Determination of the antimicrobial activity of a gel composition based on a flavonoid complex and benzidamine hydrochloride intended for the treatment of periodontal diseases in orthodontic patients

Summary. It is important to solve the problem of complex treatment and prevention of periodontal diseases in orthodontic patients by developing new drugs that have antimicrobial, anti-inflammatory, antioxidant effects and are included in effective treatment regimens. Orthodontic treatment with fixed orthodontic appliances contributes to a significant decrease in the level of individual oral hygiene against the background of oxidative stress. Therefore, the aggressiveness of periodontal pathogenic microflora increases, which contributes to the deepening of the inflammatory process in the tissues of the periodontal complex.

The aim of the study – to determine the antimicrobial activity of the developed periodontal gel composition based on flavonoid complex and benzidamine hydrochloride against microorganisms isolated from periodontal pockets of orthodontic patients with dystrophic-inflammatory diseases of periodontal tissues.

Materials and Methods. The efficacy of antimicrobial preservatives of the patented gel composition “Benzidalflaviverdine” (GCB) and the comparison drug “Cholisal" was evaluated according to the recommendations given in the State Pharmacopoeia of Ukraine (SPU). GCB samples were inoculated with suspensions of daily cultures of reference strains of *S. aureus*, *P. aeruginosa*, *C. albicans* and *Aspergillus* so that the final concentration of colony forming units (CFU) was $10^5-10^6$ per ml. Isolates obtained from the oral cavity and periodontal pockets of orthodontic patients (*S. aureus*, *S. pyogenes*, *S. mutans*, *E. faecalis*, Rothia sp, bacteria of the Actinomycetaceae family, *P. aeruginosa*, *C. albicans*) were also used. Samples were taken after 2, 7, 14 and 28 days and the concentration of CFU of microorganisms was determined. Two methods were used to determine the antimicrobial activity: the standard “well” method regulated by the SPU, as well as a modification of the suspension method for determining the specific activity of disinfectants and other antimicrobial substances and compounds. To assess the reduction in the concentration of microorganisms, the decimal logarithm of the reduction in CFU in the samples (Log10 rate of reduction) was calculated.

Results and Discussion. The results of using a modification of the suspension method, the peculiarity of which was to dilute equal proportions of GCB with a bacterial suspension, showed a well-pronounced antibacterial activity of GCB. At the same time, the activity of GCB against methicillin-resistant *S. aureus* isolate did not differ from that against sensitive isolates. The activity of GCB can be characterized as species-specific; the effect on individual isolates of the same species differed slightly. GCB activity was high against streptococci, Rothia sp, and pseudomonas (*P. aeruginosa*). For *S. aureus*, *S. pyogenes* and *C. albicans*, the dynamics of the decrease in CFU during exposure to GCB for 40 minutes was determined. It was found that the maximum
Introduction. Guided by the data of epidemiological surveys, domestic and foreign researchers have established a high prevalence of periodontal tissue diseases in young people (18–30 years). The prevalence of catarrhal gingivitis and generalized periodontitis of initial-I stage severity among such patients reaches 98 % of cases [1–3]. Despite the high level of sanitary culture, availability and variability of personal and professional oral hygiene products, over the past 20–25 years in Ukraine and around the world, periodontal diseases have become noticeably “younger” in patients with dentor alveolar anomalies (DA) [4, 5].

The adverse effect of braces in the active period of orthodontic treatment on the state of periodontal tissues of young orthodontic patients has been reported in many sources [6–8]. According to various researchers, orthodontic treatment with fixed orthodontic appliances has been shown to significantly reduce the level of individual oral hygiene against the background of oxidative stress [9–11]. As a result, the aggressiveness of periodontal pathogenic microflora increases, which contributes to the deepening of the inflammatory process in the tissues of the periodontal complex [12–17]. This fact determines the intensification of solving the problem of complex treatment and prevention of periodontal diseases in orthodontic patients by developing new drugs that have antimicrobial, anti-inflammatory, antioxidant effects and are included in effective treatment regimens.

We have developed a periodontal gel composition under the patented name “Benzidaflaziverdine” (GCB) based on two active components – a flavonoid complex (drops “Proteflazid®” LLC NKV “ECOPHARM”, Ukraine) and benzidamine hydrochloride (tablet form “T-Sept®”, ICN Polfa, Rzeszow S. A., Poland) [18]. Additional components of this composition are sodium alginate, nipagine, and water for injections. The main effect of the extemporaneous periodontal dressing is aimed primarily at the local correction of disorders of mechanisms in the lipid peroxidation-antioxidant defense system in orthodontic patients with DA who are prone to periodontal diseases. Such patients are exposed to an increased stress-modeling effect of fixed braces on periodontal tissues, which negatively affects the stabilization of treatment results after initial periodontal therapy performed before the active period of orthodontic treatment. To prevent recurrence, the treatment regimen included the inclusion of the developed GCB, which has antioxidant properties.

In this work, we focused on the study of the antimicrobial activity of GCB. One of the active components of GCB – “Proteflazid®”, has antiviral properties and is a drug with antioxidant effect. In addition, the second active ingredient, “T-Sept®” (benzidamine hydrochloride), is a non-steroidal drug with antimicrobial properties. In order to enhance the antimicrobial effect, a small dose of nipagine (0.15 %) was additionally introduced into the GCB as a preservative. In general, the effectiveness of the inclusion of components with preservative and antiseptic effects in the composition of extemporaneous periodontal gels is undeniable. It has been proven in the literature that alcohol-containing preservatives (phenylethyl alcohol) have the highest activity [19]. However, for a gel used as a drug delivery system, as a periodontal patch with a prolonged effect on periodontal tissues, the inclusion of alcohol-containing substances is undesirable. At the same time, even well-known antiseptics and preservatives can show their activity in different ways in compositions with different active ingredients [19, 20].

The aim of this study – to determine the antimicrobial activity of the developed periodontal gel.
composition based on flavonoid complex and benzidamine hydrochloride against microorganisms isolated from periodontal pockets of orthodontic patients with dystrophic-inflammatory diseases of periodontal tissues.

**Materials and Methods.** For the microbiological study, performed at the Research Institute of Epidemiology and Hygiene of Danylo Halystsky Lviv National Medical University, we used the developed GCB [18] and the comparison drug – Chop lusal gel (Jelfa S.A. Poland), which is used to treat diseases of the oral mucosa and inflammatory and dystrophic-inflammatory diseases of periodontal tissues. The following reference strains and standard samples (from the collection of live cultures stored at the Research Institute of Epidemiology and Hygiene of Danylo Halystsky Lviv National Medical University) were used in the study: *Staphylococcus aureus* ATCC 25923; *Staphylococcus aureus* ATCC 6538; *Pseudomonas aeruginosa* ATCC 27853; *Enterococcus faecalis* ATCC 12984; *Candida albicans* ATCC 885-653; *Aspergillus brasiliensis* type.

The isolates obtained from 35 orthodontic patients aged 20 to 30 years with diagnosed generalized periodontitis of initial – I stage severity were used in the study. To collect material for bacteriological examination, sterile paper pins were immersed in periodontal pockets for 30 seconds and transferred to Eppendorf tubes in 1 ml of sterile saline and transported to the bacteriological laboratory. The contents of the tubes were then inoculated into the following nutrient media: thyoglicolate medium (HiMedia, India), blood agar (Biolife, Italy), Endo agar (manufactured by “Farmaktiv” LLC, Ukraine), Sabouraud's agar (manufactured by “Farmaktiv” LLC, Ukraine), Mannitol salt agar (Biolife, Italy).

The following isolates were used: *S. aureus* – 5 typical isolates (including one methicillin-resistant); *Streptococcus pyogenes* – 5 typical isolates; *Streptococcus mutans* – 5 typical isolates; *Enterococcus faecalis* – 3 typical isolates; *Rothia sp* – 3 typical isolates; bacteria of the family *Actinomycetaceae* – 6 typical isolates; *P. aeruginosa* – 3 typical isolates; *Candida albicans* – 5 typical isolates (pseudomycelium). The efficacy of antimicrobial preservatives GCB and the comparison drug Cholisal gel, was evaluated according to the recommendations given in the State Pharmacopoeia of Ukraine (SPU) using nutrient agar (Biolife, Italy), Endo agar, yolk-salt agar, Sabouraud's agar, soy-casein agar [21, 22]. Samples of the GCB preparation were inoculated with suspensions of daily cultures of reference strains of *S. aureus*, *P. aeruginosa*, *C. albicans* and *Aspergillus* so that the final concentration of colony forming units (CFU) was $10^5$–$10^6$ per ml. After 2, 7, 14, and 28 days, samples were taken and the concentration of CFU of microorganisms was determined.

Two methods were used to determine the antimicrobial activity of the GCB: the standard «well» method regulated by the SPU, as well as a modification of the suspension method for determining the specific activity of antimicrobial substances and compounds in accordance with the requirements of DSTU EN 1040: 2004 [23] and DSTU EN 1275:2004 [24] (nutrient agar (for staphylococci and pseudomonas), nutrient agar with blood (for streptococci, actinomycetes, enterococci and rotia), Sabouraud's agar (for candida) were used).

The latter method consisted of adding a suspension of test strains of microorganisms to microtubes with a precisely defined amount (approximately 0.60-0.90 mg) of GCB samples, the comparison drug Cholisal (positive control), and sterile 85% glycerol (negative control) so that the final cell concentration corresponded to $5 \times 10^5$–$10^6$ CFU per ml. Then everything was thoroughly mixed and incubated at T $37\pm0.2{\degree}C$. After 10, 20, 30, and 40 min of incubation, samples of the reaction mixture were taken and inoculated onto appropriate agarized nutrient media. The decimal logarithm of the reduction in CFU in the samples (Log10 reduction) was calculated to assess the reduction in microbial concentration.

All studies were conducted in accordance with the principles of bioethics set forth in the Helsinki Declaration for Ethical Principles for Medical Research Involving Human Subjects and the Universal Declaration on Bioethics and Human Rights (UNESCO). In accordance with the requirements of the Bioethics Committee «On Conducting Laboratory Research of Biological Material», written informed consent was obtained from patients after providing oral and written information about the purpose and methods of the study Study protocol No. 9 of 21.12.2020 was discussed and approved by the Ethics Committee for Scientific Research, Experimental Development and Scientific Works of Danylo Halystskyi Lviv National Medical University.

The results were analyzed using statistical processing according to generally accepted methods. The creation and editing of the primary database was performed in Microsoft Excel. Statistical processing of the numerical results was performed using a standard package of application programs for biomedical research (STATISTICA 6.0). The method of analysis of variation was used to determine the
Results and Discussion. All samples of the products were checked for contamination with extraneous microflora before the study. When testing the activity of GKB preservatives, a significant decrease in the CFU concentration of bacterial and fungal test strains in the samples was observed (Table 1).

According to the results obtained, it can be stated that the tested samples of GBC fully comply with the requirements of the SPU in terms of “antimicrobial effectiveness of preservatives” for medicinal products for topical use.

When determining the spectrum of antimicrobial activity, the standard “well” method regulated by the SPU was first tested. After 24–48 hours of incubation of the crops around the wells with the comparison product “Cholisal”, clear zones of growth retardation of test strains of microorganisms were observed. According to the requirements of the SPU, this gives grounds to assert that the antimicrobial activity of this drug is sufficient. At the same time, the formation of sufficient zones of growth retardation of test strains of microorganisms was not observed around the “wells” with the introduced GCB. This led to the continuation of the study using a modification of the suspension method, the peculiarity of which was to dilute equal proportions of GCB with a bacterial suspension. The results of determining the antimicrobial effect using this method, in contrast to the results obtained by the “wells” method, showed a well-pronounced antibacterial activity of GCB (Table 2).

At the same time, the activity of GCB against methicillin-resistant S. aureus isolate did not differ from that against susceptible isolates. In general, the activity of GCB can be characterized as species-specific; the effect on individual isolates of the same species differed slightly. The high activity of GCB against streptococci and Rothia sp is noteworthy. It should also be noted the pronounced antimicrobial effect on pseudomonas (P. aeruginosa).

The results also indicate that limiting trials to only one method, even one regulated by the SPU (in our case, the standard “well” method), can lead to

Table 1. Results of the study of antimicrobial efficacy of GCB preservatives (n=4, P=95 %)

<table>
<thead>
<tr>
<th>Test strains of microorganisms</th>
<th>Microbial load after inoculation, Log10 CFU per ml</th>
<th>Log10 reduction of the initial microbial load</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 days</td>
</tr>
<tr>
<td>S. aureus ATCC 6538</td>
<td>5.80</td>
<td>2</td>
</tr>
<tr>
<td>P. aeruginosa ATCC 27853</td>
<td>5.74</td>
<td>2</td>
</tr>
<tr>
<td>C. albicans ATCC 885-653</td>
<td>5.56</td>
<td>–</td>
</tr>
<tr>
<td>A. brasilensis</td>
<td>5.60</td>
<td>–</td>
</tr>
</tbody>
</table>

Notes: 1) ND – no microorganisms are detected;
2) NI – no increase in the number of microorganisms is observed.

Table 2. Decrease in CFU concentration of gram-positive, gram-negative bacteria and fungi of the genus Candida during exposure to GCB and comparison drug “Cholisal” using a modification of the suspension method.

<table>
<thead>
<tr>
<th>Test strains of microorganisms</th>
<th>Concentration of bacteria in the reaction mixture after inoculation, Log10 CFU per ml</th>
<th>Log10 CFU reduction after exposure for 30 minutes (Log10 CFU ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GCB</td>
</tr>
<tr>
<td>S. aureus</td>
<td>5.70–5.90</td>
<td>2.03–2.22</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>5.68–5.83</td>
<td>2.28–3.04</td>
</tr>
<tr>
<td>S. mutans</td>
<td>5.68–5.83</td>
<td>1.68–1.92</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>5.62–5.79</td>
<td>1.90–2.10</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>5.69–5.88</td>
<td>2.26–2.48</td>
</tr>
<tr>
<td>Rothia sp</td>
<td>5.55–5.70</td>
<td>2.39–2.49</td>
</tr>
<tr>
<td>Actinomycetaceae</td>
<td>5.56–5.79</td>
<td>2.41–2.68</td>
</tr>
<tr>
<td>C. Albicans</td>
<td>5.0–5.1</td>
<td>1.0–2.3</td>
</tr>
</tbody>
</table>
the rejection of potentially promising drugs at the initial stages of trials. Thus, analyzing the results, we found no statistically significant differences between the absolute values of Log10 CFU reduction for GCB and the comparison drug “Cholisal”. However, for GCB, the Log10 CFU reduction value was slightly higher in almost every experiment. Confirmation of the higher actomycobacterial activity of GCB in comparison with the known analogues of periodontal gel compositions requires further research.

For *S. aureus*, *S. pyogenes* and *C. albicans*, the dynamics of CFU reduction during exposure to GCB for 40 minutes was determined. It was found that the maximum rate of Log10 reduction of CFU of *S. aureus* and *S. pyogenes* occurred in the first 10–15 minutes of incubation (Figures 1 and 2).

The Log10 reduction in the number of CFU of fungi of the genus Candida was somewhat slower (Figure 3).

However, the main decrease in the number of CFU of the test strains of microorganisms always occurred within a time period that was less than 30 minutes, the planned minimum exposure time for clinical use of the extemporaneous GCB product.

To summarize the results, the discrepancy between the antimicrobial activity of GCB obtained by the “well” method and the suspension method should be discussed first. It can be assumed that GCB and the comparison drug “Cholisal” have different consistencies (in GCB it is much thicker due to sodium alginate). The lack of sufficient activity of GCB according to the results of the diffusion method may be caused by the slow release of the gel components into the agar thickness. However, for periodontal gel compositions, such a property as a thick consistency is a good sign of prolonged action of the product, in particular under an individual periodontal guard. Thus, a higher clinical efficacy can be ensured in the treatment of catarrhal gingi-
vitis and periodontitis of varying severity, both chronic and acute [25]. The certified comparison product “Cholisal” has anti-inflammatory, analgesic and antiseptic effects on periodontal tissues. The main active ingredient in this gel for topical use is choline salicylate, which provides local anti-inflammatory and slight analgesic effects. Another component of this gel is cetalkonium chloride, which enhances analgesic and anti-inflammatory effects. The antiseptics methylparaben and propylparaben (in concentrations of 0.15 % and 0.08 %, respectively), which are part of the Cholisal gel base, have an antibacterial effect, which was confirmed in our study. However, due to the above preservatives, Cholisal gel cannot be classified as a prolonged-release drug, since in orthodontic patients with acute periodontal disease during the active period of orthodontic treatment, the effect of this gel may cause an irritating effect.

In turn, the pharmacological agent GCB developed by us contains an optimal ratio of benzidine hydrochloride as an active ingredient, which has a pronounced local analgesic, anti-exudative and antimicrobial effect. Benzidine hydrochloride belongs to non-steroidal anti-inflammatory drugs (NSAIDs) for topical use in the oral cavity in dentistry and periodontics, which are inferior to steroids in terms of the mechanism of action, but have very low toxicity and numerous advantages. Benzidine hydrochloride acts mainly on the phases of exudation and proliferation, as it acts as an inhibitor of the cyclooxygenase (COX) enzyme, which affects arachidonic acid with the formation of important mediators of inflammation and pain - prostaglandins and thromboxanes, the concentration of which increases in accordance with the severity of the disease. The lipoxygenase pathway of arachidonic acid metabolism leads to the formation of lipoxygenase – 5-LOH (lipoxygenase). Most NSAIDs are able to selectively inhibit two forms of this enzyme – COX-1 (cyclooxygenase) and COX-2, which, accordingly, reduces hyperemia, edema and pain and helps to normalize the microcirculation process [26, 27]. NSAIDs can uncouple oxidative phosphorylation, slowing the formation of macroergic bonds by affecting adenosine triphosphate in the tissues of the inflammation site. The inhibition of proliferation by NSAIDs is associated with a decrease in fibroblast activity and a decrease in collagen synthesis [28].

Thus, “T-Sept®”, a benzidine hydrochloride-based medicine, has pronounced analgesic and anti-exudative properties, and has an active anti-inflammatory effect. When applied topically in the form of a periodontal dressing with prolonged action, benzidine accumulates in inflamed tissues, where effective concentrations are achieved due to its ability to penetrate the mucous membrane [29].

The gel composition, as the second active ingredient, includes “Proteflazid®” drops, a direct-acting antiviral drug for internal use and for topical use in the form of applications to the mucous membranes, 1 ml of drops contains 1 ml of Proteflazid liquid extract (flavonoid content not less than 0.32 mg/ml in terms of rutin, carboxylic acid content not less than 0.30 mg/ml in terms of malic acid) from the herb Herba Deschampsia caespitosa L. ) and ground cinquefoil herb (Herba Calamagrostis epigeios L.) (1:1). The flavonoids contained in this preparation inhibit the replication of DNA and RNA viruses both in vitro and in vivo. In preclinical and clinical studies, the literature provides information on the antiviral effect of the drug on herpes viruses, hepatitis, papillomaviruses, HIV, influenza and acute respiratory infections. It has been proven that the mechanism of direct antiviral action is the inhibi-
tion of virus-specific enzymes such as DNA and RNA polymerases, thymidine kinase, reverse transcriptase and neuraminidase. The drug has immunotropic properties, protects mucous membranes, normalizing local immunity (lactoferrin, secretory immunoglobulin A, lysozyme and C3 component of complement). It has been established that this drug is an inducer of the synthesis of endogenous α- and γ-interferons to physiologically active levels, which increases the body's nonspecific resistance to viral and bacterial infections [30].

“Proteflazid®” has antioxidant activity, inhibits free radical processes, thereby preventing the accumulation of lipid peroxidation products, enhancing the antioxidant status of cells, reduces intoxication, promotes recovery after an infection and adaptation to adverse environmental conditions. This flavonoid complex is a modulator of apoptosis, enhances the effect of apoptosis-inducing substances and activates caspase 9, which contributes to the elimination of virus-damaged cells and the primary prevention of chronic diseases against latent viral infections, prevents disease recurrence and prolongs the period of remission [31]. Thus, in the GCB periodontal patch, developed for use primarily in the active phase of orthodontic treatment, local suppression of oxidative stress will be carried out due to this component.

Sodium alginate, included in GCB, is the sodium salt of natural alginic acid isolated from brown seaweed. Sodium alginate in powder form is slowly soluble in water, swells to form highly viscous solutions characterized by low emulsifying ability and is used as a thickener for various dosage forms (gels, emulsions). Sodium alginate is environmentally friendly, hypoallergenic, not absorbed into the bloodstream, and well tolerated by the body, which is why this compound is recommended by the WHO and FDA as an excipient in pharmaceuticals and as a food additive. Water-soluble alginates, having long chains, can easily exchange their cations for alkaline earth metal ions to form highly insoluble salts with a mesh structure, which can be the basis of a drug depot. This property is also used in various dosage forms to prolong the effect of pharmaceuticals. Alginate not only stimulate wound healing processes, but are also easily absorbed.

Nipagin (methylparaben) in the GCB acts as a preservative with antimicrobial activity. The experimentally established ratio of doses of medicinal substances in GCB is optimal.

Conclusions. 1. The developed GCB fully meets the requirements of the SPU in terms of the antimicrobial activity of preservatives.

2. Studies have shown a high antimicrobial activity of GCB, which was not inferior to the similar properties of the drug from the pharmacy chain “Cholisal”, which in turn contains such preservatives as methylparabens and 96 % ethyl alcohol.

3. Probable synergism of a low dose of nipagine introduced into the GCB and antimicrobial properties of benzidamine hydrochloride fully ensure the high antimicrobial properties of the developed GCB.

4. GCB can be recommended for use in clinical practice for the treatment of periodontal tissue diseases in orthodontic patients in preparation for the active period of orthodontic treatment, and in case of loss of remission when using braces.
LIST OF LITERATURE


7. Годований О. Заходження пародонту та аномалій і деформації зубочелюстної системи у хворих різного віку (стан проблеми та шляхи її вирішення) /
Названий текст не зберігається.