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Pharmacocorrection of Disturbances in the NO System in Experimental Chronic Generalized Periodontitis

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Abstract

BACKGROUND: In the light of modern views on the pathogenesis of inflammatory diseases of the oral cavity, a promising direction is the use of agents with metabolitotropic, endothelioprotective, and especially with antioxidant action.

AIM: The purpose of this study was to evaluate the effect of a combination of thiotriazoline and L-arginine (1:4) on the parameters of the nitroxidergic system of the blood and periodontium of rats with experimental chronic generalized periodontitis and substantiate further study of this combination.

METHODS: Real-time reverse-transcription polymerase chain reaction was used to assess the mRNA expression status of iNOS and nNOS mRNAs. The total content of reduced thiols was also determined by the reaction with Elman's reagent.

RESULTS: We found an increase in the total activity of NOS by 90.01% due to an increase in the expression of iNOS, while a decrease in the expression of its endothelial form was observed (a decrease in the expression of eNOS mRNA by 74.3%) compared with the intact group. An increase in iNOS activity led to an increase in the production of NO, which, under conditions of antioxidant deficiency, is converted into cytotoxic forms (peroxynitrite and nitrosonium ion).

CONCLUSIONS: The course administration of Mexidol (250 mg/kg) and, especially, the combination of thiotriazoline and L-arginine (1:4) (200 mg/kg) to animals with CGP, leads to a decrease in the gingival pocket to 6 mm (Mexidol) and to 4 mm against the background almost complete absence of bleeding, swelling, and tooth mobility (combination), and also led to a decrease in iNOS mRNA expression by 65.6% ($p < 0.05$).

Introduction

Despite the high achievements in the development of dentistry, periodontal disease is a prerequisite for tooth loss 5–10 times more than tooth loss from caries and its complications. According to the WHO in 2016, periodontal diseases, which lead to tooth loss, became the 11th most important among the spread of diseases on the planet [1]. In the light of modern views on the pathogenesis of inflammatory diseases of the oral cavity, a promising direction is the use of agents with metabolitotropic, endothelioprotective, and especially with an antioxidant action (Mexidol, selenites, α-tocopherol, propolis preparations, and SOD-rec) [2], [3], [4]. Recently, it has been established that an important link in the pathogenesis of inflammatory processes in the oral mucosa is the expression of proinflammatory cytokines – IL-1b, TNF-a, an increase in iNOS activity and activation of nitrosative stress, accompanied by an increase in cytotoxic forms of NO. Cytotoxic forms of NO such as peroxynitrite and nitrosonium ion lead to O-; N-; S-nitrosylation of protein molecules, reducing the processes of reparative tissue regeneration, suppressing immunity, and disrupting the molecular and biochemical mechanisms of cell signaling [5], [6].

In this regard, the drug thiotriazoline (tiazotic acid) is of interest. Thiotriazoline exhibits antioxidant activity – converts oxygen free radicals and ROS into an inactive state, indirect-reactivates antioxidant enzymes – superoxide dismutase and glutathione peroxidase, and protects endogenous antioxidants – α-tocopherol and glutathione from "overexpenditure," anti-inflammatory effect – reduces the content of circulating immune and immunomodulatory complexes, limits the release of inflammatory mediators by them, reduces the expression of the pro-inflammatory cytokine IL-1b, stabilizes the membranes of basophils, mast cells and eosinophils, increases the phagocytic

activity of macrophages, and increases the level of interferon [7]. There are experimental and clinical results of the positive use of thiotriazoline in inflammatory diseases of the oral cavity [8], [9], [10]. A promising agent for the complex therapy of periodontitis is L-arginine, a precursor of NO synthesis [11]. However, under conditions of deprivation of the thiol-disulfide system, which often occurs in inflammatory diseases of the oral cavity, the effectiveness of L-arginine becomes low due to a decrease in the bioavailability of NO and its conversion to peroxynitrite. There is evidence of antioxidant modulation by thiotriazoline in the action of basic therapy. This fact opens up prospects for the use of thiotriazoline in conjunction with L-arginine. There are convincing experimental data on high cardioprotective, endothelioprotective, and anti-ischemic activity of the combination of thiotriazoline and L-arginine in a ratio of 1:4 [12]. However, so far there are no studies on the optimization of medical treatment of chronic generalized periodontitis by administering a combination of drugs (thiotriazoline and arginine), which can lead to interruption of NO-dependent molecular biochemical mechanisms of periodontal damage. All this determines the prospects of our study.

Aim

The aim of this study was to evaluate the effect of a combination of thiotriazoline and L-arginine (1:4) on the parameters of the nitroxidergic system of blood and periodontium in rats with experimental chronic generalized periodontitis and substantiate potential further study of this combination.

Materials and Methods

The experiments were carried out on albino Wistar rats weighing 190–220 g obtained from the vivarium of the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine. The duration of the quarantine (acclimatization period) for all animals was 14 days. During quarantine, each animal was examined daily (behavior and general condition), animals were observed twice a day in cages (morbidity and mortality). Before the start of the study, animals that met the inclusion criteria for the experiment were divided into groups using the randomization method.

Ethical approval

All manipulations were carried out in accordance with the regulation on the use of animals in biomedical experiments (Strasbourg, 1986, as amended in 1998.) and the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes." The protocols of experimental studies and their results were approved by the decision of the Commission on Bioethics of ZSMU.

The experiment was reproduced for 8 weeks using a calcium peroxide-deficient diet with reduced masticatory function. In the drinking bowls for drinking water, there was a 2% solution of EDTA, and every day, the pro-oxidant Delagil (chloroquine phosphate) was administered to the animals at a dose of 30 mg/kg in the form of a 0.59% aqueous solution. Throughout the experiment, the animals received soft food [13]. After the formation of CGP, the animals received intragastrically studied preparations using a metal probe. All rats were divided into four groups (ten animals each):

1. Intact group, animals for which it is planned to administer intragastrically a solution of sodium chloride 0.9% for 30 days;
2. Control, animals with experimental CGP for which intragastrically administered sodium chloride solution 0.9% for 30 days;
3. Animals with experimental CGP, which are planned to be administered daily intragastrically thiotriazoline + L-arginine (1:4) 200 mg/kg for 30 days
4. Animals with experimental CGP, for which daily intragastric reference drug Mexidol, 250 mg/kg for 30 days.

We used the substance thiotriazoline (tiazotic acid) (Scientific and technological complex "Institute of monocrystals" of the National Academy of Sciences of Ukraine), substance L-arginine hydrochloride (Sigma-Aldridch, USA), and Mexidol (ethylmethylhydroxypyridine succinate) (Mexicor, PrJSC "Tekhnolog," Ukraine). The combination of thiotriazoline and L-arginine (1:4) was made at the Department of Pharmaceutical Chemistry of ZSMU. At the end of the experiment, rats under thiopental-sodium anesthesia (40 mg/kg) were taken out of the experiment. Blood was taken from the abdominal artery, and a section containing tissues of the supporting apparatus of the central incisors was cut out from the lower jaw, which was placed for a day in Bouin's fixator and, after standard histological wiring, the tissue was embedded in paraffin. Sections 5 microns thick were made on a rotary microtome. Real-time reverse-transcription polymerase chain reaction (RT-PCR) was used to evaluate the mRNA expression state of the iNOS and nNOS mRNAs. Blood was centrifuged (Eppendorf, Germany) [14] on a fluorescence-spectrophotometer agilent (USA), as well as nitrotyrosine by the solid-phase immunosorbent sandwich ELISA method (Catalog # HK 501-02 from Hycult Biotech) and stable NO metabolites in terms of nitrates using test systems a "Nitric oxide total ELISA" ("R&D Systems") (Catalog # SKGE001) on a full plate enzyme immunoassay analyzer (SIRIO S, Italy). The total content of reduced thiols was also determined by reaction with Elman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) (CAS № 69-78-3, Merck KGaA) [14] on an Eppendorf BioSpectometer (USA).

Polymerase chain reaction in real time

To determine the expression level of iNOS mRNA and eNOS mRNA, a CFX96 RT-PCR Detection Systems amplifier (Bio-Rad Laboratories, Inc., USA) was used. In our studies, for PCR under time conditions, there were a set of reagents manufactured by Sintol, Russia (No. R-415) that were used. The amplification composition included SYBR Green dye, SynTaq DNA polymerase with antibodies inhibiting enzyme activity, 0.2 μl each of forward and reverse-specific primers, dNTP-deoxynucleoside triphosphates, and 1 μl of matrix (cDNA). The reaction mixture was adjusted to a total volume of 25 μl by adding deionized water. Specific primer pairs (5'-3') for analysis of the studied and reference genes were selected using the PrimerBlast software (www.ncbi.nlm.nih.gov/tools/primer-blast) and manufactured by ThermoScientific, USA. Amplification took place under the following conditions: initiated denaturation 95°C – 10 min; further, 50 cycles: denaturation – 95°C, 15 s., primer annealing – 58–63°C, 30 s., and elongation – 72°C, 30 s. Registration of fluorescence intensity occurred automatically at the end of the elongation stage of each cycle through the automatic SybrGreen channel. The actin and beta (Actb) gene was used as a reference gene to determine the relative value of changes in the expression level of the studied genes.

Statistical analysis

The results of the study were calculated using the standard statistical package of the STATISTICA® for Windows 6.0 licensed program (StatSoftInc., no. AXXR712D833214FAN5), as well as SPSS 16.0, Microsoft Office Excel 2003. Distribution normality was assessed using the Shapiro–Wilk test. Data are presented as an average value. The significance of differences between the mean values was determined by Student's *t*-test with a normal distribution. In the case of a non-normal distribution or analysis of ordinal variables, the Mann–Whitney U-test was used. To compare independent variables in more than two samples, analysis of variance (ANOVA) with a normal distribution or the Kruskal–Wallis test for a non-normal distribution was used. For all types of analysis, differences $p<0.05$ (95%) were considered statistically significant.

Results and Discussion

Our studies revealed that the modeling of CGP and subsequent experimental treatment with a combination of thiotriazoline and L-arginine, as well as Mexidol, led to an improvement in the clinical picture of the disease in varying degrees of severity. Thus, typical changes were observed in the control group. In the group with CGP, which received Mexidol, gingival edema persisted, but it was smaller in size than in the control group, bleeding during probing of the periodontal pocket with a button probe persisted, the depth of the gingival pocket was 6 mm, and tooth mobility was preserved. In rats with CGP treated with a combination of thiotriazoline and L-arginine, there was a significant reduction in the size of the gingival pocket up to 4 mm and an almost complete absence of bleeding and swelling. Results, presented in Tables 1 and 2, indicate a significant violation in the nitroxidergic system of the blood and gums of rats with CGP. Thus, we found an increase in the overall activity of NOS by 90.01% due to an increase in the expression of iNOS (iNOS mRNA by 3.72 times), while a decrease in the expression of its endothelial form was observed (a decrease in eNOS mRNA expression by 74.3%) compared with intact group. An increase in iNOS activity led to an increase in NO production (as evidenced by an increase in stable NO metabolites in the blood by 116%), which, under conditions of antioxidant deficiency, incl. thiol scavengers (decrease in SH-groups by 52.2%) is converted into cytotoxic forms (peroxynitrite and nitrosonium ion). All of the above changes are the cause of nitrosative stress, as evidenced by an increase in the blood of animals with CGP of nitrotyrosine 4, 3 times compared with intact animals. Periodontal disease is known to be an inflammatory disease characterized by inflammation and loss of periodontal tissue. Periodontopathogenic bacteria and their products play an important role in its etiology. The course of the disease is determined by the interaction between periodontopathogenic bacteria and the host's immune response. Recent evidence has shown an association between the activation of phagocytes (polymorphonuclear neutrophils and macrophages) and pathways leading to periodontal injury [15]. Periodontal inflammation is initiated by chemotactic signals released by plaque bacteria, which leads to the recruitment of polymorphonuclear neutrophils into the sulcus epithelium and gingival fissure. As inflammation progresses, a huge number of phagocytes (represented mainly by PMN, but also including macrophages) will be attracted to periodontal tissues [15]. Neutrophils are believed to contribute to edema due to their interaction with the vascular endothelium during diapedesis and the production of various mediators such as arachidonic acid derivatives and chemokines [16]. In addition, neutrophils secrete ROS and serine proteases that can damage the vascular endothelium [17]. Reactive oxygen species play a role in these interactions in favor of tissue destruction [18]. In the case of periodontal disease, increased iNOS activity causes a high rate of ROS release. This causes increased oxidative stress in periodontal tissues. ROS produced on the surface of osteoclasts may play an important role in alveolar bone resorption [19], [20].

In the case of periodontal disease, increased iNOS activity causes a high rate of ROS release. This

Table 1: Parameters of the NO system and reduced thiols in the serum of animals with CGP (M ± m)

Researched indicators	Intact (n = 10)	CHP (Control) (n = 10)	CHP+thiotriazoline+L-arginine, 200 mg/kg (n = 10)	CHP+Mexidol, 250 mg/kg (n = 10)
NOS activity, nM/mg protein/hv	31.4 ± 1.68	59.7 ± 2.15	33.4 ± 2.1*1	46.5 ± 4.7
Reduced SH-groups, nm/l	351.8 ± 15.9	168.2 ± 12.6	294.0 ± 18.6*1	185.7 ± 21.5*
NO metabolites, nm/l	18.1 ± 1.8	39.1 ± 1.7	23.5 ± 1.55*1	32.4 ± 3.8
Nitrotyrosine, ng/ml	50.5 ± 3.7	217.7 ± 15.2	77.5 ± 5.7*	167.5 ± 9.7*

* – p < 0.05 in relation to the control group, 1 – p < 0.05 in relation to the mexidol group, n – the number of animals in the group.

causes increased oxidative stress in periodontal tissues. ROS produced on the surface of osteoclasts may play an important role in alveolar bone resorption [21].

The results of several studies have shown that IL-1b, TNF-a, and INF stimulate the induction of iNOS and the synthesis of NO and its cytotoxic forms involved in periodontal ligament cell apoptosis with subsequent localized microvasculopathy, ischemia, and irreversible damage to endothelial and periodontal tissues. In addition, excess NO plays a role in the onset and development of periodontal disease by re-enhancing the activity of proinflammatory cytokines [21], [22], [23], [24].

Administration of thiotriazoline and L-arginine to animals with CGP in combination leads to normalization of eNOS mRNA and iNOS mRNA expression in periodontal tissues. Thus, an increase in the expression of eNOS mRNA under the action of the composition of thiotriazoline and L-arginine by 5.6 times ($p < 0.05$) was noted and to a decrease in iNOS mRNA expression by 65.6% ($p < 0.05$). The consequence of this action was a decrease in total NOS activity by 44% and a decrease in NO production (a decrease in serum NO metabolites by 39.9% ($p < 0.05$), and as a result, nitrosative stress. Administration of a combination of thiotriazoline and L-arginine to animals with CGP causes a decrease in the serum marker of nitrosative stress - nitrotyrosine by 64.4% ($p < 0.05$) compared with the group control. The revealed decrease in iNOS mRNA expression under the action of combinations of thiotriazoline and L-arginine can be explained by the ability of thiotriazoline to reduce the level of ROS and cytotoxic forms of NO involved in the regulation of the expression of this enzyme [7], [25]. Furthermore, a decrease in iNOS RNA expression can be explained by the property of a fragment of the thiotriazoline molecule to protect sensitive cysteine residues – Cys 252, Cys 154, and Cys 61 in its DNA-binding domains from excess ROS and also participate in the restoration of these groups during reversible inactivation taking on the role of Redox Factor-1 [26], [27]. By reducing excess ROS levels, thiotriazoline, indirectly through the regulation of the transcription factor NF-kB, is able to regulate the expression of redox-sensitive genes, including those responsible for the synthesis of iNOS [28], [29]. The combination of thiotriazoline and L-arginine also affects NO transport due to the retention of SH-containing compounds. Thus, administration of a composition of thiotriazoline and L-arginine to animals with CGP significantly increases the level of SH-containing compounds by 75% in serum. Such action is provided by the following mechanism – in conditions of deficiency of thiol compounds, NO transport is disrupted, since it is attacked by such ROS with transformation into a cytotoxic product, peroxynitrite. Thiotriazoline increases the level of reduced thiols, in particular glutathione, through the activation of glutathione reductase and direct reduction of the oxidized thiol group. In addition, thiotriazoline, due to its antioxidant properties, prevents the oxidative modification of NO by oxygen radicals. Thiotriazoline is able to act as a transport molecule for NO [7]. L-arginine has a direct stimulating effect on eNOS activity and NO production. Therefore, the combined preparation of thiotriazoline and arginine has unique properties to have a protective effect on the synthesis and transport of NO, its bioavailability, which leads to the potentiation of such properties of L-arginine.

We found that the introduction of Mexidol under conditions of CGP does not affect the expression of eNOS mRNA and leads to a decrease in iNOS mRNA expression in the periodontium of experimental animals. The introduction of Mexidol leads to a significant decrease in nitrotyrosine and an increase in the number of thiol groups in the blood serum. However, according to the degree of influence on these indicators, Mexidol was inferior to the effectiveness of the combination of thiotriazoline and L-arginine; the obtained data correspond to the ideas about the action of Mexidol in chronic inflammatory processes, incl. and in periodontitis. Mexidol is an antioxidant of direct action, reduces oxidative damage to macromolecules, being a ROS scavenger, can interrupt ROS-dependent mechanisms of IL-1b expression, and thereby interrupt the mechanisms of triggering iNOS expression [30], [31].

Table 2: Parameters of eNOS mRNA and iNOS mRNA expression in gingival tissues of animals with experimental CGP (M ± m)

Group animals	eNOS mRNA expression, c.u.	iNOS mRNA expression, c.u.
Intact (n = 10)	1.00 ± 0.0112	1.00 ± 0.0108
CHP (control) (n = 10)	0.257 ± 0.001	3.72 ± 0.012
CHP+thiotriazoline+L-arginine, 200 mg/kg (n = 10)	1.456 ± 0.0012*1	1.28 ± 0.001*1
CHP+Mexidol, 250 mg/kg (n = 10)	0.361 ± 0.012	2.88 ± 0.005*

* – p < 0.05 in relation to control, 1 – p < 0.05 in relation to mildronate, n – the number of animals in the group

Conclusions

1. Calcium-deficient – pro-oxidative model of CGP leads to a typical clinical picture of periodontal disease – hyperemia, swelling of a dense consistency, the formation of a gingival pocket 8 mm deep, bleeding during probing, and tooth mobility against the background of disorders of the Nitroxidergic system

– an increase in NOS activity due to iNOS expression, an increase in NO production, which, against the background of a deficiency of thiol compounds, leads to the development of nitrosative stress.

2. The course administration of Mexidol (250 mg/kg) and, especially, the combination of thiotriazoline and L-arginine (1:4) (200 mg/kg) in animals with CGP, leads to a decrease in the gingival pocket to 6 mm (Mexidol) and to 4 mm against the background almost complete absence of bleeding, swelling, and tooth mobility (combination).
3. The course administration of a combination of thiotriazoline and L-arginine (1:4) (200 mg/kg) to animals with CGP led to a decrease in iNOS mRNA expression by 65.6% ($p < 0.05$) and an increase in eNOS mRNA expression ($p < 0.05$) 5.6 times in periodontal tissues.
4. The course administration of a combination of thiotriazoline and L-arginine (1:4) (200 mg/kg) to animals with CGP led to a decrease in total NOS activity ($p < 0.05$) and NO production ($p < 0.05$), as well as an increase in the level thiol compounds ($p < 0.05$) in blood serum
5. Mexidol did not have a significant effect on most of the studied parameters, and in terms of the degree of influence on iNOS mRNA and the level of nitrotyrosine, it was inferior to the effect of a combination of thiotriazoline and L-arginine ($p < 0.05$)
6. The results obtained are an experimental justification for further study of the drug combination of thiotriazoline and L-arginine.

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