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ORIGINAL ARTICLE

# INTERDEPENDENCE OF THE MICROBIOCENOSE COMPOSITION OF BIOPELLICLE AND THE SEVERITY DEGREE OF CHANGES IN THE MUCOSA OF THE GUMS AFTER TEN WEEKS OF EXPERIMENTAL OPIOID EXPOSURE

DOI: 10.36740/WLek202205204

**Volodymyr B. Fik[1], Ruzhena M. Matkivska[2], Yosyp M. Fedechko[3], Vasyl V. Humeniuk[1], Oksana V. Yefremova[1], Larysa Ya. Fedoniuk[4]**

[1] DANYLO HALYTSKY LVIV NATIONAL MEDICAL UNIVERSITY, LVIV, UKRAINE
[2] O.O. BOGOMOLETS NATIONAL MEDICAL UNIVERSITY, KYIV, UKRAINE
[3] ANDREI KRUPINSKI LVIV MEDICAL ACADEMY, LVIV, UKRAINE
[4] I. HORBACHEVSKY TERNOPIL NATIONAL MEDICAL UNIVERSITY, TERNOPIL, UKRAINE

## ABSTRACT

**The aim:** To study the relationship between the degree severity of changes in the tunica mucosa of the rats gums and the composition of the microbiota of tooth surface in the gingival margin under the ten-week action of the opioid.

**Materials and methods:** The study was performed on 34 male rats, weighing 160 - 255 g, aged 4.5 - 7 months. Animals were administered nalbuphine for 10 weeks, gradually increasing the dose (0.212 - 0.283 mg/kg). The research of the bacterial from the microbiotope of tooth surface in the gingival margin of rats was performed using statistical analysis of quantitative results.

**Results:** The 4 stages of development of the pathological process and changes in the qualitative and quantitative composition of the microbiocenosis in the gingival margin of the oral mucosa are determined. Depending on the severity of pathological changes in the mucous membrane of the gums of rats under the long-term action of the opioid, a significant increase in the quantitative indicators of bacterial species of pathogenic and opportunistic microbiota at degree IV of the process compared with degree I. The appearance of potential pathogens of purulent-inflammatory processes - Klebsiella and Pseudomonas aeruginosa was noted.

**Conclusions:** The relationship between the quantitative and qualitative characteristics of the microbiocenosis of tooth surface in the gingival margin and the severity of changes in the mucous membrane of the of rats gums under the ten-week action of the opioid was determined, indicating bacterial associations of dental biopellicle as an etiological factor.

**KEY WORDS:** microbiota, opioid, rats, mucosa, gums, degree of severity of the process

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## INTRODUCTION

Over the last decade, there has been an increase in illegal drug use and uncontrolled opioid abuse in patients with chronic pain, which is associated with unintentional trauma and is a major risk factor for tolerance and withdrawal, leading to overdose and death [1-5]. The attention of scientists in various fields of medicine is focused on the study of changes in organs and systems under the influence of drugs, in particular, both in clinical and experimental areas [6-9]. In clinical studies it is indicated that when exposed to opioids there are signs of immunosuppression, which cause an increased risk of infectious diseases and the development of inflammation [5, 10, 11]. Toxic effects of drugs are manifested in all organs and systems, which may have an indirect or direct effect on the organs of the oral cavity [6, 12-15].

The question of the role of bacterial flora in the etiology and initiation of periodontal disease is certainly actively studied as the improvement of microbiological methods and the accumulation of research results [16-19]. Today, one of the main hypotheses remains that dental plaque microorganisms are a determining factor in the development and progression of the inflammatory process in the periodontium, which provoke the inflammatory process and directly affect the microbial status of the oral cavity [20-23]. There is also evidence that the role of microorganisms in the development of periodontitis is unclear, although some bacterial pathogens alone or as part of microbial groups may be particularly important [24].

Therefore, in order to prevent the development of periodontal disease and the occurrence of infectious foci in the oral cavity caused by bacterial biopellicle, it is important to determine the etiology and pathogenesis of this pathology in experimental animal models in order to further extrapolate these data to the clinic [25]. However, the relationship between the species and quantitative composition of the

microbiota of tooth surface in the gingival margin and the development and progression of inflammation in the gingival mucosa under action of the opioid are controversial and needs further study using modern methods of microbiological research in the experiment.

## THE AIM

To investigate the relationship between the severity degree of pathomorphological changes in the mucous membrane of the gums and the composition of the microbiocenosis of the tooth surface in the gingival margin after ten weeks of action of the opioid in the experiment.

## MATERIALS AND METHODS

The study was performed on 28 white mature nonlinear male rats, weighing 160 - 255 g, aged 4.5 - 7.0 months. In the experiment, the animals were divided into 2 main groups. Group I: intact rats (10). Group II consisted of 18 animals, which was administered an opioid analgesic for ten weeks. Animals were administered the opioid analgesic Nalbuphine with the active substance nalbuphine hydrochloride (dilution of 1 ml of nalbuphine hydrochloride in 50 ml of 0.9% sodium chloride solution) intramuscularly, daily, once, at one time interval for 10 weeks, with a gradual increase in dose every 2 weeks: for 1-2 weeks - 0,212 mg / kg, 3-4 weeks - 0,225 mg / kg, 5-6 weeks - 0,252 mg / kg, 7-8 weeks - 0,260 mg / kg, 9-10 weeks - 0,283 mg / kg.

Microbiological studies included bacterioscopic and bacteriological methods to study the features of the microbiocenosis of the oral cavity of intact rats and rats under the influence of opioid analgesics. To conduct microbiological studies, smears were selected to study the bacterial from the mikrobiotope of the tooth surface in the area of the gingival margin, namely at the site of biopellicle formation. In bacterioscopic studies, smear material was applied to a glass slide, fixed over a burner flame, and Gram stained. In order to obtain the level of microbial colonization was carried out by sampling with a calibrated loop of primary material (0.02 ml) and transferred to a test tube with isotonic solution (1 ml). This procedure was repeated 5 times from this mikrobiotope. In 0.2 ml of saline contained the number of microorganisms that were introduced in one calibrated loop - 0.02 ml. In the laboratory, 0.2 ml of material was taken from a test tube and inoculated into 5 Petri dishes with a dense nutrient medium. Accordingly, when counting colonies, we obtained a quantitative indicator in colony-forming units (1 colony - 1 CFU). Conventional special, differential diagnostic and selective media were used: meat-peptone agar, blood agar, Endo medium, Saburo medium, yolk-salt agar, mannitol salt agar. After 24 - 48 hours the number of colonies was counted. Cultivation was performed under normal atmospheric conditions, as well as at elevated $CO_2$ levels. After counting, the characteristic isolated colonies were subcultured on inclined agar to obtain pure cultures. To create the necessary gas composition for anaerobes, the cups were placed in a microanaerostat from Bio Merieux with packages and cultured for 48 hours. at t 37 ° C, after that the number of colonies was counted.

The obtained data, for further statistical analysis, were tested for normality by calculating the coefficients of asymmetry and excess, using the Shapiro-Wilk test ($p < 0.05$). The central trend for all data was presented as M ± SD (mean ± standard deviation). To determine the significance of the difference between three or more groups of animals, ANOVA analysis of variance with the post-Hawk Tukey test was used for further pairwise comparison. If the distribution of data, according to the results of the test, differed from normal, then to establish the reliability of the difference between groups used nonparametric criteria - U-Mann-Whitney test to compare two groups and H Kraskel-Wallis for three or more independent groups followed by post-hawk analysis using Dunn's test for pairwise comparison. All statistical calculations were performed using RStudio v. 1.1.442 and R Commander v.2.4-4. Animals were kept in standard vivarium conditions and experiments were performed in accordance with international ethical principles approved by the General Assembly of the World Medical Association "About the Humane Treatment of Animals" (2000), and according to the decision of the Commission on Bioethics of Danylo Halytskyy Lviv National Medical University (protocol №5 of 24.05.2021).

## RESULTS

Bacterioscopic examination of smears from the surface of the teeth in the gingival margin of intact rats noted the same type of microbiota, which mainly detected gram-positive microorganisms Leptothrix. Cellular elements were 2-3 epitheliocytes and 3-5 leukocytes in the field of view. Bacteriological analysis of the microbiota indicated a predominance of gram-positive species, in particular non-hemolytic streptococci (45.11 ± 5.16 CFU/ml) and opportunistic species such as α-hemolytic streptococci (65.44 ± 4.36 CFU/ml), coagulase-negative staphylococci (10.33 ± 3.67 CFU/ml), enterococci (18.33 ± 2.96 CFU/ml) and gram-negative microorganisms - enterobacteria, group Escherichia coli (7.00 ± 1.50 CFU/ml).

In order to assess the etiological role of individual representatives of the microbiocenosis in the development of pathological processes in the mucous membrane of the gums, we studied the qualitative and quantitative composition of tooth surface microbiota in the gingival margin of rats after 10 weeks of experimental action of the opioid. To achieve this goal, the experimental animals were regrouped according to the severity of the changes that were visualized in the gingival margin of the oral mucosa. We have identified 4 main stages of the process: I degree - hyperemia, edema, erosion of the mucous membrane of the gums (11.1%); II degree - edema, erosions, single ulcers of the mucous membrane of the gums, the initial signs of violation of the integrity of the bottom of the gingival sulcus (16.7%); III degree - cyanotic mucosa, ulcers, recession of the gums, deepening of the gingival sulcus (27.8%); Degree IV - cyanosis and purulent-necrotic changes of the gingival mucosa, progressive deepening of the gingival sulcus (44.4%).

**Table I.** Qualitative and quantitative composition of the microbiota of tooth surface in the gingival margin of rats, depending on the severity degree of the pathological process at the end of the tenth week of opioid exposure (CFU/ml).

| Bacterial groups | The severity degree of the pathological process | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| α-hemolytic streptococci | 52.33±6.40 | 56.33±8.83 | 61.56±11.09 | 61.33±11.22 |
| Coagulase-negative staphylococci | 56.33±7.35 | 65.33±8.50 | 68.44±7.76* | 72.33±8.46* |
| Escherichia coli | 23.22±2.77 | 24.33±5.98 | 26.22±8.18 | 34.44±11.53* |
| Hemolytic Escherichia coli | 1-2 | 1-2 | 1-2 | 1-2 |
| β-hemolytic streptococci | 21.22±2.77 | 39.22±6.72* | 43.22±6.28* | 48.33±7.04* |
| Coagulaze-positive staphylococci | 9.11±3.86 | 13.33±3.74 | 21.33±5.39* | 35.44±3.94*, ** |
| Klebsiella | - | - | 1-2 | 9.22±2.05 |
| Pseudomonas aeruginosa | 1-2 | 1-2 | 4.56±2.65 | 8.11±1.83*** |
| Yeast-like fungi | 4.89±1.54 | 10.33±2.12* | 5.11±1.05** | 5.11±1.27*** |

Notes: data are presented in the form of M ± SD, where M is the average value, SD is the standard deviation; * $p<0,05$ – significant difference in values relative to the degree I, ** $p<0,05$ – significant difference in values relative to the degree II, *** $p<0,05$ – significant difference in values relative to the degree III.

Bacterioscopic examination of smears in animals with degree I revealed a moderate number of leukocytes in the field of view, as well as epitheliocytes, on the surface of which visualized the adsorption of cocci. Degree II animals showed a significant increase in white blood cell count and degeneratively altered epitheliocytes. In degree III, dense accumulations of coccal microbiota, mononuclear cells, erythrocyte autophagocytosis, and gingival epithelial cell degeneration were observed. Destroyed epitheliocytes, segmental leukocytes, erythrocyte autophagocytosis, as well as aggregation of gram-negative bacteria and gram-positive coccal microbiota were visualized in animals with degree IV smears.

In bacteriological studies, attention was paid to microbial pathogens of purulent-inflammatory process, in particular, coagulase-positive staphylococci and β-hemolytic streptococci, because coagulase-positive staphylococci are characterized by the production of toxins with necrotic and hemolytic action (hemolysins, lecithinase), and β-hemolytic streptococci produce hemolysin and protein M, which is associated with antiphagocytic and cytotoxic effects on neutrophils. In particular, the number of coagulase-positive staphylococci in degree III significantly increased 2.3 times (21.33 ± 5.39 CFU/ml), and in degree IV - 3.9 times (35.44 ± 3.94 CFU/ml), when compared with the I degree of the process ($p<0,05$). The amount of β-hemolytic streptococci significantly increased in degree II - 1.8 times (39.22 ± 6.72 CFU/ml), in degree III of the process - 2.0 times (43.22 ± 6.28 CFU/ml) and at degree IV significantly increased - 2.3 times (48.33 ± 7.04 CFU/ml), compared with degree I of the process in animals with long-term opioid action ($p<0,05$) (Table I). Such data can be regarded as evidence of a probable etiological factor, namely, these microorganisms in the development and progression of the inflammatory process in the constituent components of the gums.

According to the deepening of pathological changes in the mucous membrane of the gums of rats, the dynamics of growth in the quantitative composition of opportunistic species of microorganisms was also noted (Fig. 1). Among the bacteria we isolated, coagulase-negative staphylococci were found to predominate, which do not produce the enzyme plasmacoagulase and are part of common normobiocenoses, in particular, bacterial species Staphylococcus edermidis, Staphylococcus saprophyticus and others. Thus, the amount of Coagulase-negative staphylococci in degree III significantly increased 1.2 times (68.44 ± 7.76 CFU/ml), and in degree IV - 1.3 times (72.33 ± 8.46 CFU/ml) compared with the I degree of the process ($p<0,05$). At the same time, the amount of Escherichia coli (34.44 ± 11.53 CFU/ml) significantly increased 1.5 times in degree IV, compared with degree I ($p<0,05$).

Due to the fact that the number of α-hemolytic streptococci did not change significantly, however, there was a moderate tendency to increase in III (61.56 ± 11.09 CFU/ml) and IV (61.33 ± 11.22 CFU/ml) degree of development of the process, compared with degree I (52.33 ± 6.40 CFU/ml). However, the obtained data on the quantitative composition of α-hemolytic streptococci showed a slow and dynamic increase in the number of microorganisms, that are part of the biopellicle with odontopathogenic properties, in particular, the species Streptococcus mutans. The quantitative composition of yeast-like fungi, where the species Candida albicans predominated, had stable indicators, except for rats with degree II severity of the process, where their number increased significantly 2.1 times, compared with other degrees of severity of the process ($p<0,05$).

There was also a significant increase in the number of colonies of Pseudomonas aeruginosa by 1.8 times in degree IV, compared with degree III ($p<0,05$). It should be noted that Pseudomonas aeruginosa particularly affects the body with a weakened immune system, produces a number of toxins and enzymes that are aggressive factors in the development of sepsis and purulent-inflammatory complications. There was also a significant increase in the quantitative composition of Klebsiella (9.22 ± 2.05 CFU/

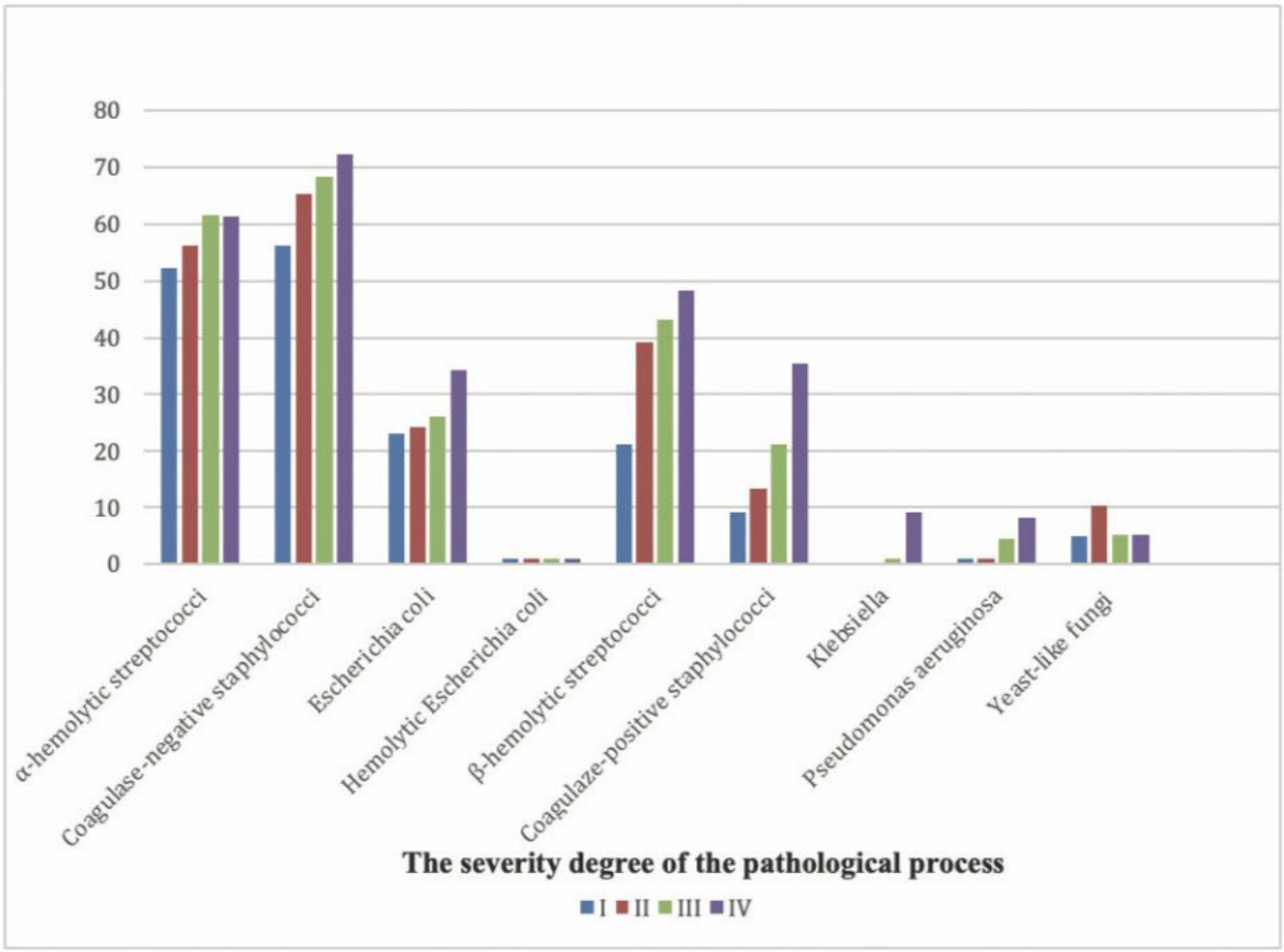


**Fig. 1.** Dynamics of quantitative indicators of microbial groups at different degrees of severity of the pathological process in the gingival margin under the action of opioids for 10 weeks (CFU/ml).

ml) at degree IV of the process, compared with degree III (1-2 colonies) and not sown in animals at degree I and II of the process. A significant increase in the number of Klebsiella colonies in degree IV inflammation indicated the development of dysbiosis involving these capsular bacteria, which have adhesive and antiphagocytic properties, which can provoke the formation of purulent foci and sepsis in the longer term opioid exposure. The number of single colonies of Hemolytic Escherichia coli with pathogenic properties was insignificant (1-2 colonies), however, their presence at all stages of the process indicated the stability of dysbiotic changes in the oral cavity of rats under the ten-week action of the opioid.

## DISCUSSION

Our results of microbiological studies with experimental opioid exposure can be compared with the results of studies obtained in patients at different stages of periodontitis, where an important role, according to researchers, is played by microbial associations in the deep gingival sulcus, which cause immunoinflammatory reactions that affects the severity of the disease [16, 19]. Besides, the ecological diversity of the periodontal environment may create certain conditions for the colonization of bacterial species, which are usually not considered resident microbiota of the oral cavity and biopellicle are a source of development and spread of systemic infections due to different groups of pathogenic microorganisms [17, 24-26].

However, without clear evidence of the etiotropicity of a particular microorganism to the development of pathological changes in the components of the periodontium, we can assume that only the "main" microbial pathogens cause progression of inflammation in the periodontium, in particular mucous membrane of the gums and oral cavity in general. This is confirmed by the fact that anaerobic bacteria may be one of the main etiological factors of periodontal disease, however, the ecology of these pathogens in the development of the pathological process is inextricably linked with other destabilizing microorganisms of the oral cavity, including Staphylococcus aureus, Staphylococcus epidermidis and Streptococcus sanguis [17-19].

It should also be noted that the microbial spectrum in the oral mucosa of addicted patients is reflected in the qualitative diversity, where a number of researchers have noted the

predominance of coccal, anaerobic, fungal microbiota and Klebsiella [12, 14, 27]. All these bacterial groups were also detected at 10 weeks of opioid action, which indicated the etiological role of these microorganisms in the progression of the inflammatory process in the paradentium. Our research suggests that there is a relationship between several microbial associations and pathomorphological changes in the paradentium area, based on which we identified 4 degrees of pathological process in the mucous membrane of the gums of rats under the ten-week action of the opioid.

## CONCLUSIONS

The relationship between quantitative and qualitative characteristics of the microbiocenosis of the tooth surface in the gingival margin and the severity of changes in the mucous membrane of rat gums after the ten-week action of the opioid was determined and this fact indicates bacterial associations of dental biopellicle as an etiological factor in the development of the inflammatory process and reflected the state of the oral microbiome in general.

Depending on the severity of the pathological process in the mucous membrane of the gums of rats with long-term action of opioids, a significant increase in the quantitative indicators of bacterial pathogenic (β-hemolytic streptococci, coagulase-positive staphylococci) and opportunistic pathogens microbiota (coagulase-negative staphylococci, Escherichia colli) at IV degree of development of process in comparison with I degree was determined. The appearance of potential pathogens - Klebsiella and Pseudomonas aeruginosa at degree IV of the process indicated profound dysbiotic changes, which in combination with other microbial associations indicated a dynamic progression of purulent-inflammatory process in the mucous membrane of the gums.

The identified changes in the qualitative and quantitative composition of microbiota of biopellicle reflect the etiopathogenesis of inflammatory diseases of the oral cavity and may serve as criteria for differential diagnosis of periodontitis in opioid-dependent individuals.

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**ORCID and contributionship:**
*Volodymyr B. Fik: 0000-0002-2284-4488* [A,B,D]
*Ruzhena M. Matkivska: 0000-0001-8260-9685* [A,C]
*Yosyp M. Fedechko: 0000-0002-2682-2729* [C,F]
*Vasyl V. Humeniuk: 0000-0003-2736-3875* [E,F]
*Oksana V. Yefremova: 0000-0002-5149-2151* [E,F]
*Larysa Ya. Fedoniuk: 0000-0003-4910-6888* [E,F]

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**CORRESPONDING AUTHOR**
**Volodymyr B. Fik**
Danylo Halytskyy Lviv National Medical University
69 Pekarska St., 79010 Lviv, Ukraine
tel: +38 0672534321
e-mail: fikvolodymyr@ukr.net

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