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RESULTS OF IMMUNOHISTOCHEMICAL STUDY OF MARKERS OF INFLAMMATION AND APOPTOSIS IN GINGIVAL BIOPSTATES IN PATIENTS WITH GENERALIZED PERIODONTITIS

Purpose of the study. Comparison of immunohistochemical indexes of expression of markers of apoptosis and inflammation in gingival biopstates of healthy persons and patients with generalized periodontitis. **Research methods.** 40 persons aged 32–45 years were studied. They were 30 patients with chronic generalized periodontitis of I–II severity and 10 persons with healthy periodontal tissues. For morphological examination, gingival biopstates were used, which were put into 4 % solution of neutral formalin during the day and poured into paraffin. Histological sections with a thickness of 4.0–6.0 microns were applied to adhesive slides. After their deparaffinization and rehydration, antigens were demasked in the citrate buffer with pH 6.0 at 121 °C for 8 minutes. The activity of endogenous peroxidase was suppressed with 3 % hydrogen peroxide for 20 minutes. It was studied cytoplasmic expression of markers COX-2, caspase-3 and intranuclear expression of markers MMP-1 and p21 (in inflammatory infiltrate), p53 and Bcl-2 (in gingival epithelium). **Scientific novelty.** At the present stage, the interest is to study the mechanisms of damage of periodontal tissues initiated by the microbial factor, which include direct cytopathic effects, in particular apoptosis and pyroptosis of periodontal epithelial and connective tissue cells. **Conclusions.** According to the results of the immunohistochemical study of gingival biopstates, there was no difference between the expression of markers of the antiapoptotic protein Bcl-2 and the marker of apoptosis p53 in patients with generalized periodontitis and healthy ones. The most indicative markers of the course of the inflammatory and destructive process in periodontal tissues were markers of inflammation COX-2 and MMP-1 and markers of apoptosis caspase-3 and p21, that allows their use for diagnostic purposes in patients with generalized periodontitis. **Key words:** generalized periodontitis, apoptosis, inflammation, immunohistochemical study.

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РЕЗУЛЬТАТИ ІМУНОГІСТОХІМІЧНОГО ДОСЛІДЖЕННЯ МАРКЕРІВ ЗАПАЛЕННЯ ТА АПОПТОЗУ В БІОПСТАТАХ ЯСЕН У ХВОРИХ НА ГЕНЕРАЛІЗОВАНИЙ ПАРОДОНТИТ

Мета дослідження. Зіставлення імуногістохімічних показників експресії маркерів апоптозу та запалення в тканинах маргінального пародонту осіб зі здоровим пародонтом та хворих на генералізований пародонтит. **Методи дослідження.** Було залучено 40 осіб віком 32–45 років, серед яких 30 хворих із хронічним генералізованим пародонтитом I–II ступеня тяжкості та 10 осіб зі здоровим пародонтом. Для морфологічного дослідження використовували біоптати ясен, які фіксували у 4 % розчині нейтрального формаліну протягом доби і заливали у парафін. Гістологічні зрізи товщиною 4,0–6,0 мкм наносили на адгезивні предметні скельця. Після їх депарафінізації та регідратації проводили демаскування антигенів в цитратному буфері з рН 6,0 при 121 °C протягом 8 хвилин. Активність ендогенної пероксидази пригнічували 3 % перекисом водню протягом 20 хвилин. Інкубацію зрізів проводили з первинними антитілами у вологих камерах при 23–25 °C протягом 30 хвилин. Вивчали цитоплазматичну експресію маркерів COX-2, каспази-3 та інтрануклеарну експресію маркерів MMP-1 та p21 (у запальному інфільтраті), p53 та Bcl-2 (в ясенному епітелії). **Наукова новизна.** На сучасному етапі інтерес представляє вивчення механізмів пошкодження тканин пародонта, ініційованих мікробним фактором, до яких належать прямі цитопатичні ефекти, зокрема апоптоз та піроптоз епітеліальних та сполучнотканинних клітин пародонта. **Висновки.** Згідно отриманих результатів імуногістохімічного дослідження біоптатів ясен достовірної різниці між експресією маркерів антиапоптотичного білка Bcl-2 та маркеру апоптозу p53 у хворих на генералізований пародонтит та здорових не виявлено. Найбільш показовими щодо перебігу запально-деструктивного процесу в тканинах пародонта виявились маркери запалення COX-2 та MMP-1, а також маркери апоптозу каспаза-3 та p21, що дозволяє їх застосування в діагностичних цілях у хворих на генералізований пародонтит. **Ключові слова:** генералізований пародонтит, апоптоз, запалення, імуногістохімічне дослідження.

Posing a problem. The study of the pathogenesis of generalized periodontitis is perspective for the development of methods of diagnosis and treatment of the disease. The direct cytopathic effects, in particular apoptosis and pyroptosis, are the least studied among the mechanisms of periodontal tissue damage which are initiated by the microbial factor [1, p. 609–619; 2, p. 1144–1154; 3, p. 945–950]. It is now established that *Treponema denticola*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Actinobacillus actinomycetemcomitans* are able to stimulate the programmed death of periodontal epithelial and connective tissue cells [4, p. 208; 5; 6].

Macroorganism's local response includes neutrophil infiltration and subsequent release of inflammation and cytokine mediators [7, p. 429–450]. Cyclooxygenase 2 (COX-2) is marker of inflammation which involved in the synthesis of prostaglandins through the conversion of arachidonic acid. This enzyme is always secreted by connective tissue cells only after induction by cytokines or growth factors [8, p. 12–15].

A decrease in immune protection and an increase in the level of inflammatory mediators lead to the progress of the inflammatory process from the gingiva to the periodontal ligament and bone tissue, accompanied by the disintegration of collagen under the action of matrix metalloproteinase (MMP). MMP-1 is one of collagenases, and its activity corresponds to the degree of stromal degradation in periodontal tissues [9, p. 6393–6400].

Pyroptosis is a pro-inflammatory programmed cell death mediated through a family of proteases, the so-called caspases, which are divided into two groups – initiating and effecting. Initiating caspases -8 and -9 are given the role of activators of subordinate effecting caspases, such as caspase-3 [3, p. 945–950]. So, caspase-3 is a performing enzyme that has the appropriate catalytic activity. Its detection in periodontal tissue indicates induction of apoptosis [10, p. 17].

Among the factors of apoptic activity in the development of the inflammatory and destructive process in periodontal tissues, there are the products of three genes that encode proteins; they are p53, p21 and Bcl-2. p53 is a protein product of tumor gene-suppressor, the expression of which initiates apoptosis. This protein affects the dynamics of cellular renewal through the induction of apoptosis in thermal stage of cell differentiation, in particular inflammatory infiltrate cells. In turn, p21 is an important cell cycle inhibitor. Its expression is one of the main targets of the transactivational action of oncoprotein

p53. p21 blocks complexes of different cycles with the necessary kinases, which are key enzymes of cell division. On the contrary, Bcl-2 is an antiapoptotic protein that reduces the risk or even prevents cell death, which is provoked by various stimuli. It is able to inhibit the process of exit of the mitochondrial cytochrome, which initiates the internal path of death [10, p. 17; 11, p. 616–623].

Based on the above, the mechanisms of apoptosis and inflammation have to play an important role in the elimination and renewal of periodontal cells. In our opinion, the immunohistochemical study of these markers in gingival bioplates in patients with periodontitis in comparison with healthy persons reveals the most indicative characteristics of the inflammatory and destructive process in periodontal tissues in order to create effective methods of diagnosis and pathogenetic treatment of the disease.

Purpose of the study. Comparison of immunohistochemical indexes of expression of markers of apoptosis and inflammation in gingival bioplates of healthy persons and patients with generalized periodontitis.

Research methods. In the study 40 young people under WHO (32–45 years), equally men and women, were taken. The main group included 30 patients with chronic generalized periodontitis of I–II severity, the control group consisted of 10 persons with healthy periodontal tissues. In the research they did not include persons with severe somatic, endocrine and oncological pathology and smokers.

For morphological examination, gingival bioplates of 2.0–2.5 mm were used. The obtained samples were fixed in a 4.0 % solution of neutral formalin during the day and poured into paraffin. Histological sections with a thickness of 4.0–6.0 microns were applied to adhesive slides SuperFrost Plus. After deparaffinization and rehydration of the sections, temperature demasking of antigens was carried out. To do this, the sections were placed in a citrate buffer with pH 6.0 and heated in an autoclave at a temperature of 121 °C for 8 minutes. The activity of endogenous peroxidase was suppressed with 3.0 % solution of hydrogen peroxide for 20 minutes. Subsequently, the incubation of sections was carried out with primary antibodies in wet chambers at a temperature of 23–25 °C for 30 minutes. As primary monoclonal antibodies to p21, p53, Bcl-2, caspase-3, COX-2 (LabVision) and polyclonal to MMP-1 (DakoCytomation) were used. The antibody titer was selected individually for each marker using Antibody Diluent (DakoCytomation) as a solvent. To identify the reaction, an ultravision imaging

system Quanto (LabVision) was used. It was used the application of 3-diaminobenzidine tetrachloride (Quanto, LabVision) as a chromogen and the control of a microscope from 20 seconds to 3 minutes. The manifestation was in the form of brown coloration of specific structures. To separate the nonspecific tissue structures, the sections were additionally stained with Myer hematoxyline for 1–3 minutes.

Cytoplasmic expression of COX-2 marker was evaluated according to three gradations of coloured cells. They were (+1) weak infiltrate, (+2) moderate infiltrate, (+3) severe inflammatory infiltrate with a positive label [8, p. 12–15]. In turn, the cytoplasmic expression of the caspase-3 marker was evaluated on a binary scale. It was (0) if coloured cells were less than 30 % of inflammatory infiltrate; (1+) – respectively, when more than 30 % cells were coloured. Levels of intranuclear expression of MMP-1 and p 21 markers (in inflammatory infiltrate), p 53 and Bcl-2 (in gingival epithelium) were divided into 4 gradations from 0 to +3. They were (0) there was no colour; (+1) coloured cells were up to 10 %, (+2) the number of coloured cells varied in the range of 10 to 30 %, and gradation (+3), where the coloured cells were more than 30 % [10, p. 17].

For statistical processing of data, a licensed software product MS Excel 2003 was used [12].

Results and discussion. According to the results of the immunohistochemical study, for the patients of the main group, an increase in the expression of COX-2 in the inflammatory infiltrate was registered (fig. 1 a). If in all the persons with intact periodontal tissues the expression of the marker of inflammation COX-2 is defined as weak (+1), then in the patients with periodontitis in 80.0 % of cases moderate expression (+2) was detected, and in 20.0 % the high (+3) level was registered.

When evaluating the cytoplasmic expression of MMP-1 in epithelial and stromal cells, it was found that in healthy individuals, 30.0 % of observations had its zero gradation (0), 60.0 % it was weak (+1), 10.0 % it was moderate (+2) (fig. 1 b). In the main group, the cases of zero result was not observed, weak (+1) expression of the marker MMP-1 was registered in 23.3 % of patients, moderate (+2) gradation was in 60.0 %, high (+3) one was in 16.7 %. Consequently, the expression of the MMP-1 marker demonstrated a reliable difference in values depending on the clinical and morphological characteristics of the gingival biotates ($p < 0.05$). Thus, the increase in the activity of MMP-1 corresponded to the development of an inflammatory and destructive process in periodontal tissues.

In gingival samples, the highest rates of expression of related markers of apoptosis (effecting caspase-3 and oncoprotein-suppressor p21) were also observed in patients with generalized periodontitis in the cells of the inflammatory stromal infiltrate and in the epitheliocytes of the multilayer flat epithelium (fig. 1 c–d). In all the patients of the control group there was the absence (0) of the expression of the caspase-3 marker and 100 % weak (+1) expression for the p21 marker. In patients with periodontitis from the main group, the negative gradation (0) of the cytoplasmic marker caspase-3 was established in 46.7 % of cases, and it was (+1) in 53.3 %. For the oncoprotein-suppressor p 21 in 40.0 % it was defined weak (+1) expression, in 40.0 % it was moderate (+2), and in the remaining 20.0 % it was high (+3). So, these markers were indicative of the development of the inflammatory and destructive process in periodontal tissues ($p < 0.05$).

Unlike markers of apoptosis caspase-3 and p21, which were actively expressed by inflammatory infiltrate cells, for p53 and Bcl-2 a positive immunohistochemical reaction was observed mainly in the basal layer of the multilayer flat epithelium (fig. 1 e–f). In the control group, in 40.0 % of observations, the expression of p53 was not detected, and in 60.0 % the weak (+1) level was established. In the main group, 36.7 % of patients had a zero gradation of this marker, in 63.3 % the gradation was weak (+1). Thus, there were no reliable differences between these markers for research groups.

The antiapoptotic protein Bcl-2 showed a decrease in expression in periodontitis patients compared to healthy ($p < 0.05$). Thus, in persons with healthy periodontal tissues, zero (0) gradation was got in 20.0 % of cases, weak (+1) one was in the remaining 80.0 %. In 60.0 % of patients with generalized periodontitis, zero (0) level of expression of the Bcl-2 marker was obtained, and in 40.0 % it was weak (+1). It should be noted that the moderate and high gradation of expression of markers p53 and Bcl-2 was not detected in our study.

Conclusions. Thus, the results of immunohistochemical studies showed that the markers of inflammation COX-2 and MMP-1 and the markers of apoptosis caspase-3 and p21 were the most demonstrative for the inflammatory and destructive process in periodontal tissues. The obtained data allow their widespread use in the diagnostic process in patients with generalized periodontitis and for the estimation of the effectiveness of the treatment.

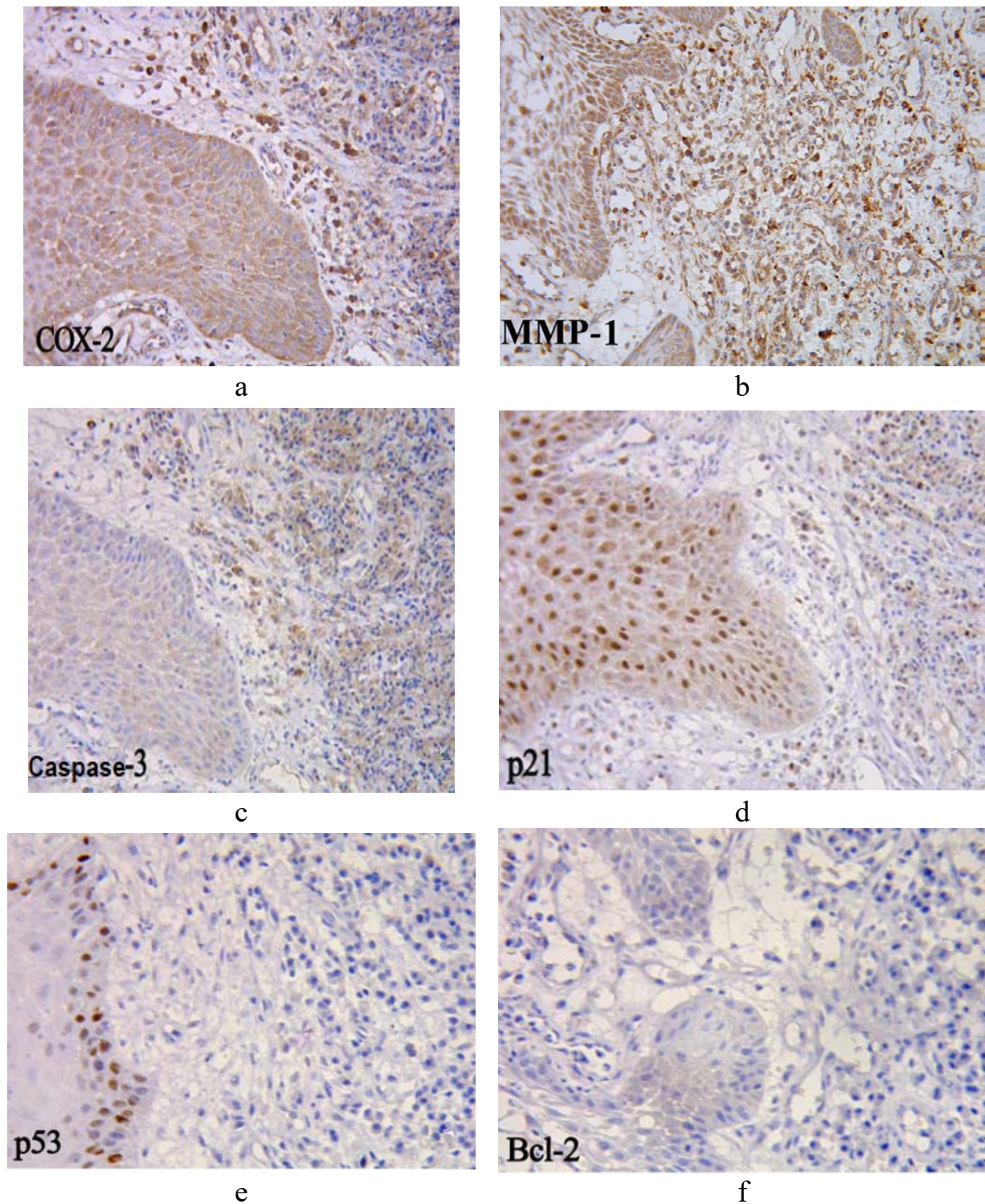


Fig. 1. Generalized periodontitis, II degree, chronic course:
a) cytoplasmic expression of COX-2 marker in the inflammatory infiltrate around the multilayer epithelium (+3);
b) mixed nuclear-cytoplasmic reaction with MMP-1 marker in inflammatory infiltrate and surrounding epithelial cells (+2);
c) cytoplasmic expression of caspase marker-3 (+1); d) mixed nuclear-cytoplasmic reaction with marker p 21 in epithelium and inflammatory infiltrate cells (+3);
e) intranuclear reaction with marker p53 exclusively in epithelial cells (+1);
f) negative reaction with Bcl-2 marker. Immunohistochemical method, additional colouring of Mayer hematoxiline (x400)

Bibliography:

1. Song B., Zhou T., Yang W. L., Liu J. & Shao L. Q. (2017). Programmed cell death in periodontitis: recent advances and future perspectives. *Oral Diseases*, 23 (5), 609–619. <https://doi:10.1111/odi.12574>
2. Listyarifah D., Al-Samadi A., Salem A., Syaify A., Salo T., Tervahartiala T., Grenier D., Nordström D. C., Sorsa T. & Ainola M. (2017). Infection and apoptosis associated with inflammation in periodontitis: An immunohistologic study. *Oral Diseases*, 23 (8), 1144–1154. <https://doi:10.1111/odi.12711>
3. Yang M. X., Ding P. H., Kang S. & Dong Y. (2021). Advances in research of pyroptosis in the mechanism of periodontal disease. *Zhonghua Kou Qiang Yi Xue Za Zhi*, 56 (9), 945–950. <https://doi:10.3760/cma.j.cn121094-20190419-00158>
4. Groeger S. & Meyle J. (2019). Oral mucosal epithelial cells. *Front Immunology*, 10, 208. <https://doi:10.3389/fimmu.2019.00208>
5. Olsen I. & Yilmaz Ö. (2019). Possible role of Porphyromonas gingivalis in orodigestive cancers. *Journal of Oral Microbiology*, 11 (1), 1563410. <https://doi:10.1080/20002297.2018.1563410>
6. Ma L., Liu H., Wang X., Jiang C., Yao S., Guo Y., Wang H., & Cao Z. (2020). CXXC5 orchestrates Stat3/Erk/Akt signaling networks to modulate P. gingivalis-elicited autophagy in cementoblasts. *Biochimica et biophysica acta. Molecular cell research*, 118923.
7. Noseykina E. M., Schepetkin I. A. & Atochin D. N. (2021). Molecular mechanisms for regulation of neutrophil apoptosis under normal and pathological conditions. *Journal of Evolutionary Biochemistry and Physiology*, 57 (3): 429–450. <https://doi:10.1134/S0022093021030017>
8. Bartold P. M., Brooks P. M., Carter C. L., Gemmell E., Marshall R. I. & Seymour G. J. (2003). COX-1 and COX-2 in human periodontal disease states, *Australian Dental Journal*. 48: 12–15.
9. Naruishi K. & Nagata T. (2018). Biological effects of interleukin-6 on Gingival Fibroblasts: Cytokine regulation in periodontitis. *Journal of Cell Physiology*. 233 (9): 6393–6400. <https://doi:10.1002/jcp.26521>
10. Bulut S., Uslu H., Ozdemir B. H. & Bulut O. E. (2006). Expression of caspase-3, p53 and Bcl-2 in generalized aggressive periodontitis. *Head Face Med*. 2: 17. <https://doi:10.1186/1746-160X-2-17>
11. Gamonal J., Sanz M., O'Connor A., Acevedo A., Suarez I., Sanz A., Martínez B. & Silva A. (2003). Delayed neutrophil apoptosis in chronic periodontitis patients. *Journal of Clinical Periodontology*, 30 (7), 616–623. <https://doi:10.1034/j.1600-051x.2003.00350>
12. Антомонов М. Ю. Математическая обработка и анализ медико-биологических данных. Киев : [б. и.], 2017. 558 с.

References:

1. Song, B., Zhou, T., Yang, W. L., Liu, J. & Shao, L. Q. (2017). Programmed cell death in periodontitis: recent advances and future perspectives. *Oral Diseases*, 23 (5), 609–619. <https://doi:10.1111/odi.12574>
2. Listyarifah, D., Al-Samadi, A., Salem, A., Syaify, A., Salo, T., Tervahartiala, T., Grenier, D., Nordström, D. C., Sorsa, T. & Ainola, M. (2017). Infection and apoptosis associated with inflammation in periodontitis: An immunohistologic study. *Oral Diseases*, 23 (8), 1144–1154. <https://doi:10.1111/odi.12711>
3. Yang, M. X., Ding, P. H., Kang, S. & Dong, Y. (2021). Advances in research of pyroptosis in the mechanism of periodontal disease. *Zhonghua Kou Qiang Yi Xue Za Zhi*, 56 (9), 945–950. <https://doi:10.3760/cma.j.cn121094-20190419-00158>
4. Groeger, S. & Meyle, J. (2019). Oral mucosal epithelial cells. *Front Immunology*, 10, 208. <https://doi:10.3389/fimmu.2019.00208>
5. Olsen, I. & Yilmaz, Ö. (2019). Possible role of Porphyromonas gingivalis in orodigestive cancers. *Journal of Oral Microbiology*, 11 (1), 1563410. <https://doi:10.1080/20002297.2018.1563410>
6. Ma, L., Liu, H., Wang, X., Jiang, C., Yao, S., Guo, Y., Wang, H., & Cao, Z. (2020). CXXC5 orchestrates Stat3/Erk/Akt signaling networks to modulate P. gingivalis-elicited autophagy in cementoblasts. *Biochimica et biophysica acta. Molecular cell research*, 118923.
7. Noseykina, E. M., Schepetkin, I. A. & Atochin, D. N. (2021). Molecular mechanisms for regulation of neutrophil apoptosis under normal and pathological conditions. *Journal of Evolutionary Biochemistry and Physiology*, 57 (3): 429–450. <https://doi:10.1134/S0022093021030017>
8. Bartold, P. M., Brooks, P. M., Carter, C. L., Gemmell, E., Marshall, R. I. & Seymour, G. J. (2003). COX-1 and COX-2 in human periodontal disease states, *Australian Dental Journal*. 48: 12–15.
9. Naruishi, K. & Nagata, T. (2018). Biological effects of interleukin-6 on Gingival Fibroblasts: Cytokine regulation in periodontitis. *Journal of Cell Physiology*. 233 (9): 6393–6400. <https://doi:10.1002/jcp.26521>
10. Bulut, S., Uslu, H., Ozdemir, B. H. & Bulut, O. E. (2006). Expression of caspase-3, p53 and Bcl-2 in generalized aggressive periodontitis. *Head Face Med*. 2: 17. <https://doi:10.1186/1746-160X-2-17>
11. Gamonal, J., Sanz, M., O'Connor, A., Acevedo, A., Suarez, I., Sanz, A., Martínez, B. & Silva, A. (2003). Delayed neutrophil apoptosis in chronic periodontitis patients. *Journal of Clinical Periodontology*, 30 (7), 616–623. <https://doi:10.1034/j.1600-051x.2003.00350>
12. Антомонов, М. Ю. (2017). *Математическая обработка и анализ медико-биологических данных*. [Mathematical processing and analysis of biomedical data]. Kyiv [in Russian].