A Comparative Study of Connective Tissue Metabolism Indices in Experimental Comorbidity-Free Periodontitis and Periodontitis Combined with Thyroid Dysfunction

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Abstract
Periodontal disease is a chronic bacterial infection characterized by persistent inflammation, connective tissue breakdown, and alveolar bone destruction. The current study aimed to compare the connective tissue metabolism indices in rats with comorbidity-free periodontitis and in animals with periodontitis in a setting of hyperand hypothyroidism. 12-14-week-old inbred white male rats (n=48) were included in the experiment. They were randomly divided into the following groups: control, animals with a model of periodontitis, animals with periodontitis in a setting of hyperthyroidism, animals with periodontitis in a setting of hypothyroidism. Serum levels of free thyroxine, free triiodothyronine, and thyroid-stimulating hormone were assayed using ELISA kits manufactured by Vector Best (Russia) to confirm the hyper- and hypothyroid status. Collagenolytic activity, the content of glycosaminoglycans, free hydroxyproline, and fucose, unbound with proteins in blood serum were assayed using the spectrophotometric method. We have found the increasing of collagenolytic activity by 46.1% (p<0.001), the content of free hydroxyproline by 74.1% (p<0.001), the content of glycosaminoglycans by 1.8 times (p<0.001), the content of fucose, unbound with proteins by 2.8 times (p<0.001) in rats with periodontitis vs. the control group. The development of periodontitis in a setting of thyroid dysfunction leads to an even more significant increase in the destruction of connective tissue, which is confirmed by a significant increase in the content of studied indices vs. euthyroid animals, both in hyperthyroidism and hypothyroidism.

Keywords: Connective tissue, periodontitis, thyroid dysfunction.

Introduction
Periodontitis is a chronic non-communicable disease (NCD) that shares social determinants and risk factors with the major NCDs that cause around two-thirds of deaths, such as heart disease, diabetes, cancer, and chronic respiratory disease [1]. The Global Burden of Disease Study indicates that severe periodontitis is the 6th most prevalent disease worldwide, with an overall prevalence of 11.2% and around 743 million people affected [2]. Overall, it affects about 20-50% of the population around the globe [3] and is the most common oral condition of the human population [4]. In Europe, epidemiological evidence indicates that mild gingival inflammation and mild to moderate loss of periodontal attachment in specific sites are prevalent in the adult population, but epidemiological studies available from most Eastern European countries are limited [5].

The periodontium is a unique organ that consists of two soft connective tissues (gingival and periodontal ligament) and two calcified components (cementum and alveolar bone) [6]. The extracellular matrix (ECM) of the connective tissue (CT) is represented by fibrous structures as well as by a gel-forming medium formed by glycosaminoglycans (GAGs) [7]. Sulfated glycosaminoglycans (GAGs) - chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate provide stabilization and cementation of fibrous structures, protect cells from the penetration of microorganisms and their toxins, regulate water-salt metabolism in tissues, participate in intercellular signaling and regulate the activity of growth factors, including the fibroblast growth factor [8, 9].

Periodontal disease is a chronic bacterial infection characterized by persistent inflammation, connective tissue breakdown, and alveolar bone destruction [10, 11]. The aggression of periodontopathogenic microflora is associated with the presence in the shell of bacteria of proteolytic enzymes and endotoxins. Microbial enzymes (hyaluronidase, chondroitin sulfatase, protease, glucuronid-
dase, collagenase) cause the depolymerization of proteoglycans and GAGs of the periodontium and disruption of their resynthesis, resulting in endotoxin invasion of tissue [12]. Therefore, the depolymerization of CT biopolymers is an essential link in the pathogenesis of the periodontal disease.

Metabolic processes occurring in CT and its remodeling are largely determined by the functional state of hormonal systems [13], including thyroid hormones.

Therefore, this paper aims to compare the connective tissue metabolism indices in rats with comorbidity-free periodontitis and in animals with periodontitis in a setting of hyper- and hypothyroidism.

Material and Methods

Animals

12-14-week-old inbred white male rats (n=48) with a body weight of 180-200 g were included in the experiment. The animals were kept under standardized conditions, with controlled light cycle (12/12) and unlimited access to water and food throughout the period of the experiment.

Study groups

The animals were randomly divided into the following groups: Group I: control animals were administered intragastric 1% starch solution (n=12); Group II: animals with a model of periodontitis (n=12). During two weeks, the rats in this group were administered 40 μL (1 mg/mL) of E. coli lipopolysaccharide (LPS) (manufactured by Sigma-Aldrich, USA) into gingival tissues every other day [14]. Group III: rats with periodontitis in a setting of hyperthyroidism (n=12). To create an experimental model of thyroid hyperfunction, the animals received daily intragastric doses of L-thyroxine in a 1% starch solution at 10 μg/day per 100 g of body weight for 21 days [15]. Starting from day 8 of the experiment, the rats were given LPS into gingival tissue for two weeks; Group IV included rats with periodontitis in a setting of hypothyroidism (n=12). To create an experimental model of thyroid hypofunction [15], the animals received daily intragastric doses of methimazole at 1 mg/day per 100 g of body weight for 21 days. The rats were given LPS into the gingival tissue for two weeks starting from day 8 of the experiment. The rats were euthanized under deep thiopental-sodium anesthesia on day 22 from the onset of the experiment. Blood serum was used for further investigation.

All manipulations with experimental animals were performed according to provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [16]. The Bioethics Commission of I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine approved the protocol of the experiment (Excerpts from Minutes No. 59, dated 23.10.2019).

Serum levels of free thyroxine (FT4), free triiodothyronine (FT3), and thyroid-stimulating hormone (TSH) were assayed with ELISA kits manufactured by Vector Best (Russia) to confirm hyper- and hypothyroid status.

Collagenolytic activity, the content of glycosaminoglycans and fucose, unbound with proteins, were determined using the methods of P.N. Sharaev and co-authors [17-19]; the content of free hydroxyproline was determined using the method of S.S. Tetyanets [20].

Statistical processing of digital data was carried out using the software Excel (Microsoft, USA) and STATISTICA 6.0 (Statsoft, USA). The distribution of data was analyzed according to the assessment of normality by the Kolmogorov-Smirnov criterion. The obtained values had a normal distribution, so the difference between the groups was analyzed using the Student’s t-criterion. All data were presented as M (mean) ± m (standard error). A probability level (p-value) of less than 0.05 was considered to be statistically significant.

Results

The analysis of data indicated that collagenolytic activity in the blood serum of rats with periodontitis was increased by 46.1% (p<0.001) vs. control group (Table 1). In rats with periodontitis in a setting of hyperthyroidism, this index has increased by 2.3 times (p<0.001) vs. control group. It should be noted that the collagenolytic activity in the blood serum of hyperthyroid rats significantly exceeded this index (by 55.6%) in rats with periodontitis without concomitant pathology and by 17.2% (p<0.01) in rats with periodontitis in a setting of hypothyroidism.

In rats with a model of periodontitis in a setting of hypothyroidism, the collagenolytic activity increased by 1.9 times (p<0.001) vs. control group and significantly exceeded the index of animals with periodontitis without concomitant pathology by 32.8% (p<0.001).

Activation of collagenolysis is also evidenced by an increased content of free hydroxyproline. Thus, this index in blood serum of rats with periodontitis increased by 74.1% (p<0.001), in rats with periodontitis in a setting of hyperthyroidism by 2.5 times (p<0.001), in rats with periodontitis in a setting of hypothyroidism by 2.1 times (p<0.001) vs. control group. It should be noted that the content of free hydroxyproline in blood serum of hyperthyroid rats significantly exceeded this index (by 45.2%) in rats with periodontitis without concomitant pathology and by 19.8% (p<0.001) in rats with periodontitis in a setting of hypothyroidism.

In rats with a model of periodontitis in a setting of hyperthyroidism, the content of free hydroxyproline in blood serum significantly exceeded the data of rats with periodontitis without concomitant pathology by 21.2% (p=0.001).

Glycoproteins and proteoglycans are the major proteins in the periodontal connective tissue. The content of glycosaminoglycans (GAGs) in blood serum can be considered as a biochemical marker of proteoglycans decomposition [12].
Table 1: The indices of connective tissue metabolism in blood serum of rats with periodontitis without comorbidities and in a setting of hyper-and hypothyroidism (M ± m, n=12).

| Index                        | Group of animals | Periodontis in a setting of hyperthyroidism | Periodontis in a setting of hypothyroidism |
|------------------------------|------------------|---------------------------------------------|-------------------------------------------|
|                              | Control          | Periodontis                                 |                                           |
| Collagenolytic activity, µmol/l×hour | 5.40 ± 0.18    | 7.89 ± 0.45 p<0.001                         | 12.28 ± 0.38 p<0.001                      |
|                              |                  |                                             |                                           |
| Free hydroxyproline, µmol/l  | 11.9 ± 0.33      | 20.80 ± 0.76 p<0.001                         | 30.20 ± 0.80 p<0.001                      |
| Glycosaminoglycans, µmol/l   | 42.38 ± 1.65     | 77.81 ± 2.29 p<0.001                         | 109.83 ± 4.97 p<0.001                      |
| Fucose, unbound with proteins, µmol/l | 79.93 ± 3.28  | 227.39 ± 10.70 p<0.001                       | 389.55 ± 15.40 p<0.001                     |

Note: p1 – significant differences compared to control animals; p2 – significant differences between the group of periodontitis with the group of periodontitis combined with hyperthyroidism; p3 – significant differences between the group of periodontitis with the group of periodontitis combined with hypothyroidism; p4 – significant differences between the group of periodontitis combined with hyperthyroidism with the group of periodontitis combined with hypothyroidism.

The analysis of data indicated that the modeling of periodontitis in rats has led to the increased content of GAGs in blood serum by 1.8 times (p<0.001) vs. control group. In rats with periodontitis in a setting of hyperthyroidism, this index has probably increased by 2.6 times (p<0.001) vs. control group. It should be noted that the content of GAGs in blood serum of hyperthyroid rats significantly exceeded this index (by 41.2%) in rats with periodontitis without concomitant pathology.

In the case of periodontitis in a setting of hyperthyroidism, the content of fucose, unbound with proteins in blood serum increased. Collagenolytic activity, and content of free hydroxyproline in blood serum increased. Collagen is the main structural protein of the periodontal intracellular matrix of CT. An enzyme collagenase, which is synthesized by CT cells (fibroblasts and macrophages) and is found in four isoforms, regulates collagen metabolism. Collagenase activity depends on the ratio in the intracellular matrix of its activators and inhibitors. Plasmin, kallikrein, and cathepsin B play a special role in its activation in inflammatory processes [21].

Activation of collagenolysis in experimental periodontitis reflects an increase in catabolic processes in the CT structures of the periodontium, which contribute to the violation of its supporting function, characterized by a decrease in the content of collagen in periodontal tissues [22].

The increase of catabolic processes in the CT of the periodontium in the case of experimental periodontitis was also confirmed by the increase of glycoproteins degradation marker (the content of fucose, unbound with proteins) and an increase of GAGs content (proteoglycans degradation marker). This leads to the disorganization of not only a setting of hypothyroidism, this index significantly exceeded this index (by 34.0%) in rats with periodontitis without concomitant pathology.

Discussion

Our research has found that in rats with periodontitis, collagenolytic activity, and content of free hydroxyproline in blood serum increased. Collagen is the main structural protein of the periodontal intracellular matrix of CT. An enzyme collagenase, which is synthesized by CT cells (fibroblasts and macrophages) and is found in four isoforms, regulates collagen metabolism. Collagenase activity depends on the ratio in the intracellular matrix of its activators and inhibitors. Plasmin, kallikrein, and cathepsin B play a special role in its activation in inflammatory processes [21].

Activation of collagenolysis in experimental periodontitis reflects an increase in catabolic processes in the CT structures of the periodontium, which contribute to the violation of its supporting function, characterized by a decrease in the content of collagen in periodontal tissues [22].

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the collagen structures of the CT but also the depolymerization of the organic matrix components and disruption of their resynthesis.

The depolymerization of CT biopolymers and their impaired resynthesis is an important link in the pathogenesis of inflammatory and dystrophic periodontal diseases. On the one hand, this is facilitated by the production of exotoxins and histolytic enzymes (hyaluronidases, chondroitin sulfatases, proteases, glucuronidases, collagenases) by pathogenic microorganisms, which causes depolymerization of collagen, proteoglycans, and glycoproteins. On the other hand, CT destruction is associated with endogenous activation of matrix metalloproteinases (MMPs), plasmin, serine proteinases of polymorphic nuclear leukocytes, and their phagocytic activity in response to the production of proinflammatory cytokines [8, 23, 24].

The destruction of CT biopolymers can also be associated with the development of oxidative stress in periodontal tissues, which causes oxidative modification of proteins and carbohydrates, apoptotic changes, or induces the production of histolytic enzymes due to the activation of redox-sensitive transcription factors (NF-κB) [25-27]. NF-κB-dependent processes associated with the activator of the NF-κB receptor (RANK), its ligand (RANKL), and the false receptor osteoprotegerin are important regulators of the resorptive activity of osteoclasts [28].

The development of periodontitis in a setting of thyroid dysfunction leads to an even greater increase in the destruction of CT, which is confirmed by the significant predominance of the catabolic indices vs. euthyroid animals, both under hyperthyroidism and hypothyroidism. It should be noted that when comparing CT catabolism indices in both under hyperthyroidism and hypothyroidism, it is possible to observe the dominance of the catabolic indices vs. euthyroid animals, both in hyperthyroidism and hypothyroidism. This indicates that thyroid dysfunction leads to an even greater increase in the catabolism of connective tissue, which is confirmed by the significant increase in the content of connective tissue degradative marker – the content of fucose, unbound with carbohydrates, responsible for connective tissue and further alveolar bone destruction by activation of the resident cells of periodontium [37]. Monea et al. suggested that cytokines IL-6 and TNF-α produced in thyroid disorders play a major role in the initiation and amplification of the inflammatory cascade in the periodontal tissues. The endotoxins produced by the bacteria in dental plaque combines with these cytokines, further aggravating the inflammatory cascade by the production of more cytokines responsible for MMPs activation and periodontal breakdown [38]. Babior B.M. suggested that polymorphonuclear leukocytes (PMNs) play a major role in bacterial phagocytosis by the respiratory burst mechanism through the nicotinamide adenine dinucleotide phosphate-oxidase complex and leads to the production of reactive oxygen species (ROS) which generates oxidative stress within periodontal tissues. These ROS lead to bone resorption by acting at the ruffled border of osteoclasts [39]. Similarly, Mezosi E. et al. have shown that thyroid hormones stimulate free radical production and impaired phagocytosis by PMNs, mainly in hypothyroid patients [40]. Kanatani M. et al. showed a negative effect of thyroid dysfunction on IL-6 and TNF-α, which are responsible for osteoclast differentiation and function independent of the RANKL mechanism [41].

**Conclusion**

Our studies showed the increase of connective tissue catabolism in rats with periodontitis, as evidenced by an increase in collagenolytic activity, in the free oxyproline content, in the proteoglycans degradation marker – the content of glycosaminoglycans, in the glycoproteins degradation marker – the content of fucose, unbound with proteins.

The development of periodontitis in a setting of thyroid dysfunction leads to an even greater increase in the destruction of connective tissue, which is confirmed by a significant increase in the content of connective tissue catabolism indices vs. euthyroid animals, both in hyperthyroidism and hypothyroidism.

**Conflict of Interest**

The authors declare that there is no conflict of interest.

**References**

1. Tonetti MS, Jepsen S, Jin L, Otomo-Corgel J. Impact of the global burden of periodontal diseases on health, nutrition and wellbe-
19. Sharaev PN, Strelkov NS, Kildiyarova RR. The method of determination of fucose, unbound with proteins. Clinical Laboratory Diagnosis and Therapy. 1997; 4: 17-18. (in Russian)

20. Shevchenko NS, Lebec IS, Khaskalda DA. Changes of connective tissue biochemical indexes in children and adolescents with inflammatory diseases of joints. // Bulletin of the V.N. Karazin Kharkiv National University. 2016; 28: 11-15. (in Ukrainian)

21. Shvets IE, Bandirovskiy VL. The dynamics of metabolic changes in the periodontal tissues of experimental animals. Clinical and experimental pathology. 2014; 13 (4): 158-161. (in Ukrainian)

22. Silva N, Abusleme L, Bravo D, Dutzan N, Garcia-Sesnick J, Vernal R, Hernández M, Gamonal J. Host response mechanisms in periodontal diseases. J. Appl. Oral Sci. 2015; 23(3): 329-355.

23. Zherebiatiev A, Kamysnyi A. Expression levels of proinflammatory cytokines and NLRP3 inflammasome in an experimental model of oxazolone-induced colitis. Iran J Allergy Asthma Immunol. 2016; 15(1): 39-45.

24. Topol I, Kamysny A. Study of expression of TLR2, TLR4 and transcription factor NF-kB structures of gait of rats in the conditions of the chronic social stress and modulation of structure of intestinal microflora. Georgian medical news. 2013; 12(225):115-122.

25. Marushchak M, Krynitsa I, Petrenko N, Klishch I. The determination of correlation linkages between level of reactive oxygen species, contents of neutrophiles and blood gas composition in experimental acute lung injury. Georgian Medical News. 2016; 4(253): 98-103.

26. Marushchak M, Krynitsa I, Milevska L, Miz A, Mialiuk O. The changes of activity of effector caspase cascade components in case of alimentary obesity in rats. Bangladesh Journal of Medical Science. 2017; 16 (2): 252-258.

27. Ambili R, Janam P A critique on nuclear factor-kappa B and signal transducer and activator of transcription 3: The key transcription factors in periodontal pathogenesis. J. Indian Soc. Periodontol. 2017; 21(5): 350-56.

28. Drobnik J, Cisek J, Slotwinska E, Skruczynski L, Rozanski J. The expression of mRNA of cytokines and of extracellular matrix proteins in triiodothyronine-treated rats hearts. Mol Cell Biochem. 2009; 319(1-2): 151-62.

29. Zhang L, Bowen T, Gennnan-Jones F, Paddon C, Giles P, Webber J, Steadman R, Ludgate M. Thyrotropin Receptor Activation Increases Hylaurozon Production in Preadipocyte Fibrolasts. The journal of biological chemistry. 2009; 284(39): 26447-26455.

30. Amerin M, Tahajodi S, Hushmand Z, Mahdavi Shahri N, Nikravesh MR, Jalali M. The effect of maternal thyroid disorders (hyperthyroidism and hypothyroidism) during pregnancy and lactation on skin development in wistar rat newborns. Iran J Basic Med Sci. 2013;16(5): 665-674.

31. Cardoso LF, Maciel LM, Paula FJ. The multiple effects of thyroid disorders on bone and mineral metabolism. Arq Bras Endocrinol Metab. 2014; 58(5): 452-463.

32. Ziegelhofer-Mihalovicova B, Breist W, Baba HA, Rabler B, Zimmer HG. The expression of mRNA of cytokines and of extracellular matrix proteins in triiodothyronine-treated rats hearts. Mol Cell Biochem. 2003; 247: 61-88.

33. Dolidze NM, Karynitsa I, Komarova I.V., Sirotenko L.A., Spirdonov A.V., Anikevych K.S. New approaches to complex correction hypothyroidism and its related complications. Problems of endocrine pathology. 2016; 4: 71-81 (in Russian).

34. Moysseeva EH. Metabolic homeostasis and immune reactivity of the organism in the dynamics of inflammation in periodontal tissues. Extended abstract of Doctor's thesis. Moscow, 2008; 45 p. (in Russian)

35. Ratushenko VO. Functional role of thio-disulphide system in experimental hypo- and hyperthyroidism. Odessa Medical Journal 2010; 2(18): 17-20. (in Ukrainian)

36. European convention for the protection of vertebrate animals used for experimental and other scientific purposes. Council of Europe. Strasbourg. 1986; 123.

37. Sharaev PN, Pishkov VN, Zvorygina NG. Determination of collagenolytic activity of blood plasma. Laboratory science. 1987; 5: 1-6.

38. Sharaev PN, Strelkov NS, Kildiyarova RR. The method of determination of fucose, unbound with proteins. Clinical Laboratory Diagnostics. 1997; 4: 17-18. (in Russian)

39. Sharaev PN, Strelkov NS, Kildiyarova RR. The method of determination of glycosaminoglycans in biological fluids. Laboratory science. 1987; 5: 330-332. (in Russian)
hypothyroid patients with periodontal diseases. Indian J Dent Res. 2017; 28:16-21.

38. Monea A, Elod N, Sitaru A, Stoica A, Monea M. Can thyroid dysfunction induce periodontal disease? Eur Sci J. 2014; 10: 74-83.

39. Babior BM. NADPH oxidase: An update. Blood. 1999; 93:1464-1476.

40. Mezosi E, Szabo J, Nagy EV, Borbely A, Varga E, Paragh G, Varga Z. Nongenomic effect of thyroid hormone on free-radical production in human polymorphonuclear leukocytes. J Endocrinol. 2005; 185: 121-129.

41. Kanatani M, Sugimoto T, Sowa H, Kobayashi T, Kanzawa M, Chihara K. Thyroid hormone stimulates osteoclast differentiation by a mechanism independent of RANKL-RANK interaction. J Cell Physiol. 2004; 201: 17-25.