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EFFECT OF N-CHLOROTAURINE ON BIOFILMS OF PATHOGENS ASSOCIATED WITH COMPLICATIONS OF DENTAL IMPLANTATION

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Dental implants can be colonized by bacteria, leading to implant-related infections. N-chlorotaurine (NCT) is a promising remedy against biofilms. The present study aimed to investigate in vitro activity of NCT against long-term biofilms on titanium material used for implants. We studied the activity of 1% NCT against isolates obtained from patients with dental implantation failure. The study of biofilm-forming properties was performed in vitro on titanium disks, incubation was up to 8 weeks. To study the immediate effect of NCT on a biofilm, the XTT-reductase test was applied. The studied 1%-solution of NCT has demonstrated considerable bactericidal activity against all test bacteria already after 30 min of the study initiation. The best results were achieved for gram-positive bacteria, especially for Streptococcus spp. Results obtained in experiments with gram-negative bacteria also were promising. But microbial load below the detection limit was achieved later in comparison to gram-positive bacteria (p<0.01). On all weeks, after 30-min exposure, the bacterial load comprised 5.2 (0.3) log10 CFU/ml for Enterobacteriaceae and 5.2 (0.2) log10 CFU/ml for Acinetobacter spp. But after an additional 30 min of cultivation, the microbial load fell below the detection limit (p<0.01). The studied concentration of NCT also significantly reduced metabolism in microbial cells. High values for most of the studied isolates have been already achieved within the first 30 min of incubation. Subsequent incubation also promoted metabolic turnover; in comparison to 30 min after exposure, prolonged incubation within 60 min led to up to 85.0% reduction of metabolic activity (p<0.05). However, 3h exposure didn`t bring any statistically significant results. NCT in a millimolar concentration can efficiently limit the vital activity of microorganisms on biofilms produced in vitro.

Key words: *biofilms, N-chlorotaurine, microorganisms, bactericidal activity, bacteria*

Connection of the publication with planned research works.

The work was carried out as part of the research work of the department of microbiology, virology, immunology, epidemiology and medico-biological physics and informatics and the department of biochemistry and medical chemistry of the Dnipro State Medical University, the title «Multifunctional polymer materials with powerful antimicrobial properties for antiseptic treatment of wound surfaces, air and water disinfection» (state registration number 0120U101548).

Introduction.

Dental implants are widely used in modern medical practice to replace missing teeth because of their stability and comfort characteristics [1]. However, they can be colonized by bacteria, leading to implant-related infections. These germs can form biofilms, an organized and long-lived community impregnable for mechanical cleaning, antimicrobial compounds, and immune factors. Peri-implant infection may be a reason for implantation failure [1, 2, 3]. The application of anti-adhesive strategies for titanium implant surfaces is critical for resisting microbial adherence. Based on the results of a recent meta-analysis, it can be concluded that there is evidence in favour of systematic antibiotic use in patients who underwent dental implants since such an approach significantly reduces the risk of implant failure. Simultaneously, antibiotic use does not exert a significant preventive effect against postoperative infection [3, 4]. Furthermore, it may provide selective pressure on antimicrobial resistance. Therefore, the use of alternative strategies to control microbial growth could be beneficial for the prevention and treatment of peri-implant infection [5, 6].

A remedy against biofilms resulting from implant extraction is highly desirable. N-chlorotaurine (NCT) is a substance of special interest [5, 7]. NCT is an N-chloro derivative of the amino acid taurine and is a long-lived oxidant produced by activated human granulocytes and monocytes. It has anti-inflammatory properties and is involved in the termination of inflammation. The successful synthesis and possibilities of the long-termed storage of the crystalline sodium salt facilitated its development as an endogenous antiseptic. Furthermore, NCT has microbicidal activity against a wide range of microorganisms. Except for direct killing activity, after sublethal incubation, the compound also possesses a post-antibiotic effect and leads to loss of virulence in different pathogens, including yeasts. NCT was proved to be well tolerated by human tissue in clinical studies with instillation into the eyes, outer ear canal, nasal and paranasal sinuses, oral cavity and urinary bladder and skin ulcerations [7, 8]. Lackner et al. (2022) also demonstrated broad virucidal activity of NCT at pH 7.0 against viruses causing acute respiratory tract infections, including SARS-CoV-2, influenza viruses, and respiratory syncytial virus. Moreover, it was suggested that NCT participates in downregulation of chemokines and proinflammatory cytokines, including interleukins like IL-6, and therefore a have a good potential in control of cytokine storm in SARS-CoV-2 [9].

Lorenz K. et al (2007) studied the activity of NCT on oral plaque regrowth and vitality using a local application (mouth rinses). They found that 2% and 3% solutions of NCT significantly reduce the vitality of plaque bacteria. However, discolouration of the tongue and an unpleasant taste were recorded in participants [10]. In a more recent study, Arnitz R et al. (2018) showed that a 1% solution of NCT is tolerated better [11].

In chronic pathology, persisting biofilms appear to be associated with antibiotic resistance and poor clinical outcomes. The increasing resistance to antiseptic products (hypochlorite, chlorhexidine, and iodine) of biofilms grown for up to 3 weeks with no further change of susceptibility up to 8 weeks was demonstrated [5, 12]. Knowledge about the activity of NCT against long-term biofilms is growing and lower concentrations are of interest nowadays.

The aim of the study.

The present study was conducted to investigate *in vitro* activity of NCT against long-term biofilms on titanium material used for implants in dentistry.

Object and research methods.

N-chlorotaurine. The study used a buffer solution of NCT with a final concentration of 1%, pH 7.1. The concentration was chosen due to the well-described clinical tolerability by different routes of administration (inhalation and skin) and good microbicidal activity against several pathogens associated with the pathology of the respiratory system and skin infections [5, 7].

Tested isolates. For the current study of the effect of NCT on a biofilm, clinical isolates of *Streptococcus spp.,* coagulase-negative *Staphylococcus spp.* (CoN)*, Staphylococcus aureus, Klebsiella sp., Acinetobacter sp.* and *Candida sp.*, associated with complications of dental implantation, were selected.

Biofilm formation. Titanium disks (surface area 109 mm^2) were soaked in chlor-containing sterilant according to manufacturer recommendations, gently washed with sterile saline solution and then autoclaved. Sterile disks aseptically were placed into a 6-well plate containing 2 ml of tryptic soy broth (TSB) inoculated with 0.1 ml aliquots of 2×10^5 CFU/ml of a corresponding microbial strain. The tubes were incubated at 37°C for up to 8 weeks with daily shaking. TSB was exchanged every 72 hours. The study of the antimicrobial properties of the studied isolates was performed one time per week.

After each interval, the extracted disks were immersed into sterile TSB and sonicated for 5 min in an ultrasound water bath to secede the remaining live bacteria from the disk. Subsequently, 10-µl aliquots of these solutions were spread on Mueller-Hinton agar (MHA) or MHA + 5% defibrinated horse blood + 20 mg/L β-NAD (MH-F) plates, undiluted and after 10-fold and 100-fold dilution in saline, an established detection limit was at 100 CFU/ml. The plates were incubated at 37°C, and CFU was counted after 72 h.

In the following experiments, the biofilm-covered disks were rinsed in the sterile saline solution and then immersed in wells with 1% NCT solution and incubated at 37°C for 30 min, 1 h, or 3 h. Subsequently, 10-µl aliquots of these solutions were spread on MHA, undiluted and after 10-fold and 100-fold dilution in saline.

Negative controls in phosphate-buffered saline (PBS) were performed in parallel.

Metabolic activity of the tested cultures. To investigate the impact of NCT on microbial metabolism within biofilms, the XTT assay was performed. The study measured the reduction of 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxamide to the orange formazan product. Biofilms were grown at 37°C in polystyrene 96-well plates and then washed with PBS. Further, the biofilms were treated at 37°C with 1% of NCT for 30 min, 1 h, or 3 h. Subsequently, they were washed three times with PBS, XTT was added, and the plates were incubated at 37°C in the dark for 2 h. The supernatants were transferred to new 96-well plates, and the absorbance was measured at 550 nm. All values were calculated in comparison to a negative control (PBS) [5].

Statistics. The experiments were performed in 3 independent repetitions for each part. The results are presented as mean values and standard deviation – M (SD). Student's unpaired t test or one-way analysis of variance (ANOVA) followed by Dunnett's and Tukey's multiple comparison tests were carried out for the comparison of test samples with controls and for different incubation times, respectively. The level of significance was estimated as $p<0.05$ [13].

Research results and their discussion.

Biofilm killing activity of NCT against clinical isolates associated with complications of dental implantation. The microbial load in untreated biofilms reached 8.3 CFU/mL. The studied 1%-solution of NCT has demonstrated considerable bactericidal activity against all test bacteria already after 30 min of the study initiation.

The best results were achieved for gram-positive bacteria. After the initial exposure, the growth of *Streptococcus spp.* was below the detection limit in all weeks of the study. There were several isolates of *Staphylococcus spp.,* including *S. aureus*, which retained a vital activity and gave growth within the first weeks of the study. However, for mentioned isolates, the desirable effect was achieved after following 30 minutes of cultivation. The obtained data is demonstrated in **fig. 1.**

Results obtained in experiments with gram-negative bacteria also were promising. But microbial load below the detection limit was achieved later in comparison to gram-positive bacteria (*p<0.01*). On all weeks, after 30-min exposure, the bacterial load comprised 5.2 (0.3) $\log_{10}$ CFU/ml for *Enterobacteriaceae* and 5.2 (0.2) log CFU/ml for *Acinetobacter spp.* But after an additional 30 min of cultivation, the microbial load fell below the detection limit (*p<0.01*). The obtained data is demonstrated in **fig. 2.**

The results show that prolonged cultivation didn`t add clearance benefits. There was no positive growth after already after 60 minutes after the study onset.

The impact of 1% NCT on fungi of the genus *Candida* also wasn`t changing sufficiently during all week of the study. However, the required time of exposure comprised about 3 hours.

Metabolic activity of the tested cultures. The metabolic failure after incubation in 1% NCT within 30 min, 1h and 3h was recorded by XTT assay. The XTT reductase test is used for the evaluation of living cells number in biofilms. XTT is a type of tetrazolium salts, the solutions of which are transparent and non-toxic to cells. When metabolic activators are added, viable cells convert XTT to formazan, which has a well-recorded colour. The

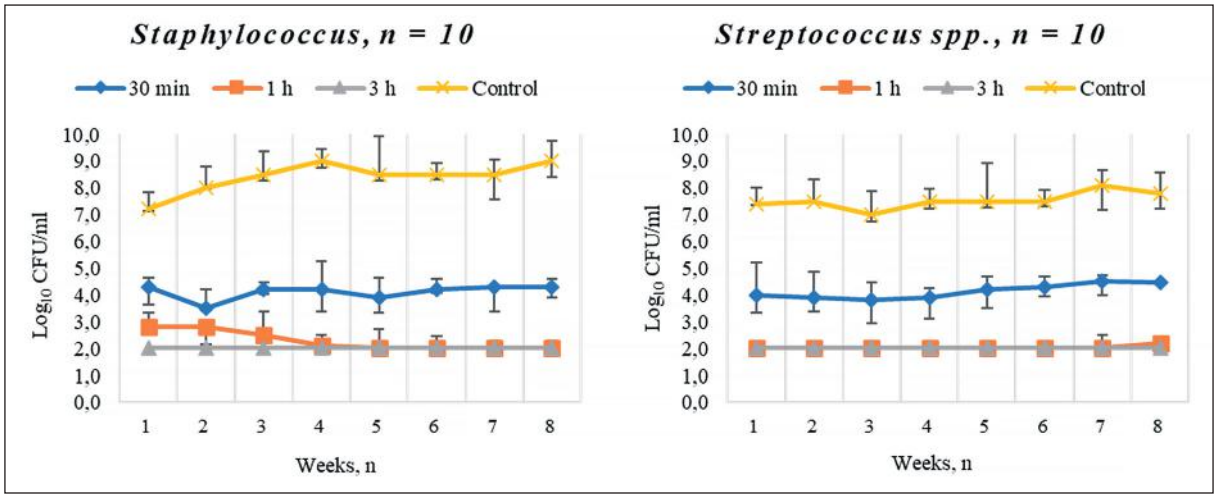

Figure 1 – Bactericidal activity of 1% NCT at pH 7.0 and 37°C against biofilms of gram-positive clinical isolates grown for 8 weeks. The vital activity was studied weekly by quantitative cultural assays. M (SD) log_{10} CFU/mL; *p<0.001* for all values of 30 min, 1h and 3h NCT and versus control; *p>0.05* for hebdomadal changes in microbial load.

method is quite simple and has a high throughput, does not violate the integrity of cells, and gives numerical values [14].

The studied concentration of NCT significantly reduced metabolism in microbial cells. High values for most of the studied isolates have been already achieved within the first 30 min of incubation. Subsequent incubation also promoted metabolic turnover; in comparison to 30 min after exposure, prolonged incubation within 60 min led to up to 85.0% reduction of metabolic activity (*p<0.05*). However, 3h exposure didn`t bring any statistically significant results. The obtained data is demonstrated in **fig. 3.**

In our study, we cultivated the long-term biofilm on titanium disks incubated with TSB containing clinical isolates of bacteria associated with dental implantation failure. As was shown before, titanium discs are a useful model for in vitro testing of the bactericidal activity of active chlorine compounds, particularly, different concentrations of NCT. The chosen metal alloys are major components of dental implants [15].

It was shown that low concentrations of NCT have good bactericidal activity. Nagl M. et al. (2000) showed that 200 to 500 µM of NCT killed *Escherichia coli* after 2 h of incubation at pH 6.6 and 37°C [16]. Kusminyh S. et al. (2021) showed the potentiated activity of 1% NCT in an acidic environment against pathogens associated with secondary peritonitis [17]. The researcher also noticed the postantibiotic effect of the compound [16, 17]. Further studies demonstrated the potentiated activity of NCT after shorter exposure. Anich C. et al (2021) showed the bactericidal activity of NCT against multidrug resistant (MDR) gram-positive and gram-negative clinical isolates. 1% NCT (55 mmol) reduced the burden of methicillin-resistant *S. aureus*, linezolid-resistant *Staphylococcus epidermidis*, vancomycin-resistant, and linezolid- and vancomycin-resistant *Enterococcus faecium*, MDR *E. coli*, *Pseudomonas aeruginosa*, *A. baumannii* and *K. pneumoniae* by at least 2 log_{10} steps after 15 min of exposure and completely or nearly to the detection limit after 30 min at pH 7.1 and 37°C [18].

Débora C. Coraça-Huber et al. (2014) in studies with millimolar concentrations found a time – and concen-

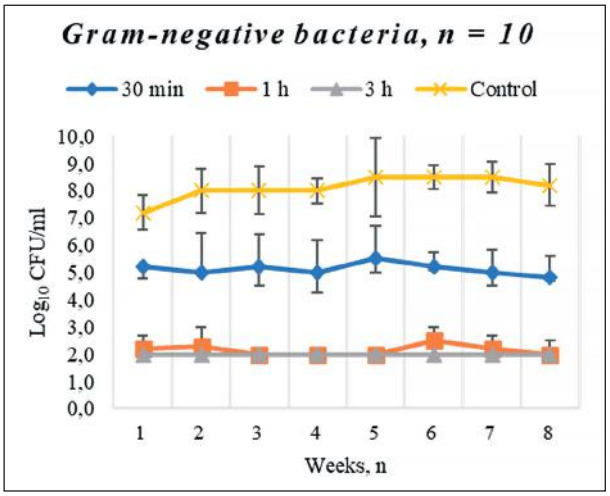

Figure 2 – Bactericidal activity of 1% NCT at pH 7 and 37°C against biofilms of gram-negative clinical isolates (*K. pneumoniae, A. baumannii*) grown for 8 weeks. The vital activity was studied weekly by quantitative cultural assays. M (SD) log_{10} CFU/mL; *p<0.001* for all values of 30 min, 1h and 3h NCT and versus control; *p>0.05* for hebdomadal changes in microbial load.

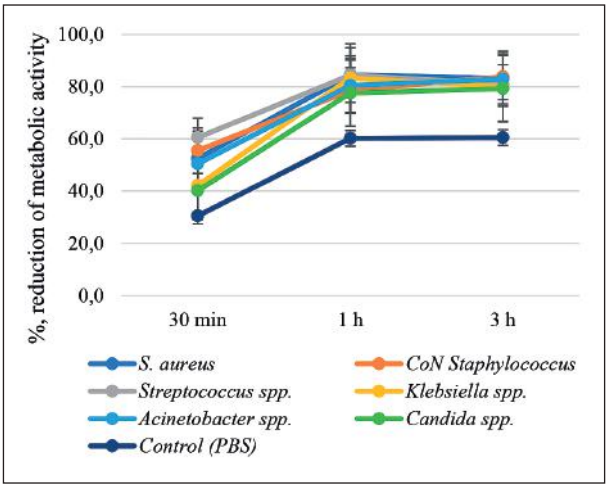

Figure 3 – The XTT reductase test is used for the study of metabolic turnover in formed biofilms. M (SD), %; *p <0.01* for time points 30 minutes and 1 hour; *p<0.01* in all time points for studied isolates and control; p>0.05 for time points 1 hour and 3 hours.

tration-dependent antibiofilm activity at concentrations between 0.1% and 1% (5.5 to 55 mM). The bactericidal activity against *S. aureus* and *S. epidermidis* in biofilm appeared approximately 3 to 6 times slower than that against planktonic bacteria. Remarkably, they demonstrated viable counts of all tested bacteria in the biofilm were reduced by at least 1 power of 10 after 15 min of incubation in 1% NCT [5]. In our study, we also evaluated the impact of 1% concentration on long-term biofilm and achieved the desired results. The 30-min exposure provided significant elimination of the microbial load (in comparison to the control), however, the prolonged cultivation (up to 60 min) was required for the complete elimination of bacterial burden.

In a recent study, Grimus V et al. (2021) demonstrated the activity of NCT against long-term biofilms of bacteria and yeasts. Monospecies biofilms of *S. aureus*, *P. aeruginosa*, and *Klebsiella variicola* grew to approximately 1×10^6 CFU/mL and *C. albicans* to 1×10^5 CFU/mL. In the study, viable counts of bacteria were reduced by 2.8 to 5.6 log10 in 1% NCT after 60 min (0.9 to 4.7 $\log_{10}$ after 30 min) with gram-negative bacteria being more susceptible than *S. aureus*. A significant reduction of *C. albicans* by 2.0 to 2.9 $\log_{10}$ occurred after 4 h incubation [19]. In our study, the effectiveness of microbial killing in 1% NCT was commensurable with their time points. However, clinical isolates of gram-positive bacteria were more susceptible, especially in the case of *Streptococcus spp.*

A comparison of quantitative killing assays with XTT-reductase tests showed the expected result. The microbial metabolism was affected within minutes after the beginning of exposure and therefore earlier than the loss of vital activity. The same findings were obtained in the study mentioned above [5].

Conclusions.

NCT in a millimolar concentration can efficiently limit the vital activity of microorganisms on biofilms produced in vitro. Therefore, the compound looks like a promising candidate for studies in clinical settings to control microbial colonization and biofilm formation in dental implants.

Prospects for further research.

A study of the bactericidal properties of lower concentrations of NCT on polymicrobial biofilms associated with dental implantation complications could open new prospects for the compound. Studies in vivo with local applications of NCT also are of interest.

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ВПЛИВ N-ХЛОРТАУРИНУ НА БІОПЛІВКИ ПАТОГЕНІВ, АСОЦІЙОВАНИХ З УСКЛАДНЕННЯМИ ДЕНТАЛЬНОЇ ІМПЛАНТАЦІЇ

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Резюме. Дентальні імплантати широко використовуються в сучасній медичній практиці для заміни втрачених зубів, є стабільними та комфортними у використанні. Однак вони можуть бути колонізовані бактері-

ями, що призводить до появи пери-імплантатних інфекцій. N-хлоротаурин (NCT) є перспективним засобом проти біоплівок.

Метою цього дослідження було дослідити *in vitro* активність NCT проти довготривалих біоплівок на титановому матеріалі, який використовується для імплантатів у стоматології.

Об'єкт і методи дослідження. Ми вивчали активність 1% NCT щодо ізолятів, отриманих від пацієнтів з невдачею дентальної імплантації. Дослідження біоплівко-утворювальних властивостей проводили in vitro на титанових дисках, інкубація до 8 тижнів. Після кожного інтервалу часу диски виймали з бульйону та обробляли 1% 1 NCT протягом 30 хвилин, 1 години та 3 годин. Для вивчення впливу NCT на біоплівку застосовували XTT-редуктазний тест.

Результати досліджень. Досліджуваний 1% розчин NCT виявляв значну бактерицидну активність відносно всіх досліджуваних ізолятів бактерій вже через 30 хв від початку дослідження. Найкращі результати були досягнуті для грампозитивних бактерій, особливо для *Streptococcus spp.* Було декілька ізолятів *Staphylococcus spp.,* у тому *числі Staphylococcus aureus,* які зберігали життєдіяльність і давали ріст протягом перших тижнів дослідження. Однак для згаданих ізолятів бажаний ефект досягався після 30 хвилин культивування. Результати, отримані в експериментах з грам-негативними бактеріями, також були багатообіцяючими. Але мікробне навантаження нижче межі виявлення було досягнуто пізніше порівняно з грам-позитивними бактеріями (*p<0,01*). Протягом усіх тижнів після 30-хвилинної експозиції бактеріальне навантаження становило 5,2 (0,3) $\log_{10}$ КУО/мл для *Enterobacteriaceae* та 5,2 (0,2) $\log_{10}$ КУО/мл для *Acinetobacter spp.* Але після додаткових 30 хвилин культивування мікробне навантаження впало нижче межі виявлення (*p<0,01*). Досліджена концентрація NCT також значно знижувала метаболізм у мікробних клітинах. Високі значення для більшості досліджених ізолятів були досягнуті вже протягом перших 30 хвилин інкубації. Подальша інкубація також сприяла метаболічному провалу; тривала інкубація протягом 60 хвилин призвела до подальшого зниження метаболічної активності на 85,0% (*p<0,05*). Однак 3-годинна експозиція не дала статистично значущих результатів.

Висновки. NCT у мілімолярній концентрації може ефективно обмежувати життєдіяльність мікроорганізмів на біоплівках, отриманих in vitro.

Ключові слова: біоплівки, N-хлортаурин, мікроорганізми, бактерицидна активність, бактерії.

EFFECT OF N-CHLOROTAURINE ON BIOFILMS OF PATHOGENS ASSOCIATED WITH COMPLICATIONS OF DENTAL IMPLANTATION

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Abstract. *Background.* Dental implants are widely used in modern medical practice to replace missing teeth because of their stability and comfort characteristics. However, they can be colonized by bacteria, leading to implant-related infections. N-chlorotaurine (NCT) is a promising remedy against biofilms.

The present study aimed to investigate in vitro activity of NCT against long-term biofilms on titanium material used for implants in dentistry.

Object and research methods. We studied the activity of 1% NCT against isolates obtained from patients with dental implantation failure. The study of biofilm-forming properties was performed in vitro on titanium disks, incubation was up to 8 weeks. After each time interval, the disks were removed from the broth and treated with 1%1 NCT for 30 minutes, 1 hour and 3 hours. To study the immediate effect of NCT on a biofilm, the XTT-reductase test was applied.

Research results. The studied 1%-solution of NCT has demonstrated considerable bactericidal activity against all test bacteria already after 30 min of the study initiation. The best results were achieved for gram-positive bacteria, especially for *Streptococcus spp*. There were several isolates of *Staphylococcus spp.,* including *Staphylococcus aureus*, which retained a vital activity and gave growth within the first weeks of the study. However, for mentioned isolates, the desirable effect was achieved after following 30 minutes of cultivation. Results obtained in experiments with gram-negative bacteria also were promising. But microbial load below the detection limit was achieved later in comparison to gram-positive bacteria (*p<0.01*). On all weeks, after 30-min exposure, the bacterial load comprised 5.2 (0.3) $\log_{10}$ CFU/ml for *Enterobacteriaceae* and 5.2 (0.2) $\log_{10}$ CFU/ml for *Acinetobacter spp.* But after an additional 30 min of cultivation, the microbial load fell below the detection limit (*p<0.01*). The studied concentration of NCT also significantly reduced metabolism in microbial cells. High values for most of the studied isolates have been already achieved within the first 30 min of incubation. Subsequent incubation also promoted metabolic turnover; in comparison to 30 min after exposure, prolonged incubation within 60 min led to up to 85.0% reduction of metabolic activity (*p<0.05*). However, 3h exposure didn`t bring any statistically significant results.

Conclusions. NCT in a millimolar concentration can efficiently limit the vital activity of microorganisms on biofilms produced in vitro.

Key words: biofilms, N-chlorotaurine, microorganisms, bactericidal activity, bacteria.

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Conflict of interest:
The authors declare that there is no conflict of interest.

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THE DEPENDENCE OF THE ORAL BIOFILM ON THE GALVANISM OF THE ORAL CAVITY

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Dentistry uses fixed prostheses as a tertiary prevention. Prostheses in the oral cavity are foreign bodies. Therefore, they lead to a violation of the biological balance. It can cause complications during treatment.

The purpose of the work was to determine the influence of galvanism on the composition of the microbiocenosis of the oral cavity in patients who used orthopaedic structures made of different materials, depending on the design features of the prosthesis.

An examination of 81 persons who were prosthetics with fixed structures was carried out. They determined the composition of periodontal plaque bacteria. A plaque sample was taken from the surface of the cervical area of the vestibular surface of orthopaedic structures (crowns) close to the gingival margin (without touching or injuring it) on the maxilla and mandible to do this. The multiplex polymerase chain reaction method was used in real-time to determine microflora's bacterial mass and quantitative ratios. Biopotentials of the oral cavity were also determined. The potential difference was evaluated between the extreme points of the metal prosthesis (metal-metal) and the metal inclusion and the mucous membrane of the oral cavity.

The presence of galvanism was established in all patients with fixed orthopaedic structures. It was determined that individual representatives of the microflora have different sensitivities to changes in the electrical potential of the oral cavity. The microbiome as a whole is a stable entity with a certain homeostasis.

These phenomena should be considered when conducting orthopaedic treatment and recommending the patient's hygiene after orthopaedic treatment.

Key words: *fixed orthopaedic structures, oral microbiome, galvanism of the oral cavity.*

Connection of the publication with planned research works.

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Introduction.

In the natural environment, microorganisms exist in two forms: free-floating or planktonic and fixed (sessile) – as part of biofilms. Most often, bacteria form microbial communities, in particular on the surface and cavities of the human body. These formations are called microbiomes or biofilms [1, 2]. Biofilms are structured systems that differentiate functions between the representatives that inhabit them. Biofilms are surrounded by a polymer matrix, are constantly renewed, have internal and external intercellular interactions [3].

The oral cavity is one of the complex and stable biotopes, which is very favourable for the growth and maintenance of biofilms. It maintains constant temperature, acidity, humidity and other parameters. Tissues have a complex microanatomy [4].

With age, due to various reasons, a person loses teeth. This problem is solved with the help of orthopaedic treatment. The most popular type of dental prostheses are fixed orthopaedic structures. The need for dental prosthetics among the adult population of Ukraine is relatively high – about 80%. But orthopaedic structures in the oral cavity lead to a violation of the biological balance. The nature and severity of these changes depend both on the material and technology of manufacturing prostheses and on the influence of