). GSH content in blood serum in patients of both age groups was significantly lower than in the control group. Specifically, GSH content in blood serum in patients of young age group was 1.45 times ($P < 0.05$) and in patients of the middle age group 1.59 times lower than in healthy men ($P < 0.001$). In blood lymphocytes these changes were less expressed, however statistically significant. Changes in the GSH/GSSG ratio were significant only in blood serum. Specifically, the GSH/GSSG ratio in blood serum in patients of the young age group was 1.69 times ($P < 0.05$) and in patients of the middle age group in 1.95 times lower than in healthy men ($P < 0.05$).

Fig. 3. The GSH content in blood serum (*a*) and blood lymphocytes (*b*) of men with erectile dysfunction due to combat trauma: x ± SE, n = 48–68; * – P < 0.05; *** – P < 0.001 changes are statistically significant compared to healthy men

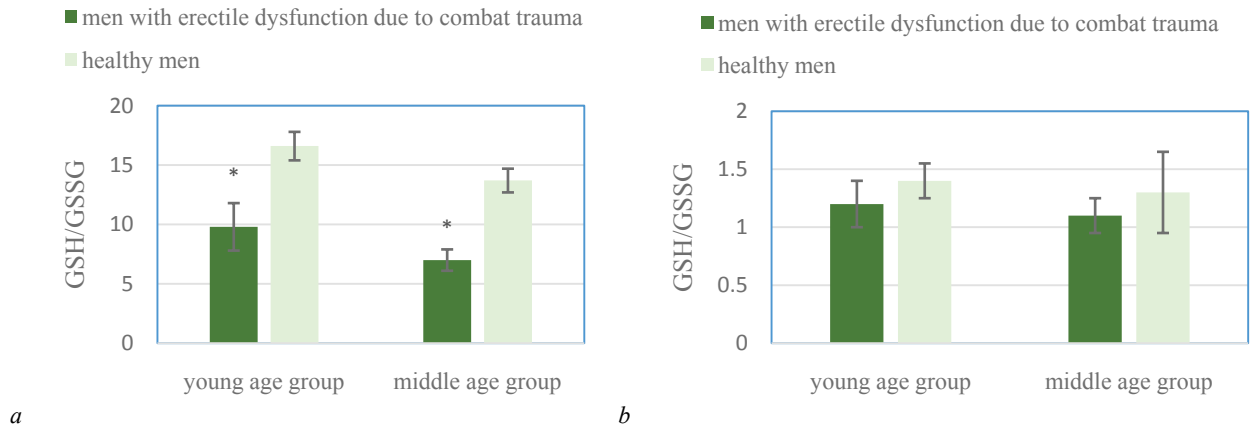
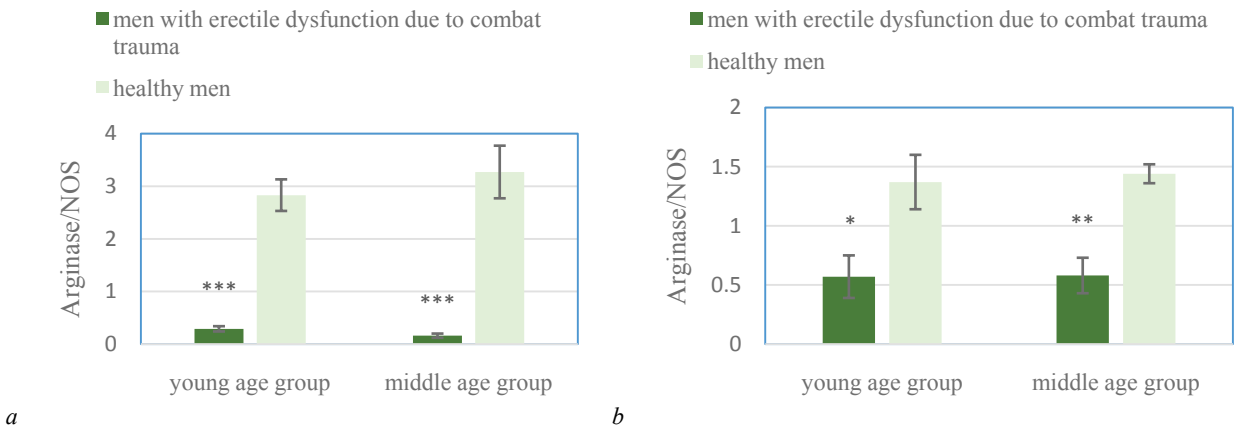


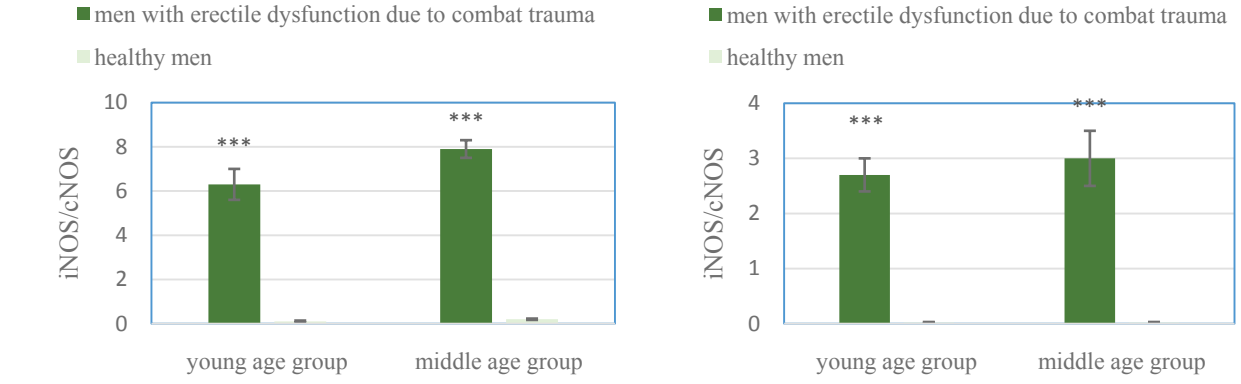
Fig. 4. The GSH/GSSG ratio in blood serum (*a*) and blood lymphocytes (*b*) of men with erectile dysfunction due to combat trauma: x ± SE, n = 48–68; * P < 0.05 changes are statistically significant compared to healthy men

Arginase activity in the blood serum and lymphocytes of men with erectile dysfunction due to combat trauma was significantly lower than in the clinically healthy men. The arginase/NOS ratio in blood serum was 9.75 times lower ($P < 0.001$) in the young age group and 20.45 times lower in the middle age group ($P < 0.001$) compared to healthy men (Fig. 5). In blood lymphocytes changes in arginase/NOS ratio were less expressed, however significant in both age groups ($P < 0.05$; $P < 0.01$). Finally, we studied the activity of inducible (Ca^{2+}-independent) and constitutive (Ca^{2+}-dependent) isoforms of NOS. We found that iNOS/cNOS ratio drastically increases in blood serum and blood lymphocytes in men with erectile dysfunction due to combat trauma of both age groups (Fig. 6).



a *b*

Fig. 5. The arginase/NOS ratio in blood serum (*a*) and blood lymphocytes (*b*) of men with erectile dysfunction due to combat trauma: x ± SE, n = 48–68; * – P < 0.05; ** – P < 0.01; *** – P< 0.001 changes are statistically significant compared to healthy men



a *b*

Fig. 6. The iNOS/cNOS ratio in blood serum (a) and blood lymphocytes (b) of men with erectile dysfunction due to combat trauma: x ± SE, n = 48–68; *** – P < 0.001 changes are statistically significant compared to healthy men

Discussion

An important factor in the pathogenesis of a number of diseases is the ratio of the antioxidant system to the pro-oxidant indicators, which is known as the antioxidant status of the cell. It is known that the pro- and antioxidant systems are in a state of dynamic equilibrium, and a violation of this equilibrium causes oxidative stress. Most pathological conditions and diseases are characterized by an increase in free radical processes and a decrease in antioxidant capacity (Ayaz et al., 2015; Adeoye et al., 2018; Agarwal et al., 2018; Chaudhary et al., 2023). The MDA content, which is the end product of lipid peroxidation, is considered as a marker of oxidative stress, which reflects the degree of lipid damage due to the effect of ROS (Fafula et al., 2023; Haro Girón et al., 2023). The obtained data are consistent with results which show increased MDA level in patients after an earthquake and may suggest increased oxidative stress in these patients (Atli et al., 2016).

It was found that decrease in MDA levels could increase NO-cGMP level, which in turn promotes the erection mechanism (Sheweita et al., 2015). Free radical processes occur most intensively in pathozoospermic samples of men with leukocytospermia. In our opinion, this established fact is explained by the fact that leukocytes can generate ROS in high concentrations (Henkel et al., 2011; Fafula et al., 2023). The increase in the concentration of ROS in leukocytospermia is due to the direct interaction of leukocytes with spermatozoa or the release of soluble products by leukocytes that act on spermatozoa. In addition, high levels of free radicals, produced as a result of the pathogenic influence of microorganisms, can reduce the fertilizing ability of spermatozoa both by damaging sperm membranes and their DNA (Viloria et al., 2010; Chaudhary et al., 2023).

Considering that GSH not only protects cells against free radicals, but generally determines the redox characteristics of the intra-cellular environment, a decrease in GSH content indicates inhibition of antioxidant capacity. This leads to an increase in the formation of toxic ROS, depletion of the cell pool of bioantioxidants and an increase in lipid peroxidation. The above mentioned may cause a violation of the structure and functions of cells. The antioxidant properties of GSH are due to its direct interaction with toxic ROS and the functioning of glutathione-dependent enzymes, such as glutathione peroxidase and glutathione-S-transferase, which uses GSH as a substrate. Therefore, depletion of its pool leads to inhibition in activity of glutathione-dependent enzymes – peroxidase and glutathione-S-transferase. Disturbances in the glutathione system in spermatozoa are one of the pathogenetic mechanisms associated with infertility (Fafula et al., 2017). A decrease in the activity of enzymes of the glutathione system indicates the depletion of cell compensatory mechanisms. Determining the parameters of the glutathione antioxidant system in various forms of pathospermia can be an important auxiliary criterion in the diagnosis of male infertility and the evaluation of the effectiveness of its treatment with specific antioxidant agents. Also, determining the content of GSH, the GSH/GSSG ratio, the activities of glutathione-dependent enzymes can be used as an additional biochemical indicator to assess the fertilization potential of spermatozoa.

The decrease in GSH content in the spermatozoa of infertile men, obtained in our experiments earlier, was due to decreased glutathione reductase activity, indicating the inactivation of GSH regeneration. It is known that glutathione reductase uses as a substrate NADPH, the content of which decreases under conditions of oxidative stress. It is known that the formation of GSH occurs not only as a result of its de novo biosynthesis, but also with the participation of glutathione reductase, an enzyme that provides the reduction of GSSG to GSH in the presence of NADPH as a source of reducing equivalents. The consequence of GSH reduction can be the accumulation of free radicals, which increases with the subsequent formation of the OH radical and damage to high molecular structures, in particular DNA (Viloria et al., 2010; Gavriliouk et al., 2015). The activity of inducible (Ca^{2+}-independent) de novo synthesis of NO significantly increases in the serum and blood lymphocytes of men injured as a result of

hostilities. Activation of iNOS has a more pronounced character both in blood serum and blood lymphocytes of men of the older age group, which may be due to the activation of inflammatory or other processes that stimulate the development of oxidative-nitrative stress or a decrease in the body's antioxidant reserves with age. Under physiological conditions, iNOS activity in serum and blood lymphocytes of both age groups was insignificant.

According to the data we obtained, the state of the NO synthase system in men of the control group was characterized by the pre-dominance of cNOS activity. This can be explained by the absence of factors that activate iNOS in healthy men with preserved fertility, primarily bacterial lipopolysaccharides, proinflammatory cytokines, etc. It is believed that generation of "basal" (physiological) NO occurs by cNOS, while iNOS provides excessive NO production in cells in various pathological conditions. However, separate studies have confirmed the involvement of iNOS also in the physiological NO synthesis (Bryan et al. 2009). We found inhibition of cNOS activity and an increase in the activity of its Ca^{2+}-independent inducible isoform in blood samples of men injured as a result of hostilities, which indicates dysmetabolic changes in the synthesis of NO, namely its hyperproduction. Similar changes in arginase-NO-synthase activity were found in peripheral blood lymphocytes of patients with ovarian cancer and spermatozoa in human subjects with different fertility potential (Iakubets et al., 2013; Fafula et al., 2018). Hyperproduction of NO with the participation of iNOS can induce the formation of ROS, the formation of highly toxic derivatives of NO, in particular peroxynitrite, and contribute to the development of oxidative and nitrative stress. It is known that peroxynitrite activates the transcription factor NF-κB, which, in turn, leads to an increase in the expression of iNOS and as a consequence to the subsequent formation of NO in high cytotoxic concentrations (Siomek, 2012).

Hyperproduction of NO leads to the excessive formation of free radicals, in particular ROS and active forms of nitrogen, through which the prooxidant effect of NO is realized (Weidinger & Kozlov, 2015). This leads to the activation of lipid peroxidation processes, protein modification, inhibition of biosynthesis, and a decrease in the reparative capacity of DNA. It has been established that high levels of NO lead to a sharp increase in Ca^{2+}, prevent oxygen absorption, reduce the formation of ATP, and lead to depletion of cellular energy reserves.

Conclusion

It was established that in the blood serum and blood lymphocytes of men with erectile dysfunction due to combat trauma processes of lipid peroxidation were intensified and the GSH level was reduced. The GSH/GSSG ratio was reduced only in blood serum. It was found that the oxidative stress is associated with development of nitrative stress. The arginase/NOS ratio was shifted towards increased NOS activity. Activation of iNOS was accompanied by significant inhibition of cNOS.

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No potential conflicts of interest relevant to this article were reported.

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