The Influence of Chronic Hyperhomocysteinemia on Connective Tissue Disorders in Case of Lipopolysaccharide-Induced Periodontitis

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Abstract

The progression of destructive phenomena in periodontal tissues depends on many factors, including many systemic pathologies that act as comorbidities and contribute to destruction of the periodontium that leads toward tooth loss. One of such comorbidities may be chronic hyperhomocysteinemia (HHcy).

This study aimed to determine the features of connective tissue metabolism in rats with lipopolysaccharide (LPS)-induced periodontitis combined with chronic thiolactone HHcy. 48 white non-linear mature rats were divided into 4 groups: control; animals with a model of LPS-induced periodontitis; rats with chronic thiolactone HHcy; animals with a model of periodontitis combined with HHcy. The rats were sacrificed the day after the last LPS injection or the day after the last homocysteine thiolactone administration. Connective tissue metabolism was determined by the collagenolytic activity, the content of glycosaminoglycans (GAGs), fucose, unbound with proteins, and free hydroxyproline in blood serum.

It was found that chronic thiolactone HHcy enhances the destruction of connective tissue in case of periodontitis, which is confirmed by the significant excess of collagenolytic activity (by 2.5 times; p=0.009) and the content of GAGs (by 61.7%; p=0.007) in the blood serum of animals with combined pathology in relation to animals with LPS-induced inflammation without concomitant pathology.

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Introduction

Considered as the most prevalent inflammatory disease in the world, periodontal disease affects nearly 50% of adult population and 60% of aged population globally.[1] Periodontitis as a progressive disease induced by dental plaque bacteria results in gradual destruction of the periodontal tissues.[2] The development of destructive changes in periodontal tissues and alveolar bone in case of periodontitis is due to the level of metabolic disorders in their organic and mineral components. There is evidence that the dynamics of pathological changes in case of periodontitis is determined by the level of impaired connective tissue (CT) metabolism, the state of osteoblast synthetic activity, as well as the degree of violation of calcium-phosphorus metabolism.[3,4]

The progression of destructive phenomena in periodontal tissues depends on many factors, including many systemic pathologies that act as comorbidities and contribute to destruction of the periodontium that leads toward tooth loss.[2,5] Comorbidities are characterized by a mutually burdened course of diseases, the development of complications, worse prognosis, and an increase in material costs for treatment[3]. One of such comorbidities may be chronic hyperhomocysteinemia (HHcy).[6]

The role of increased level of homocysteine (Hcys) in the progression, and maintenance of the periodontal diseases remains not fully understood. Probable mechanisms of the negative impact of chronic HHcy on the

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course of periodontitis may include the activation of oxidative stress, which primarily causes damage to the endothelium as well as the development of its dysfunction and the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells – NF-kβ, which is known to stimulate the synthesis of cytokines, chemokines, leukocyte adhesion molecules, which in turn enhances the leukocyte migration into the vessel wall, thus increasing leukocyte cytotoxicity.[7,8,9,10]

This study aimed to determine the features of CT metabolism in rats with lipopolysaccharide-induced periodontitis combined with chronic thiolactone HHcy.

Materials and methods

Animals and study design. The study included mature inbred white male rats (n=48) with a body weight of 180-200 g. During the period of the experiment, animals were kept in controlled-temperature (22±2ºC) room with an adjustable light cycle (12/12) and unrestricted access to water and food at the animal facility of I. Horbachevsky Ternopil National Medical University, Ternopil. Animal treatment and all experimental procedures were performed in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (European convention for the protection of vertebrate animals used for experimental and other scientific purposes 1986). The Bioethics Commission of I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine approved the protocol of the experiment (Excerpts from Minutes No. 64, dated 17.05.2019).

The animals were randomly divided into the following groups: group 1: control (n=12); group 2: animals with a model of periodontitis (n=12). For two weeks, the rats in this group were injected 40 μL (1 mg/mL) of E. coli lipopolysaccharide (LPS) (manufactured by Sigma-Aldrich, USA) into gingival tissues every other day[3]; group 3 – rats with chronic thiolactone HHcy (n=12). Homocysteine thiolactone was administered intragastrically (100 mg/kg of body weight in 1 % solution of starch) once a day for 42 days[11]; group 4 – animals with a model of periodontitis combined with HHcy (n=12). In animals of this group chronic thiolactone HHcy was caused as described above. From the 29th day after the start of HHcy induction, animals were injected into the gum tissue with LPS for 14 days in parallel with thiolactone homocysteine in accordance to the above scheme.

The rats were euthanized under deep thiopental-sodium anesthesia by cardiac puncture the day after the last LPS injection (group 2 and 4) or the day after the last homocysteine thiolactone administration (group 3). Blood samples were used for further investigations.

To confirm the development of chronic HHcy, the total Hcys level in the blood serum of the rats was determined by a solid-phase enzyme-linked immunosorbent assay using Axis-Shield (Great Britain) reagent kit according to the manufacturer's protocol on a Multiscan FC analyzer (Thermo Scietific, Finland). The Hcys level was expressed in μmol/L.

Collagenolytic activity, the content of glycosaminoglycans (GAGs) and fucose, unbound with proteins, were determined using the methods of P.N. Sharaev and co-authors[12,13,14]; the content of free hydroxyproline was determined using the method of S.S. Tetyanets.[15]

Statistical analysis. The experimental data were processed and analyzed using MS Office 2016 EXCEL (Microsoft Corp., USA) and Statistica 7.0 software (StatSoft Inc., USA). The distribution of data was analyzed in accordance with the assessment of normality by the Kolmogorov-Smirnov test. The obtained values had not a normal distribution, so comparison of three or more groups on a quantitative basis was carried out using Kruskal-Wallis test followed by the Mann-Whitney test for pairwise comparison of groups, taking into account the Bonferroni correction. All data were presented as a median and quartiles (lower and upper) – Me (Lq; Uq). A probability level (p-value) of less than 0.05 was considered to be statistically significant. The relationship between the studied indices was established based on the results of the correlation analysis using the Spearman's correlation coefficient. The linear correlation coefficient (r) and its probability (p) were calculated. The relationship was considered very weak at a correlation coefficient r 0.10-0.30, weak – at r 0.31-0.50, moderate – at r 0.51-0.70, strong – at r 0.71- 0.90, very strong – at r 0.91-0.99. The vector of the relationship, either positive or inverse (negative), was also assessed.

The correlation coefficient was evaluated as statistically significant at $p<0.05$.

Results

The results of our study demonstrated that Hcys level in blood serum of rats with LPS-induced periodontitis increased by 52.1% vs. the control group (Table 1).

However, these changes were not statistically significant ($p=0.215$). In rats with LPS-induced periodontitis combined with chronic thiolactone HHcy this index increased by 4.2 times ($p<0.001$) vs. control group, which is 2.7 times higher than the data obtained for only LPS-induced periodontitis. It should be noted that in animals with chronic thiolactone HHcy, Hcys level in blood serum demonstrated a 3.8 times increase ($p<0.001$) vs. control group and did not significantly differ from the data obtained from rats with LPS-induced periodontitis combined with chronic thiolactone HHcy.

During the study of indices of CT metabolism, it was found that the activity of collagenolysis in the blood serum of rats with LPS-induced periodontitis significantly increased by 80.0% compared to controls (table 2). In rats with LPS-induced periodontitis combined with chronic thiolactone HHcy, this index increased by 4.5 times ($p<0.001$) vs. controls, which is 2.5 times ($p=0.009$) higher than the data in case of only LPS-induced periodontitis.

The activation of collagenolysis is also evidenced by an increase of free oxyproline content. Thus, this index in the blood serum of rats with LPS-induced periodontitis significantly increased by 93.2% compared to the control group. In rats with LPS-induced periodontitis combined with chronic thiolactone HHcy, this index increased by 3.5 times ($p<0.001$) vs. control group, which is 81.4% ($p=0.003$) higher than the data in case of only LPS-induced periodontitis.

It should be noted that in animals with isolated chronic thiolactone HHcy, the collagenolytic activity of blood serum increased by 39.2% and free oxyproline content – by 22.5% compared to the control group, but these changes were not statistically significant.

The content of GAGs in the blood serum of rats with LPS-induced periodontitis increased by 80.9% ($p<0.001$) compared to controls. In rats with LPS-induced periodontitis combined with chronic thiolactone HHcy, this index increased by 2.9 times ($p<0.001$) compared to the control group, which is 61.7% ($p=0.007$) higher than the data in case of LPS-induced periodontitis without concomitant pathology. In animals with isolated chronic thiolactone HHcy, the content of GAGs in the blood serum increased by 30.1%, but these changes were not statistically significant.

The degree of glycoprotein degradation was assessed by the content of fucose not bound to proteins in the blood serum. It was found that the content of fucose not bound to proteins in the blood serum of rats with LPS-induced periodontitis increased by 2.9 times ($p<0.001$) compared to the control group. In rats with LPS-induced periodontitis combined with chronic thiolactone HHcy, this index increased by 4.9 times ($p<0.001$) compared to the control group, which is 69.2% higher than in case of LPS-induced periodontitis without concomitant pathology, but these changes were not statistically significant ($p=0.215$).

Analyzing the correlative linkages between serum level of HCys and indices of CT destruction in rats with only LPS-induced periodontitis a strong direct correlation between serum HCys level and GAGs content was found (table 3). At the same time in rats with LPS-induced periodontitis combined with chronic thiolactone HHcy a number of significant correlations were found: a strong direct correlation between serum HCys level and free hydroxyproline content; a moderate direct correlation between serum HCys level and GAGs content; a moderate direct correlation between serum HCys level and collagenolytic activity.

Discussion

The periodontium is a specialized organ comprising soft connective tissues (gingival and periodontal ligament) and calcified components (cementum and alveolar bone).[16] In the CT, the extracellular matrix (ECM) consists of fibrous structures surrounded by a gel-forming medium made of GAGs.[17] Sulfated GAGs molecules, such as chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratosulfate, stabilize and cement fibrous structures; they also shield cells from microorganisms and toxins, are involved in tissue water-salt regulation, and intercellular signal transduction. An important role of GAGs is mediation of the activity of growth factors, for

instance the fibroblast growth factor.[18,19] Proteolytic and lyzing enzymes produced by bacteria, for instance protease, glucuronidase, hyaluronidase collagenase, and chondroitin sulfatase, depolymerize proteoglycans and GAGs while interfering with their re-synthesis, facilitating infiltration of endotoxins into periodontium tissue.[3] Thus, the breakdown of CT biopolymers is an crucial factor contributing to the pathogenesis of the periodontitis.

Collagen is the main structural protein of the periodontal ECM. Collagen metabolism is regulated by an enzyme collagenase, synthesized in humans by fibroblasts and osteoblasts of CT and represented by four isoforms. Collagenase activity responds to the ratio of its activators and inhibitors in the intracellular matrix. In inflammatory processes, a special role in collagenase activation is played by proteases such as plasmin, kallikrein, and cathepsin B.[20] Our study found that in rats with LPS-induced periodontitis, both collagenolytic activity and the amount of free blood serum hydroxyproline have increased. This activation of proteolytic processing of collagen in LPS-induced periodontitis indicates an increase in catabolic processes in the periodontium CT structures, and the collagen decrease in periodontal tissues contribute to the weakening of their supporting function.[21]

The upsurge of catabolic processes in the periodontium CT in the cases of LPS-induced periodontitis was also corroborated by both the increase in glycoproteins degradation marker (unbound fucose levels) and in proteoglycans degradation marker (GAGs levels). This indicates breakdown of not only CT collagen structure but also the depolymerization of ECM components and disruption of their re-synthesis.

While the destruction of CT biopolymers is associated with endogenous activation of matrix metalloproteinases (MMPs), plasmin and serine proteinases, it's another key contributor is neutrophil phagocytic activity triggered by the release of pro-inflammatory cytokines.[22] Oxidative stress in periodontal tissue also plays a crucial role in biopolymer breakdown by causing oxidative modification of proteins and carbohydrates, apoptotic changes, or inducing histolytic enzymes expression by the activating of redox-sensitive transcription factors such as NF-κB.[3] NF-κB-mediated processes, which involve the receptor activator of the NF-κB receptor (RANK), receptor activator of nuclear factor kappa-B ligand (RANKL), and decoy receptor osteoprotegerin (OPG) are all important regulators of the resorptive activity of osteoclasts.[23]

Chronic thiolactone HHcy amplifies CT breakdown in LPS-induced periodontitis, which is confirmed by the significant increase in collagenolytic activity and GAGs content in rats with combined pathology compared to the animals with LPS-induced inflammation without concomitant pathology. Correlation analysis between HCys levels and CT catabolism serum markers in rats with LPS-induced periodontitis against the background of chronic thiolactone HHcy revealed a number of significant correlations, which also confirms the effect of elevated HCys levels on disorganization of collagen structures and CT matrix depolymerization.

It is likely that increased destruction of CT on against background of chronic thiolactone HHcy is caused by HCys promoting NF-κB production[24], which in turn mediates chronic inflammatory processes. Fibroblasts trigger various NF-κB-induced genes by reacting to TNF-α or IL-1, together with chemokines, which results in more inflammatory infiltrates and MMPs that initiate CT destruction.[25] Also HCys was shown to increase phosphorylation of p38 mitogen-activated protein kinases (MAPK) mediated by RANKL.[26] The p38 MAPK pathway is a stress response signaling pathway that is activated by intracellular and extracellular stresses, including exposure to inflammatory cytokines or other biosynthetic stresses. Thus it is involved in variety of biological responses as well as molecular regulation[27, 28, 29] and exert regulatory and biological effects on periodontium metabolism. Bode MK et al. found a correlation between HCys and C-terminal telopeptide of type I collagen in blood serum.[30] Moreover, increased CT destruction in the comorbid course of periodontitis (against the background of chronic thiolactone HHcy) can be caused by increased expression of MMP9 in mitochondrial matrix, which controls the properties of CT (collagen/elastin ratio).[31] Studies also suggest that the presence of thiolactone HCys in tissues inhibits mRNA expression of protein-lysine-6-oxidase, a key enzyme for post-translational modification of collagen and elastin precursors, which would cause destabilization of CT

structure.[32]

Conclusions

LPS-induced inflammation of periodontal tissues in rats is accompanied by CT catabolism, as evidenced by a significant increase of collagenolytic activity, increased content of free oxyproline, increased content of glycoprotein degradation marker – free fucose and increased content of proteoglycan breakdown marker – GAGs in the blood serum. Chronic thiolactone HHcy enhances the destruction of CT in case of periodontitis, which is confirmed by the significant excess of collagenolytic activity (by 2.5 times; p=0.009) and the content of GAGs (by 61.7%; p=0.007) in the blood serum of animals with combined pathology in relation to animals with LPS-induced inflammation without concomitant pathology. A correlation analysis between the level of Hcys and indices of connective tissue catabolism in blood serum of rats with LPS-induced periodontitis combined with chronic thiolactone HHcy revealed a number of significant correlations, which confirms the effect of increased Hcys levels on disorganization of collagen structures and depolymerization of the components of the organic matrix of CT.

Declaration of Interest

The authors report no conflict of interest.

Ethics

The Bioethics Commission of I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine approved the protocol of the experiment (protocol N 64 from 17 May 2021)

Parameter	Experimental group			
	Control	Periodontitis	HHcy	Periodontitis + HHcy
	1	2	3	4
HCys, μmol/l	7.10 (6.35; 7.35)	10.80 (9.80; 11.40)	27.05 (24.60; 29.15)	29.60 (27.85; 31.90)
	Kruskal-Wallis criterion: H=26.41; p<0.001*			
	p_{1-2}=0.215 p_{1-3}<0.001* p_{1-4}<0.001*	p_{2-3}=0.068 p_{2-4}=0.001*	p_{3-4}=0.999	–

Note 1. p_{1-2}, p_{1-3}, p_{1-4} – the probability of differences between control and experimental groups; p_{2-3} – the probability of differences between the group with periodontitis and group with HHcy; p_{2-4} – the probability of differences between the group with periodontitis and group with periodontitis combined with HHcy; p_{3-4} – the probability of differences between the group with HHcy and group with periodontitis combined with HHcy. Note 2. * – statistically significant results.

Table 1. Level of HCys in blood serum of rats with LPS-induced periodontitis without comorbid pathology and combined with chronic thiolactone HHcy.

Parameter	Experimental group			
	Control	Periodontitis	HHcy	Periodontitis + HHcy
Collagenolytic activity, μmol /l×hour	6.25 (5.80; 6.90)	11.25 (10.55; 12.45)	8.70 (7.95; 9.75)	28.33 (24.86; 29.65)
	Kruskal-Wallis criterion: H=43.75; p<0.001*			
	p_{1-2}<0.001* p_{1-3}=0.193 p_{1-4}<0.001*	p_{2-3}=0.265 p_{2-4}=0.009*	p_{3-4}<0.001*	–
Free hydroxyproline, μmol/l	11.80 (11.55; 12.70)	22.80 (19.80; 23.30)	14.45 (13.60; 15.00)	41.35 (39.85; 42.00)
	Kruskal-Wallis criterion: H=42.65; p<0.001*			
	p_{1-2}<0.001* p_{1-3}=0.512 p_{1-4}<0.001*	p_{2-3}=0.132 p_{2-4}=0.003	p_{3-4}<0.001*	–
Glycosaminoglycans, μmol/l	42.15 (37.95; 45.65)	76.24 (68.68; 81.73)	54.85 (50.85; 58.20)	123.25 (121.45; 139.80)
	Kruskal-Wallis criterion: H=42.95; p<0.001*			
	p_{1-2}<0.001* p_{1-3}=0.424 p_{1-4}<0.001*	p_{2-3}=0.148 p_{2-4}=0.007*	p_{3-4}<0.001*	–
Fucose, unbound with proteins, μmol/l	82.01 (73.78; 88.78)	238.90 (192.55; 253.06)	93.34 (90.27; 100.38)	404.10 (350.90; 423.75)
	Kruskal-Wallis criterion: H=42.16; p<0.001*			
	p_{1-2}<0.001* p_{1-3}=0.692 p_{1-4}<0.001*	p_{2-3}=0.109 p_{2-4}=0.215	p_{3-4}<0.001*	
Note 1. p_{1-2}, p_{1-3}, p_{1-4} – the probability of differences between control and experimental groups; p_{2-3} – the probability of differences between the group with periodontitis and group with HHcy; p_{2-4} – the probability of differences between the group with periodontitis and group with periodontitis combined with HHcy; p_{3-4} – the probability of differences between the group with HHcy and group with periodontitis combined with HHcy. Note 2. * – statistically significant results.				

Table 2. The indices of connective tissue metabolism in blood serum of rats with LPS-induced periodontitis without comorbid pathology and combined with chronic thiolactone HHcy.

Parameters		Experimental group		
		Periodontitis	HHcy	Periodontitis + HHcy
HCys, μmol/l	Collagenolytic activity, μmol/l×hour	r=0.36; p=0.254	r=0.49; p=0.103	r=0.66; p=0.019*
	Free hydroxyproline, μmol/l	r=0.39; p=0.209	r=0.58; p=0.047*	r=0.76; p=0.004*
	Glycosaminoglycans, μmol/l	r=0.77; p=0.004*	r=0.65; p=0.023*	r=0.63; p=0.028*
	Fucose, unbound with proteins, μmol/l	r=0.30; p=0.337	r=0.61; p=0.035*	r=0.54; p=0.225
Note. * – statistically significant results.				

Table 3. Correlative linkages between the level of HCys in blood serum and indices of connective tissue metabolism in case of periodontitis without comorbid pathology and combined with chronic thiolactone HHcy (r_{xy})

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