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ORIGINAL PAPER

The effect of thiocetam on the parameters of the nitric oxide system under the conditions of the experimental periodontitis and immobilization stress formation

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ABSTRACT

Introduction and aim. The aim of this work is to study the parameters of the nitric oxide (NO) system in the blood of guinea pigs under the conditions of the experimental periodontitis (EP) and immobilization stress (IS) formation and to evaluate the effectiveness of thiocetam use.

Material and methods. Experimental studies were performed on 50 guinea pigs (males, body weight 0.18–0.21 kg) which were divided into five groups (10 in each): the first group were intact animals as control; the second experimental group were animals with experimental periodontitis under conditions of immobilization stress (3rd day), the third group included guinea pigs with EP and IS on the 5th day of the combined model process, group IV – animals with EP and IS 15th day (without administration of thiocetam) and group V – animals on the 15th day of experiment with EP and IS after use of thiocetam.

Results. As a result of this research, changes in the activity of the NO system in the blood were observed, namely an increase in the level of stable metabolites and an increase in the activity of total NO-synthase, which is accompanied by a compensatory inhibition of the L-arginine activity, and these indicators were most pronounced in the late stages of EP and IS formation.

Conclusion. The use of thiocetam showed a corrective effect on the changed variables of NO metabolism in the peripheral blood of guinea pigs under the conditions of the EP and IS development.

Keywords. L-arginine, nitric oxide, periodontitis, stress, thiocetam

Introduction

Generalized periodontitis is a specific dystrophic inflammatory process (reflex neurovascular dystrophy of periodontal tissues) that occurs as a result of the various exogenous and endogenous factors interaction. It is characterized by progressive destruction of periodontal tissues with subsequent tooth loss. In particular, according to WHO data, periodontal disease is found in 80% of children and 95% of adults. It is a social problem that in the most able-bodied population aged 35–44, the preva-

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lence of periodontal lesions reaches 92–98%.[1,2] Progressive generalized periodontitis is one of the main causes of tooth loss among the adult population.[3]

When dystrophic inflammatory diseases of the periodontium occur, the interaction between conditionally pathogenic microorganisms of dental plaque (biofilm) and the patient's body (host) is important in its pathogenesis.[4] Its implementation is largely determined by the immune response of the host organism.[4,5] General somatic diseases, smoking, and psychological stress have a significant impact on this interaction.[6] The impact of stressful factors causes changes in the parameters of the functional activity of the body's physiological systems, including various adaptive changes in the immune system and periodontal tissues.[1,6,7] The degree of stressor tissue damage depends on the features of the neurohumoral mechanisms of tissue metabolism regulation.[6]

The relevance of the problem is due not only to a significant percentage of the periodontal diseases spread among the population worldwide, but also to the insufficiency of early diagnosis and the effective treatment of patients with this pathology.[7] For the early detection of periodontitis, a thorough examination is required, which includes collection of information, examination, probing the depth of the gingival pockets, as well as radiography. Considering the polyetiological nature and complex pathogenesis of generalized periodontitis, complex and multicomponent treatment regimens are used to treat patients.

In particular, Kuvaeva demonstrated the positive effect of the nitric oxide (NO) modulator in the form of arginine gel in the treatment of experimental periodontitis in rats.[8] Bedeniuk showed the corrective effect of a selective inhibitor and -NOS – aminoguanidine, which was used at a dose of 20 mg/kg in rats for 14 days together with the antioxidant lycopene at a dose of 100 mg/kg for generalized periodontitis.[9] Several scientists used L-arginine as a drug with a corrective effect on impaired levels of the nitric oxide system during adrenaline-induced myocardial damage and immobilization stress.[10]

Oleshchuk recommended the application of such modulators for the synthesis of NO:[11]

1. L-arginine, administered intraperitoneally at 25 mg/kg in the form of a 2.5% aqueous solution once a day for 7 days,

2. Glutargin is administered intraperitoneally at 45 mg/kg in an equimolar dose in terms of L-arginine once a day for 7 days,

3. N-nitro-L-arginine methyl ester was administered 10 mg/kg as a 1% solution intraperitoneally once a day for 7 days.

The full range of treatment measures is quite long, expensive and not always sufficiently effective,[4,5] therefore the issue of treatment regarding the expediency of using expert recommendations in the doctors' daily practice is extremely relevant and requires special research.

In recent years, researchers have paid considerable attention to studying the role of NO, which lies in providing it with optimal work of the whole organism. The important role of nitric oxide in the regulation of various body systems has been experimentally demonstrated, therefore it shows that most chronic diseases are directly related to the deterioration of the functional state of systems and organs responsible for the synthesis of NO in the body (blood vessels endothelium, nervous and connective tissue cells – neurons and macrophages).[12]

Bedeniuk established disorders in the microcirculation system in patients with chronic periodontitis with concomitant atrophic gastritis.[9] The direct cause of microcirculatory disorders is endothelial dysfunction, largely dependent on the bioavailability of nitric oxide produced by the endothelial form of NOS. In inflammatory lesions, hypersecretion of inducible NO usually occurs. It leads to the production of excess NO, which can play the role of an important effector in the development mechanisms of periodontal tissue inflammation. In addition, it has been shown that during periodontitis, so-called nitrooxidative stress develops, in which nitric oxide interacts with superoxide anion, resulting in the formation of peroxynitrite. The latter is associated with the altering effect of NO on biological macromolecules. Especially on proteins and lipids, which in turn causes an imbalance in the processes of inactivation of reactive oxygen forms, which leads to disruption of the structure and function of cell membranes and ends in cell death and the development of inflammation.[13]

Our previous studies also revealed the development of oxidative stress and an imbalance of cytokine status in experimental periodontitis (EP) and immobilization stress (IS). Savelieva showed that NO is a factor initiating and maintaining the development of inflammation in periodontal tissues in patients with chronic generalized periodontitis, and changes in the nitric oxide system play a significant role in the mechanisms of periodontitis formation.[14]

Thus, NO can be considered as one of the most important regulators of the body's general adaptive capabilities, which ensures its optimal adaptation to external influences of various character and, as a result, maintenance of the necessary level of health in general.[15] Almost every extreme state of the body and pathological process is directly or indirectly related to the multifunctional characteristics of NO. Therefore, further study of the nitric oxide effects in medicine and, through the process of research, obtaining new scientific facts about the peculiarities to the biosynthesis of this compound and how its molecular and biochemical effect will allow to develop a new strategy and tactics for correcting and treating pathologies of various genesis.[16]

It is known that the production of NO by macrophages under periodontal inflammation stimulates the phagocytosis reaction together with other radicals, that is, NO can also perform a useful function in periodontitis, acting as a non-specific factor of protection against bacteria.[17] At the same time, NO deficiency contributes to the reproduction of pathogens in the periodontal tissues, which leads to this pathological process developing into chronical. However, a sharp increase in the level of NO plays a negative role, as it initiates a number of free radical mechanisms that cause the destruction of tissues, including periodontal connective tissue.[18]

Proceeding from the above, it became necessary to carry out the pharmacological correction of the revealed disturbed metabolic processes concerning the nitric oxide system in EP and IS with the help of the drug thiocetam, which belongs to the group of cerebroactive agents, possessing nootropic, anti-ischemic, antioxidant, membrane-stabilizing properties, as well as improving the rheological properties of blood due to the activation of the fibrinolytic system, it stabilizes and reduces areas of necrosis and ischemia and eliminates the stress consequences.[19,20]

It is known that thiocetam has as its main active substance a morpholinium salt of tiazotic acid (1 mL of a solution containing morpholinium salt of tiazotic acid) in terms of 100% of the substance – 25 mg, which is equivalent to 16.6 mg of tiazotic acid, and piracetam – 100 mg. It should be emphasized that thiocetam contains two components: the first, piracetam, is an antioxidant, that stabilizes cell membranes, stimulates alternative metabolism pathways in hypoxia, and improves microcirculation. The second component is thiotriazolin, which has antioxidant, membrane-stabilizing, immunomodulating, cytokine-correcting, and anti-inflammatory properties, and is hepatoprotective and cardioprotective.

The positive antioxidant and immunocorrective effect of thiotriazolin was established by Shchepanskyi and Reheda in guinea pigs with bronchial asthma and experimental periodontitis and pneumonia.[21,22]

To date, the influence of thiocetam on disturbed indicators of the NO system in EP and IS is unknown. But taking into account the thiotriazolin component of this drug and its wide mechanism of action, which is covered in the article, it is possible to express an opinion about the mediated effect of thiocetam on the specified markers of the nitric oxide system in EP and IS.

The mechanisms underlying the stress impact on the periodontitis pathogenesis, the specifics of the combined pathology course, still require a more detailed study. In particular, the assessment of the nitrooxidative stress intensity, which to a certain extent determines the manifestation of resorptive processes in the connective tissue, becomes important both in studying the formation mechanisms and in correcting the periodontitis treatment.

Aim

The aim of the work is to study the parameters of the nitric oxide system in the blood of guinea pigs under the conditions of the EP and IS formation and to evaluate the effectiveness of the thiocetam use.

Material and methods

Experimental studies were performed on 50 guinea pigs (males), body weight 0.18–0.21 kg, which were divided into five groups (10 in each): the first were intact animals as control; the second (experimental) group were animals under the conditions of experimental periodontitis and immobilization stress development (3rd day), the third group included guinea pigs with EP and IS on the 5th day of the combined model process, group IV – animals with EP and IS 15th day (without using of thiocetam) and group V – animals on the 15th day of experiment with EP and IS after the use of thiocetam.

It is known that guinea pigs serve as a classic object for modeling inflammatory and allergic processes. Therefore, these animals were used to reproduce this experimental model of disease and stress.

Fixed days were chosen: the 3rd, 5^{th,} and 15th day for the experiment under conditions of the development of EP and IS separately and in their combination before and after treatment with thiocetam, which corresponded to the stages of the acute inflammatory response, which included the development of the disease (3rd), the height of the disease (5th), convalescence (15th day) and stages of stress: the 3rd day corresponded to the anxiety stage: the 5th day – the stage of resistance and the 15th day – the stage of exhaustion.

Experimental periodontitis was modeled by the method of Jogan, which consisted of animals that were on a diet that included 1 g of dry lyophilized cattle liver, 10 g of dry skimmed milk, and 20 g of crackers.[23] The diet is designed for one guinea pig per day. Morphologically, the presence of periodontitis in animals was not confirmed, since this is a long-known, tested experimental model.

Immobilization stress was reproduced by the method of Horizontov.[24] We selected fixed days (3$^{rd.}$, 5th and 15th) for studies that corresponded to the classic stages of acute inflammation. To correct disorders in group V, the drug thiocetam was administered at a rate of 250 mg/kg intramuscularly from the 6th day of the experiment for 10 days. The application of the specified dose of thiocetam was based on experimental scientific studies, which used thiocetam at 250 mg/kg of body weight once a day for the treatment of cranioskeletal trauma complicated by blood loss with functional and morphological disorders of the liver in rats with different resistance to hypoxia and established the effect and improvement of endogenous intoxication and cytolysis indicators, reduced dystrophic-necrotic processes in the liver.[19,20]

They also took into account the fact that thiocetam has the main active ingredient morpholinium salt of tiazotic acid, which is similar to that contained in thiotriazolin, and the latter is used in various doses of 100–250 mg/kg of body weight in various inflammatory and allergic processes.[21,22,25]

All experiments on laboratory animals carried out with following the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986), Council Directive 2010/63/EU, the Law of Ukraine 3447- IV "Protection animals from the cruelty," the general ethics of animal experimentation adopted by the first national Congress on bioethics in Ukraine. The study protocol was approved by the Ethical Committee of Danylo Halytsky Lviv National Medical University (protocol No 35; 05.10.2022).

Activity of NO synthase (NOS) was detected by VV Sumbaev method.[26] The total activity of nitric oxide synthase was determined by the intensity of NADPH·H^+ use in a reaction medium containing 0.6 mL of 5 mM KH_2PO_4, 0.6 mL of 1 mM $MgCl_2$, 0.6 mL of 10 mM $CaCl_2$ in Tris-HCl buffer pH=7.4, 0.6 mL of 4 mM aqueous solution of L-arginine, 0.4 mL of 1.0 mM NADPH·H^+ solution. The reaction was started by adding 0.3 mL of experimental biopsy material (tissue homogenate, erythrocyte hemolysate) to the reaction mixture. The control tube contained a similar set of reagents, except for a solution of L-arginine, instead of which 0.6 mL of distilled water was added. The reaction was stopped by adding 8 mM $HClO_4$ solution to the reaction mixture.

A decrease in the absorbance of solutions was recorded at a wavelength of 340 nm. Nitric oxide synthase activity was expressed as nmol of NADPH·H^+, which was oxidized within 1 minute per 1 mg of protein.

L-arginine content in the blood serum was determined by the Aleinikov method.[27] Up 0.5 mL of a 5% trichloroacetic acid solution was added to 0.5 ml of blood serum and centrifuged for 10 minutes at 3000 rpm. 0.5 mL of the supernatant was taken and 1 mL of a 5% NaOH solution, 0.05 ml of a 0.02% alcohol solution of α-naphthol, 0.05 ml of a hypobromide reagent, and 0.2 mL of a 10% urea solution were added and made up with distilled water up to 4 mL. After 20 minutes, they were evaluated by spectrophotometry using at l=500 nm. The experimental sample and control were evaluated by spectrophotometry against distilled water. The control contained the same reagents as the experiment; instead of serum, distilled water was added. Arginine concentration was determined using a pre-constructed calibration graph.

Stable NO metabolites were determined by the Schmidt protocol.[28] The content of total NO products in the studied biological samples was determined using the Griess reagent, spectrophotometrically measuring the staining products at wavelengths l=550 nm. From the measured optical density values, the average value was found and the concentration of stable nitric oxide products was determined using a pre-constructed calibration curve.

This method was carried out as follows: 0.2 mL of the test sample was placed in a centrifuge tube, and 0.2 mL of 4% sodium hydroxide solution was added and incubated, stirring, in an ice bath for 10 minutes. After this, 0.4 mL of distilled water and 1.2 mL of a 4% solution of zinc sulfate were added and kept in a water bath with ice. In 10 min. centrifuged for 20 min. at a temperature of 0±4 °C at a speed of 15000 rpm. To 1.4 mL of the selected supernatant was added 1.4 mL of Griess reagent (1:1), which included: 0.1% N-(naphthyl)ethylenediamine hydrochloride and 1% sulfanilic acid, prepared in 5% orthophosphoric acid. The sample with the added reagent was placed for 15 min. in a dark place for color development, then absorbance was measured using a spectrophotometer at l=550 nm.

The control is an 8% protein solution, processed according to the experimental method. Recalculation was carried out according to the calibration graph obtained with standard solutions with a concentration of total nitric oxide metabolites from 1 to 250 μmol/L.

All numerical results were subjected to statistical processing using the arithmetic mean (M), error of the arithmetic mean (m), and Student's test. Calculations were performed using statistical and graphical analysis tools for Microsoft Excel spreadsheets in the Microsoft Office software package.

Results

When studying the activity of stable metabolites, total NOS (endothelial and inducible) and L-arginine in the blood of guinea pigs with EP and IS, it was recorded that at all stages of their formation there were likely changes in variables compared to the control group. Having compared the variables of nitric oxide metabolism in guinea pigs with EP and IS between different groups of animals, we also found shifts in all the indicators we had determined in different periods of its formation.

In the dynamics of EP and IS formation, it was noticed that stable metabolites grow practically linearly already at the initial stage and reach their maximum at the latest term of the experiment. When comparing the groups, we observe that the studied indicator is almost at the same level on the 5th day compared to the 3rd day of the experiment ($p_1 \leq 0.05$). Subsequently, the level of stable metabolites increases by 7.2% ($p_1 \leq 0.05$) on the 15th day of EP and IS compared to the second group (Table 1).

When studying the activity level of total nitric oxide synthase (endothelial and inducible) in the blood of guinea pigs with EP and IS, an increase in its level was found (on 3rd, 5th and 15th days), both in comparison with the control group and between different groups of animals. There is a significant increase in nitric oxide synthase in all studied days compared to the group II of animals: by

10% ($p_1 \leq 0.05$) and 11.8% ($p_1 \leq 0.05$) on the 5th and 15th days respectively, under EP and IS (Table 1, Fig. 1). These changes lead to excessive formation of nitric oxide and its active derivatives in the blood, which, in turn, causes inhibition of enzyme function, DNA damage, activation of free radical processes, i.e., in high concentrations, initiates the processes of oxidative and nitrosative stress.

Table 1. The parameters of the nitric oxide system in the blood of guinea pigs under the conditions of the EP and IS formation. (M ± m, n=40)[a]

Form of experiment	Duration of experiments in days	Number of animals	Stable metabolites NO mmol/l	Total activity NOS, nmol NADPH(H+) / (min/ml)	L-arginine mg/ml
Intact animals, control		10	17.1±2.4*	0.62±0.11*	41.3±5.0*
Guinea pigs with EP and IS	3	10	27.7±3.2*	1.1±0.02*	18.4±4.4*
	5	10	28.5±3.3*	1.21±0.03*	17.3±3.2*
	15	10	29.7±3.4*	1.23±0.03*	15.2±3.4*

[a] * – changes are likely with respect to the values of the control group (p≤0.05)

For a more complete assessment of the nitric oxide system parameters, the level of L-arginine activity was studied and its regression in the dynamics of EP and IS formation was found (Fig. 1).

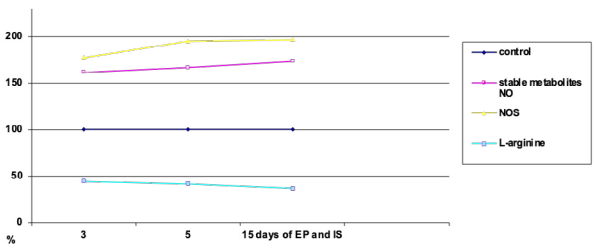

Fig. 1. The level of nitric oxide system indices in guinea pigs' blood in the EP and IS formation dynamics (% of control)

L-arginine is one of the most versatile metabolic amino acids which is a precursor to the synthesis of polyamines, proline, glutamate, creatine, agmatine, and urea and is an important component of the metabolic processes of maintaining optimal nitrogen balance in the body, as it is a precursor of NO and participates in transportation and detoxification excess NO in the body. The part of L-arginine that was not subject to metabolism is used as a substrate for NO production. Under physiological conditions, synthesis of NO from L-arginine occurs with the help of NO-synthase enzymes, and the main supplier of endogenous arginine is protein metabolism in the body. NO in the brain acts as a neurotransmitter, in the work of the immune system – as a mediator of the immune response.[16,29]

As a result of our work, it was noted that the level of the studied indicator decreased in comparison between groups of animals. Thus, on the 5th day of the development of the combined model process, L-arginine was reduced by only 6.0% ($p_1 \leq 0.05$) compared to the 3rd day of EP and IS. Later, on the 15th day, a similar pattern was noted: a gradual decline of L-arginine by 17.4% ($p_1 \leq 0.05$), respectively, against the second group of animals (Table 1, Fig.1).

Administration of thiocetam at the rate of 250 mg/kg intramuscularly from the 6th day of the experiment for 10 days led to a partial correction of the variables of the nitric oxide system, namely a decrease in the content of stable metabolites by 35.3% ($p_1 \leq 0.05$) and total activity NOS by 42.6% ($p_1 \leq 0.05$) and a rather significant increase in the content of L-arginine in the blood by 81.5% ($p_1 \leq 0.05$) compared to the group of guinea pigs with EP and IS before therapy (Fig. 2), which indicates a positive corrective effect of this medicinal product on the tested tests.

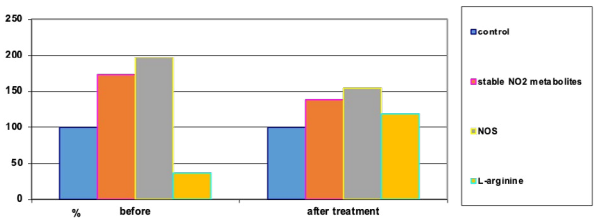

Fig. 2. The effect of thiocetam on the level of the parameters of the nitric oxide system in guinea pig blood under EP and IS formation dynamics

As a result of the research, changes in the activity of the NO system in the blood were observed, namely an increase in the level of stable metabolites and an increase in the activity of total NO-synthase, which is accompanied by a compensatory inhibition of the L-arginine activity, and these indicators were most pronounced in the late stages of the EP and IS formation.

Discussion

NO is a unique molecule for biological organisms, and has also very important effects on various pathologic and physiologic mechanisms. Many cell types can generate NO during different pathological and physiologic conditions. Recently, many authors have been reported the connections between NO and inflammatory diseases. Periodontal pathologies are characterized with specific periodontal inflammation and host tissues damage around the teeth, and additional factors such as force application on teeth. However, the production of NO and the role of NO in the development of periodontal pathologies are unclear. On the other hand, many evidences from previous studies indicates a close relationship between NO and periodontal pathologies.[30]

Inflammatory damage also usually involves overexpression of the NOS inducible form, which results in the production of excessive amounts of NO. This may play an important effector role in the inflammation mechanisms generated by bacterial endotoxin. Thus, the so-called nitrooxidative stress develops along with the inflammation.[9]

We have established that EP associated with IS causes such pathophysiological features of disorders of the nitric oxide system, which consist in a gradual progressive increase in the indicators of stable NO metabolites and total NOS activity against the background of a decrease in the L-arginine content, most pronounced on the 14th and 24th century, period of the experiment, corresponding to the stages of the inflammatory process (height and recovery) and the stage of stress (resistance and exhaustion) relative to the control group, which may indicate activation of the inflammatory process in the periodontium. The decrease in L-arginine content is explained by its excessive consumption for the synthesis of nitric oxide during EP and IS. In our opinion, changes in NO levels during periodontitis and stress are associated with impaired microcirculation in periodontal tissues, the influence of hypoxia and the inflammatory process, and the action of pro-inflammatory cytokines on them, which have been proven in several scientific studies.[9,21]

Thiocetam acts as an antioxidant and anti-ischemic agent, helps to improve microcirculation, normalizes bioenergetic processes, increases the body's resistance to hypoxia, inhibits the formation of reactive oxygen species, improves the rheological properties of blood by activating the fibrinolytic system, stabilizes and reduces areas of necrosis and improves cytolysis rates, endogenous intoxication; reduces degenerative processes in the liver during injury against the background of blood loss.[19,20]

This drug, by correcting the above pathological processes, can indirectly have a positive effect on impaired indicators of the NO system, since its use involves not only studying the effect on markers of the NO system but also elucidating its effect on immune indicators, lipid peroxidation processes, cytokine status, proteolysis processes in EP and IS.

Thiocetam was started to be administered from the 6th day of EP and IS, since during this period the disturbances of metabolic and immune processes were most pronounced, as established in our previous studies (development of oxidative stress, increase in the level of anti-inflammatory cytokines against the background of a decrease in anti-inflammatory cytokines, inhibition of cellular under conditions of stimulation humoral immunity, proteolysis processes against the background of inhibition of antiprotease potential), which dominated on the 5th, and especially on the 15th day of the experiment.

Conclusion

Under the conditions of the experimental periodontitis and immobilization stress development, a multidirectional vector of changes in the metabolism variables of the NO system is observed – an increase in the content of stable nitric oxide metabolites and the total activity of nitric oxide synthases against the background of the decrease in the L-arginine concentration, which were most pronounced on the 15h day of the experiment. This indicates the production of an excessive amount of NO, which can play the role of an important effector in the mechanisms of inflammation generated by bacterial endotoxin. Increased synthesis of NO can exhibit a cytotoxic effect associated with the formation of peroxynitrite, leading to necrosis or apoptosis, i.e. during inflammation, the so-called nitrooxidative stress develops, which will lead to the progression of the disease.

The use of thiocetam showed its corrective effect on the changed variables of NO metabolism in the peripheral blood of guinea pigs under the conditions of the EP and IS development.

Declarations

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Author contributions

Conceptualization, M.R. and P.O.; Methodology, M.R.-F.; Software, S. R.; Validation, S.R., P.O. and M.K.; Formal Analysis, L.F.; Investigation, P.O.; Resources, V.F.; Data Curation, M.R.-F.; Writing – Original Draft Preparation, S.R.; Writing – Review & Editing, P.O.; Visualization, M. K.; Supervision, M. R.; Project Administration, M.R.; Funding Acquisition, P.O., L.F., M.R.-F., M.K.

Conflicts of interest

The authors declare no competing interests.

Data availability

The datasets used and/or analyzed during the current study are open from the corresponding author on reasonable request.

Ethics approval

The study protocol was approved by the Ethical Committee of Danylo Halytsky Lviv National Medical University (protocol No 35; 05.10.2022).

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