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Original article

Antimicrobial Activity of Chlorhexidine and Cerium Oxide Nanoparticles Composition

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SUMMARY

Introduction. Antiseptics are non-specific antimicrobial drugs that are widely used in dentistry. The "gold standard" in periodontology is chlorhexidine digluconate (CHG). A widespread use of CHG-containing products for daily care in medicine and dentistry and other fields leads to acquiring resistance to CHG in microorganisms.
Methods. A macro method of serial dilution was used for the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) on clinical strains of *Streptococcus mutans (S. mutans)* and *Staphylococcus epidermidis (S. epidermidis*) obtained from the patients with associated dental plaque-induced gingivitis, whereas museum strains of *Escherichia coli (E. coli)* ATCC25922 and *Candida albicans (C. albicans)* ATCC10231 were used as inoculum.
Results. The MIC and MBC of CHG, cerium oxide nanoparticles (CeNPs) and the solution of the CeNPs and CHG were tested. It was found that CeNPs itself had a weak inhibitory and bactericidal effect on microorganisms. The composition of CHG and CeNPs had significantly higher MIC and MBC for clinical cultures *S. mutans* and *S. epidermidis*; museum strains of *E. coli* ATCC25922 and *C. albicans* ATCC10231 were compared with CHG alone.
Conclusion. This method significantly enhanced bactericidal and bacteriostatic activity of chlorhexidine digluconate against clinical and museum strains of microorganisms.

Keywords: **periodontitis, nanoparticles, gingivitis, antiseptics, cerium oxide**

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INTRODUCTION

Inflammatory periodontal diseases are one of the most prevalent chronic pathological conditions in the human population worldwide. They affect all components of the periodontium such as the gingiva, tooth cement, alveolar bone and periodontal ligament. It was considered that chronic periodontitis is predominantly of concern in older adults. Aggressive periodontitis occasionally occurs in children under the background of systemic diseases (1). Even among young adults, the prevalence of periodontal diseases is still high, up to 74%, with a domination of generalized severe forms in patients with systemic conditions such as diabetes mellitus or obesity (2, 3).

Mostly, the etiological factors of periodontal disease are pathogenic and opportunistic micro-organisms of the oral cavity. Today, there are two views on the occurrence of gingivitis and periodontitis associated with dental plaque. There are several hypotheses how plaque causes the disease. According to the non-specific plaque hypothesis, the main role belongs to the size of the plaque and the total number of microorganisms in it (4). The second is the theory of specific dental plaque, where the main role in the occurrence of diseases is associated with a certain type of microorganisms (5). Currently, the most recognized is the theory of multiple pathogen theory, associated with the emergence of periodontal pathology involving a certain complex of microorganisms. However, the severity and manifestation of periodontal disease also depends on a host response to a certain microbial pathogen, which is reflected by the level of oral mucosa colonization resistance. Thus in patients with normal body mass index who have high colonization resistance and obese individuals who have compromised colonization resistance of oral mucosa, the same level of oral hygiene (microbial load) leads to different manifestations of gingivitis that was significantly more severe in all obese individuals (6).

However, one of the main points of treatment of all periodontal diseases is the elimination of the local causative factors, with dental plaque being one of them. Effective antimicrobial therapy requires both the influence of physical factors (ultrasound, scaling, scaler, curet treatment, air abrasive treatment, laser and diodynamotherapy) and chemical antimicrobial specific (antibiotics) and non-specific antimicrobial (antiseptics) agents. Dental plaque is a consortium of resistant microorganisms that has little permeability to any antimicrobial drug.

Antiseptics are a group of non-specific antimicrobial drugs that used widely in dentistry. The "gold standard" in periodontology is chlorhexidine digluconate (CHG) which is applied in various concentrations that depend on the dosage form: rinses - 0.05 - 0.2%, gels - 1%, varnishes - 40%. Due to the widespread usage of CHG-containing products for daily care at home, in medicine and dentistry and other fields, there is much evidence of acquiring resistance to the action of CHG in numerous microorganisms such as: *Escherichia coli* (*E. coli), Salmonella spp., Staphylococcus aureus (S. aureus), Streptococcus spp., Enterobacter spp., Pseudomonas spp., Proteus spp* and also the oral cavity microflora (7). There is evidence that clinical strains of *S. aureus,* develop resistance to CHG, which the author explained by changes in the locus of the bacterium qac gene encoding proteins of the channels of the efflux system (QAC-specific efflux pumps), which allows bacteria to get rid of antimicrobial compounds (8). However, the fundamental mechanisms of antimicrobial resistance remain unknown.

Due to the abovementioned statements, there is a need to tackle this issue, possibly by the invention of new effective antimicrobial drugs or increase in the activity of the existing ones.

The aim of the study was to find new solutions for enhancing antimicrobial activity of chlorhexidine digluconate for use in periodontology.

METHODS

This research involved a collection of clinical samples of bacterial species from the crevicular fluid of 22 patients with gingivitis during the treatment at the Department of Therapeutical Dentistry of Poltava State Medical University, Poltava, Ukraine. Before the treatment and sample harvesting, all patients signed an informed consent statement. The design of the study was approved by the Commission on Bioethics of Poltava State Medical University (protocol No. 197). The study was performed according to the Good Clinical Practice guidelines and according to the Declaration of Helsinki.

Isolation and identification of bacterial strains

A microbiological analysis of crevicular fluid in young patients (18 - 22 years old) with gingivitis associated with dental plaque was performed. The collection of clinical material was conducted at the state dental facility "Poltava Regional Center of Dentistry". The criteria for inclusion of patients into the study were the presence of gingivitis, associated with dental plaque and the age of patients, which ranged from 18 - 22 years. Exclusion criteria: administration of antibiotics or usage of local antimicrobial drugs during the last three months, the presence of fixed orthodontic appliances, fixed prosthetic construction, the presence of general somatic pathology and pregnancy. Before treatment, all patients were informed about their participation in the study and signed a written informed consent form. The Bioethics Commission of Poltava State Medical University approved the research protocol. Crevicular fluid samples were taken in the morning on an empty stomach, with prior treatment of the vestibular surface of teeth and gums with a sterile gauze swab moistened with a sterile solution of water for injections. The collection of the samples was performed using sterile paper endodontic pins DentsplyTM, (№20.04 according to ISO), 1 cm long. After saturation with the crevicular liquid, the pin was put into a sterile container Eppendorf Tube TM with sterile solution for injection, after which they were thoroughly washed there. No later than 20 minutes after collection, standard dilutions were inoculated on the selective and differential diagnostic medias including Salt Egg Yolk Agar Base, Endo medium, Saburo, blood agar, sugar agar (HIMEDIA, India); bacteria were cultured in aerobic condition at 37 ^{0}C. The preparation of nutrient media was done according to the manufacture's instructions. On the obtained cultures, we determined the total microbial number of bacteria obtained from crevicular fluid. Quantitative results were expressed in colony-forming units - CFU/ml. Identification of the obtained pure cultures to the genus was performed using morphological, tinctorial, cultural and biochemical characteristics.

Macroscopy of the microorganism's colonies on nutrient media was done, and the following morphological features of colonies were evaluated: the size of colonies, shape, surface, margins, opacity, color. Cultural traits were also assessed: the presence of α-hemolysis or β-hemolysis. Classification of the colonies to the genus staphylococci or streptococci (staf +, strepto -) was performed by determining the production of catalase, as staphylococci are positive for this enzyme and streptococci are negative (9). To assess the tinctorial and morphological features, microscopic examination of the colonies of microorganisms were performed, previously fixed and stained using simple and complex staining methods, including the Gram staining method (9). The samples were examined by light microscopy under immersion lens (x 90). Streptococcal and staphylococcal cultures were identified to the genus using a bacteriological analyser bioMerieux Vitek 2, France.

Determination of microorganisms' sensitivity to antibacterial drugs

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the antiseptic chlorhexidine bigluconate 0,05% ("Chervona Zirka") that belongs to the detergent group, nanoparticles of cerium oxide 2 - 7 nm (CeNPs) (concentration of the solution 140 μg/ml) were tested. In addition, the solution of the CeNPs and CHG in a ratio of 1:1 by volume were tested. CeNPs "Cerera" were kindly provided by prof. Mykola Spivak (Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine). CeNPs, stabilized with sodium citrate are registered in Ukraine under the name "Cerera" as a biologically active supplement (the registered number is TYY 10.8-2960512097-004: 2015). Both solutions were taken in such ratio convenient for preparation in clinical practice.

Both minimum inhibitory and bactericidal concentrations were determined by ISO 20776-1:2019 standard, involving susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices.

Part 1: Broth micro-dilution reference method for testing the *in vitro* activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases was performed.

The MIC of each substance was estimated using the macro method of serial dilutions in broth according to the standard serial dilution method with a culture of streptococcal and staphylococcal microorganisms isolated from the crevicular fluid of patients with associated dental plaque gingivitis. The last tube with a transparent medium indicated an

inhibition of bacterial growth under the influence of the minimum inhibitory concentration in the test tube.

The MBC was found by seeding the material from all dilution tubes to the particular agar sectors in Petri dishes. The MBC was considered the last dilution without bacterial growth on the agar sector. Clinical cultures of *Streptococcus mutans (S. mutans*) and *Staphylococcus epidermidis (S. Epidermidis)*, obtained from patients with associated dental plaque gingivitis and museum strains of *E. coli* ATCC25922 and *Candida albicans (C. albicans)* ATCC10231, purchased at the Gromashevsky Institute of Epidemiology and Infectious Diseases, were used as inoculum. We isolated 8 cultures of *S. mutans* from 19 diverse patients with gingivitis and 8 cultures of *S. Epidermidis* from 10 patients with gingivitis.

In total, 10 serial dilutions were performed in each group of drugs, where the clinical culture of streptococci and staphylococci was used as an inoculum, as well as two serial dilutions with each culture of museum strains: *E. coli* ATCC25922 and *C. albicans* ATCC10231. All clinical strains of were assessed as sensitive to CHG, thus, to the solution of CHG and CeNPs. The main issue of the study was to explore how nanoparticles influence antimicrobial activity of the solution. The serial dilutions were done for CeNPs and 0.05% CHG. The concentrations of CHG and CeNPs in the first tube were 16 mg/mL - 46.6 μg/mL, respectively. In every following dilution, the concentration of each medication in the solution was reduced twice.

This method was patented with the patent №134206 of Ukraine, "The novel method of antiseptics bactericide activity enchantment".

Statistical analysis

GRAPHPAD PRISM 8.0.1 was used for data statistical processing. All results were described as average and standard deviation. For data analysis, we used a one-factor analysis of variance (one-way ANOVA) for unrelated samples and Bonferroni corrections for multiple comparisons were done. The difference between groups was considered statistically significant at $p < 0.05$.

RESULTS

In the samples of crevicular fluid of patients with gingivitis, we identified the genus using the bacteriological analyzer bioMerieux Vitek 2, France. Clinical strains of *S. mutans* (alpha-hemolytic, lecitovitellase-) and *S. epidermidis* (beta-hemolytic, lecitovitellase -) were used as the inoculum.

CeNPs alone had a weak antimicrobial activity on all strains of microorganisms.

In contrast, CHG demonstrated a MIC in the 7 - 8 dilution and MBC in the 6 - 7 dilution.

Table 1. *MIC (minimum inhibitory concentration) and MBCs (minimum bactericidal concentration) of CeNPs (Cerium oxide nanonarticles 2-7 nm) (μg/mL), CHG (chlorhexidine digluconate) (mg/mL) and the effect of the solution of CeNPs (μg/mL) and CHG (mg/mL) on clinical strains of S. mutans and S. epidermidis, museum strains of E. coli and C. albicans*

Microorganisms	Antiseptic	MIC (CHG) mg/mL	MIC (CeNPs) μg/mL	MBC (CHG) mg/mL	MBC (CeNPs) μg/mL
Clinical strains of *S. mutans* and *S. epidermidis*	CHG	0.44 ± 0.05	-	0.87 ± 0.09	-
	CeNPs	-	65.63 ± 4.38	-	70
	CHG+CeNPs	0.09 ± 0.09	0.23 ± 0.029*	0.33 ± 0.043*	0.91 ± 0.12*
Museum strains of *E. coli*	CHG	0.58 ± 0.08	-	1.11 ± 0.1	-
	CeNPs	-	70	-	70
	CHG+CeNPs	0.12 ± 0.03*	0.37 ± 0.032*	0,6 ± 0.051*	1.73 ± 0.17*
Museum strains of *C. albicans* ATCC10231	CHG	0.39	-	0.78	-
	CeNPs	-	70	-	70
	CHG+CeNPs	0.08	0.18	0.31	0.87

Notes * $P < 0.01$ compared to CHG and CeNPs

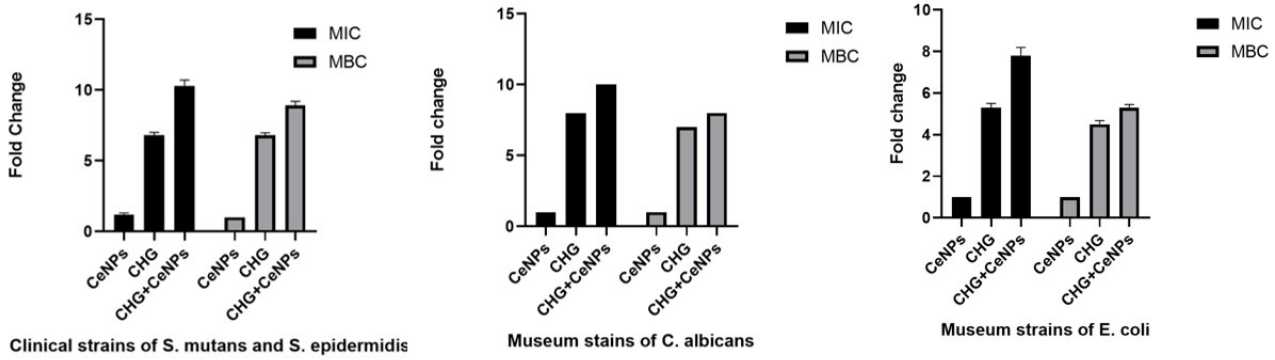

Figure 1. *Fold change in MIC and MBCs of CeNPs and CHG, and the effect of the solution of CeNPs and CHG on different microorganisms*

Simultaneous usage of CeNPs and CHG enhanced significantly the antimicrobial properties of the composition by 1.5 – 1.7 times compared with CHG usage alone. Detailed results and concentration of CHG and CeNPs for each strain are shown in Table 1. Fold change in MIC and MBC enhancement for CeNPs and CHG composition are shown in Figure 1.

DISCUSSION

Despite significant progress in the study of pathogenesis, prevention and treatment of oral cavity diseases, they remain one of the most widespread diseases all over the world in all age groups. Thus, the prevalence of caries varies from 24% to 99% and the prevalence of periodontal disease is 12% - 87% in different populations (10 – 12).

In most treatment guidelines on gingivitis and periodontitis associated with dental plaque, the treatment of choice is a combined effect of physical, mechanical and chemical factors on dental plaque, because the influence of only one of them cannot effectively deplete such a stable consortium of microorganisms as a biofilm. The "gold standard" in periodontology is CHG, which is applied in various concentrations that depend on the dosage form: oral wash - 0.05% - 0.2%, gels - 1%, varnishes - 40%. It has been recorded for the last 35 years that many microorganisms, including the oral cavity biotope, have acquired resistance to CHG because of the wide usage of CHG-containing soaps, detergents, personal hygiene products, hand washes (13). Disadvantages of high dosage and frequent usage of CHG in toothpastes and mouthwash involve the occurrence of dysgeusia, the development of teeth discoloration, acquired bacterial resistance (14). We propose to solve this problem by enhancing the antimicrobial activity of CHG by its simultaneous use with CeNPs, that itself has weak antimicrobial activity, but along with CHG significantly improves the antimicrobial activity and reduces CHG adverse effects by reducing CHG concentration in the solution.

In addition to the antimicrobial properties of CeNPs, they have antioxidant properties. The prospects for their biomedical application stem from two main factors - oxygen non-stoichiometry and relatively low toxicity. The first factor determines the ability of nanoparticles to catalytically participate in the redox processes in human cells, especially in the neutralization of reactive oxygen species (15). The second promotes the safe use of CeNPs in clinical medicine. The specific properties of CeNPs include the ability to regenerate oxygen non-stoichiometry, which is the ability of the CeNPs in a relatively short span to restore to their original state, after participation in the redox process. That enables them to participate again in redox processes, in contrast to traditional antioxidants (16).

As the size of the CeNPs decreases, their superficial surface area increases. As a result, the percentage of atoms increases, being on the surface of nanoparticles and characterized by higher activity. It was clearly shown that cerium oxide nanoparticles with a higher superficial surface area have a promising biological activity, which is determined by

the size of the superficial surface area and not the mass of nanoparticles (17).

CeNPs have powerful antioxidant properties because they can inactivate the superoxide anion radical and hydroperoxyl radicals. In biological systems, the superoxide dismutase enzyme inactivates superoxide anion radical. The ability to perform the function of superoxide dismutase was one of the first discovered enzyme-like properties of CeNPs, and the mechanisms in the case of natural enzyme and CeNPs were similar (18).

We have shown that intraoral administration of CeNPs as the experimental correction of periodontal diseases in the monosodium glutamate-induced obese rats increased the activity of antioxidant enzymes and inhibited the oxidative and nitrosative stress in the periodontal tissues (19). Also, intraoral administration of CeNPs has shown significant efficacy for treating gingivitis in young obese patients (20).

In addition to powerful antioxidant properties, nanoparticles have their own weak bactericidal and bacteriostatic properties. The effect of CeNPs on the morphology and cell wall of *E. coli* was detected (21). CeNPs readily penetrate into the cytoplasm of *E. coli* (unlike mammalian cells) and inhibit cellular respiration and glucose metabolism. The presence of sodium citrate as a stabilizer makes nanoparticles targeted to eukaryotic mitochondria and bacterial cell mesosomes. Under the influence of CeNPs on the bacteria, the cell wall remained morphologically unchanged, but electron microscopy revealed emerging "knob-like protrusions", which were associated with possible membrane destruction (22).

It has been reported that CeNPs accumulate directly on the *E. coli* cell wall (23). However, *C. albicans* under the stress condition forms the layer of exopolysaccharides, which makes a direct interaction impossible. In this case, CeNPs reveal indirect bactericide effect through reactive oxygen species (24).

The low toxicity of CeNPs and numerous additional therapeutic effects, in addition to antimicrobial, makes this substance promising for the use in the treatment of patients with periodontal disease. It has also been proven that CeNPs promote the differentiation and growth of keratinocytes, fibroblasts, endotheliocytes, which is important for the stimulation of regeneration in periodontal soft tissues after treatment (25).

For CHG, the MIC on the clinical strains of S. mutans and S. epidermidis was 0.44 ± 0.05 mg/ml, whereas the the MBC was 0.087 ± 0.09 mg/ml. CeNPs had weak antimicrobial activity. The MBC and MIC were 65.63 ± 4.38 μg/ml and 70 μg/ml, respectively.

In contrast, the solution of CHG and CeNPs was tested, whereby MIC of the solution for CHG was 0.088 ± 0.01 mg ml and 0.23 ± 0.029 μg/ml for CeNPs. The MBC of the solution was registered with the concentration of CHG of 0.33 ± 0.043 mg/ml and CeNPs of 0.91 ± 0.12 μg/ml.

Thus, a simultaneous use of CeNPs enhances MIC by 1.5 times and MBC of CHG by 1.3 times on clinical strains of *S. mutans* and *S. epidermidis*. The same effectiveness was observed for museum cultures of *E. coli* ATCC25922 and *C. albicans* ATCC10231.

The idea of improvement of antiseptics' antimicrobial properties is not novel. Some of them have their own advantages and disadvantages. Therefore, this problem has not yet been tackled. Diverse scientists use certain methods and approaches of CHG antimicrobial properties enhancement. For example, the combination of CHG with crude eucalyptus oil and 1,8-cineol (which is a major constituent of eucalyptus oil) showed a synergistic antimicrobial activity against a wide range of microorganisms in the planktonic and biofilm modes of growth (26).

Active nanocarriers for CHG based on sterically stabilized shellac nanoparticles with dual surface functionalization greatly enhance the antimicrobial action of CHG. The enhancement of the CHG antimicrobial activity was thought to be due to the increased electrostatic adhesion between the cationic surface of the octadecyl trimethyl ammonium bromide coated, CHG-loaded shellac nanoparticles and the anionic surface of the cell walls of the microorganisms, ensuring direct delivery of CHG with a high concentration locally on the cell membrane (27). A combination of chitosan nanoparticles and CHG acted synergistically, inhibiting and eliminating significant growth of colonies of *Enterococcus faecalis* (*E. faecalis*) on growth media and infected collagen membranes (28). The aforementioned methods also have high effectiveness, but the method proposed in this study is simple, does not require special equipment, and has additional antioxidant and healing properties.

Our method demonstrates promising results and the solution of CHG and CeNPs can be successfully used for treating periodontal disease. In addition to the antibacterial effect, the composition exhibits antioxidant properties and stimulates the regeneration of connective tissue.

CONCLUSION

A simultaneous use of the solution of chlorhexidine digluconate and nanoparticles of cerium oxide significantly enhances the antimicrobial effect of the composition. Due to the high level of antibiotic resistance of oral cavity microorganisms, this composition can be used to solve this problem. In addition to antimicrobial activity, it has antioxidative and regenerative effects, which are important for shortening of the recovery period after periodontal treatment.

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Antimikrobna aktivnost hlorheksidina i sastav čestica cerijum-oksida

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SAŽETAK

Uvod. Antiseptici su nespecifični antimikrobni lekovi sa širokom primenom u stomatologiji. „Zlatni standard" u parodontologiji predstavlja hlorheksidin-diklukonat (engl. CHG). Široka primena proizvoda koji sadrže CHG u dnevnoj nezi u medicini i stomatologiji, kao i u ostalim oblastima, dovodi do rezistencije mikroorganizama na CHG.
Metode. Upotrebljena je makrometoda serijskog rastvaranja radi određivanja minimalne inhibitorne koncentracije (engl. MBC) na kliničkim sojevima *Streptococcus mutans (S. mutans)* i *Staphylococcus epidermidis* (*S. epidermidis*), koji su dobijeni od bolesnika sa gingivitisom izazvanim dentalnim plakom, dok su referentni sojevi *Escherichia coli (E. coli)* ATCC25922 i *Candida albicans (C. albicans)* ATCC10231 upotrebljeni kao inokulum.
Rezultati. Testirani su MIC i MBC hlorheksidin-diklukonata, nanočestice cerijum-oksida (CeNPs), kao i rastvor CeNPs-a i CHG-a. Utvrđeno je da CeNPs ima slabo inhibitorno i baktericidno dejstvo na mikroorganizme. Sastav CHG-a i CeNPs-a imao je viši MIC i MBC za kliničke sojeve *S. mutans* i *S. epidermidis*; referentni sojevi *E. coli* ATCC25922 i *C. albicans* ATCC10231 upoređeni su samo sa CHG-om.
Zaključak. Ova metoda je u znatnoj meri povećala baktericidna i bakteriostatska svojstva hlorheksidin--diglukonata kod kliničkih i referentnih sojeva mikroorganizama.

Ključne reči: **periodontitis, nanočestice, gingivitis, antiseptici, nanočestice cerijum-oksida**