Wiadomości Lekarskie
Medical Advances

VOLUME LXXVI, ISSUES 9, SEPTEMBER 2023
Official journal of Polish Medical Association has been published since 1928

ISSN 0043-5147
E-ISSN 2719-342X

ALUNA Publishing

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ORIGINAL ARTICLE

COMPARATIVE STUDY OF BIOTOLERANCE CHARACTERISTICS OF DIFFERENT GELS COMPOSED OF BENZYDAMINE AND FLAVONOIDS THAT WERE DEVELOPED FOR TREATMENT OF PERIODONTAL DISEASES IN ORTHODONTIC PATIENTS

DOI: 10.36740/WLek202309114

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ABSTRACT

The aim: Different gels composed of benzydamine and flavonoids that were developed for treatment of periodontal diseases in the orthodontic patients will be compared regarding their effects on survival of mammalian cells of various tissue origin and their DNA intactness.

Materials and methods: Effect of different variants of patented gel composition «Benzidaflaziverdine» including a gel base and «Proteflazid®» containing flavonoids and benzydamine hydrochloride in powder form («T-Sept®») towards survival (MTT) of murine BALB-3T3 fibroblasts, J774.2 macrophages, human HaCaT keratinocytes was studied. Their effect on nativity of DNA of J774.2 macrophages was evaluated using DNA-comet assay.

Results: Three gel compositions were used. Sample 1 was prepared on gel basis including benzydamine in liquid form and demonstrated inhibitory effect towards pseudonormal murine BALB-3T3 fibroblasts and murine J774.2 macrophages, however, normal human HaCaT keratinocytes were resistant to its action. Sample 2 included BH in powder form and it did not affect significantly HaCaT keratinocytes and BALB-3T3 fibroblasts, but it suppressed J774.2 macrophages. Sample 3 («Benzidaflaziverdine») was developed and patented by us as a gel composed of benzydamine in powder form and flavonoid drops «Proteflazid®». It did not suppress tested mammalian cells and was not genotoxic (measured as % of DNA in comet tail and Olive Tail Moment) for murine J774.2 macrophages.

Conclusions: Inclusion of flavonoids in gel composition «Benzidaflaziverdine» blocked cytotoxic and genotoxic actions of benzydamine. Developed gel composition might be efficient in clinical periodontology, in particular, for treatment of periodontal diseases in orthodontic patients.

KEY WORDS: periodontium, gel composition, flavonoids, mammalian cell lines, biotolerance

Wiad Lek. 2023;76(9):2000-2007

INTRODUCTION

The orthodontic anomalies and malocclusions rank third after caries and periodontal diseases (40 - 85% of cases), thus, this problem is both of medical and social significance [1-3]. The results of epidemiological studies show that the polyetiological nature of orthodontic anomalies and malocclusions and untimely diagnosis at the stage of formation in temporary and mixed occlusion encourages the development of their more severe forms in a permanent occlusion. Along with the orthodontic anomalies, the periodontal diseases is recognized as a multifactorial one that continue leading the list of causes of tooth loss in the adult patients [4].

Patients with the orthodontic anomalies of the periodontal pathology require standard hardware treatment lasting from 6 months to 2.5 years [5, 6]. In turn, the active period of the orthodontic treatment using braces can cause an recurrence of the periodontal disease during which a number of processes occur in the oral cavity associated with the lipid peroxidation (LPO), exposure to metals and long retention period which indicates the need for effective and targeted preventive and treatment measures, primarily before and during active phase of the orthodontic treatment [7-9].

Thus, a search for effective remedies of treatment and prevention of the periodontal diseases in the orthodontic patients continues. It is important that local medicines in the form of pastes, ointments, gel compositions should have an intense anti-inflammatory, immunomodulatory, anti-edematous, antiexudative, antimicrobial, fungicidal

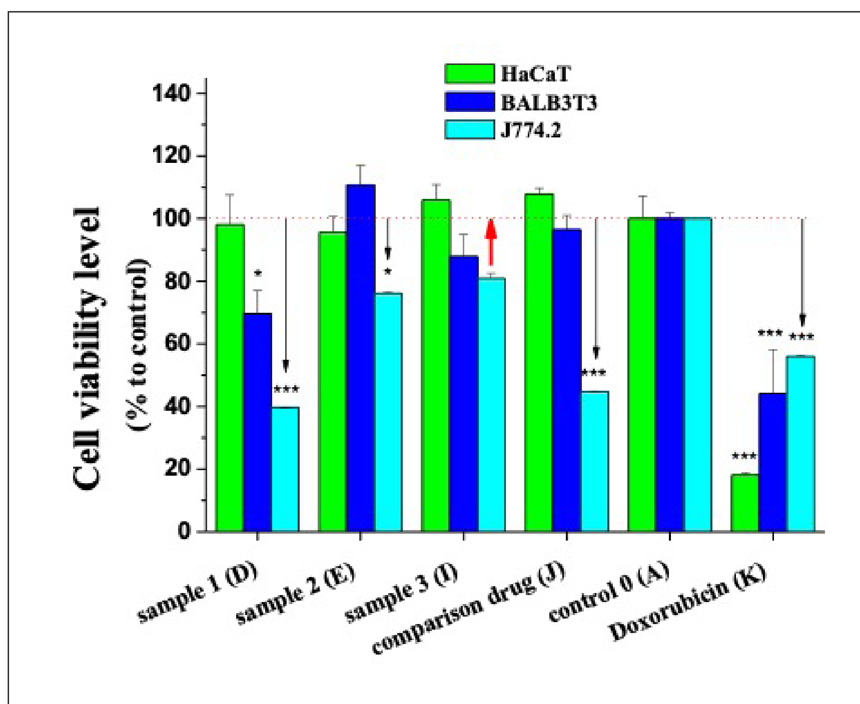

Fig. 1. Comparison of the viability rates (MTT-test, M ± m) of various lines of pseudonormal mammalian cells: murine fibroblasts of BALB-3T3 line, human keartinocytes of HaCaT line and murine macrophages of J774.2 line.
Notes: Control 0 – intact native cells, A (Table I); Comparison drug – «Cholisal» (Elfa, Poland), gel was added in the final concentration = 1%, J (Table I); Toxicological (positive) control – Doxorubicin, 1 µg/ml (Kyivmedpreparat, Ukraine), K (Table I)
Significance of difference with the non-treated control cells:
* $P<0.05$; *** $P<0.001$.

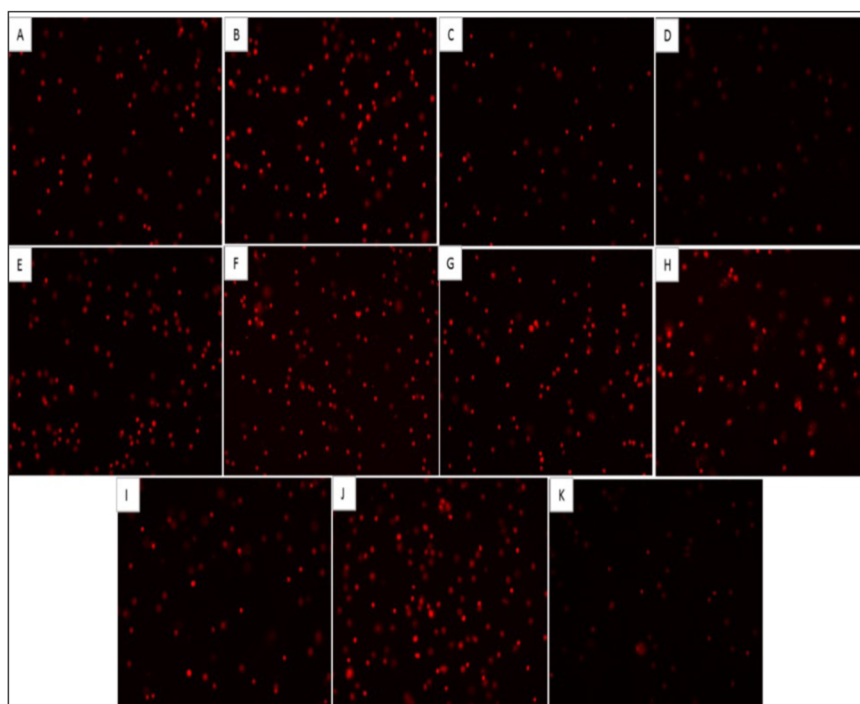

Fig. 2. The results of DNA comet study in murine J774.2 macrophages treated for 72 h with various gel compositions and other substances (A – control (native cell culture), B – DMSO (0,1%), C – gel base, D – Sample 1, E – Sample 2, F – gel base + BH (in solution and powder), G – gel base + «Proteflazid®», H – gel base + BH (in solution and powder) + «Proteflazid®», I – Sample 3 (patented gel composition), J – «Cholisal» (final concentration = 1%), K – Doxorubicin (1 µg/ml). Microscopic magnification x100.
Note: Final concentration of studied substances in culture medium with cells was 1%.

and antioxidant properties due to the improved composition and quantitative ratio of components that allow a reduction of treatment terms [10].

Flavonoids are known to possess a pronounced antioxidant effect. The phenolic structure of flavonoids allows them to interact with free radicals, reducing the intensity of lipid peroxidation (LPO) and inhibiting the malondialdehyde, that leads to a reduction of the level of local stress in tissues [11, 12].

In order to treat the periodontal diseases in the orthodontic patients, we have developed on the basis of the above drugs and patented the extemporaneous gel composition «Benzidaflaziverdine». This composition includes the BH (tablet form «T-Sept®»), «Proteflazid®» drops, as well as gel-based sodium alginate, nipagin and water for injections [13].

THE AIM

Different gels composed of benzydamine and flavonoids that were developed for treatment of the periodontal diseases in the orthodontic patients

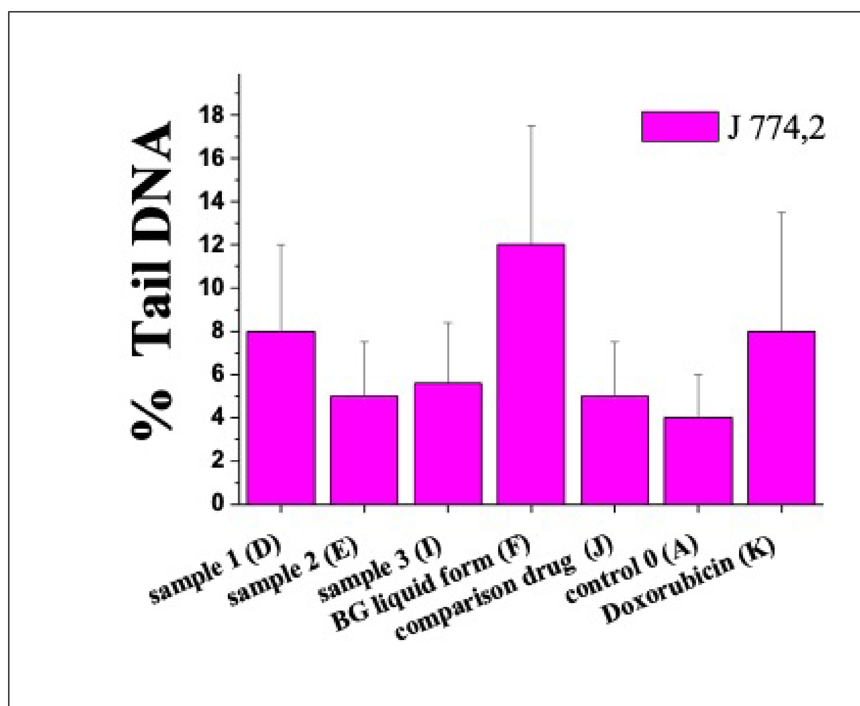

Fig. 3. The results of testing genotoxic effect of studied samples of various gel compositions introduced in the medium (0.5% dose in volumetric units) with cultured murine macrophages of J774.2 line (results are presented as a M ± m of 250 comets for each sample).
Quantitative evaluation of the results shown in Fig. 2.
Note: Control 0 – intact native cell culture, A (Table I); Comparison drug – «Cholisal», Elfa, Poland, (gel was added in the final concentration = 1%)), J (Table I); Toxicological (positive) control – Doxorubicin, 1 μg/ml (Kyivmedpreparat, Ukraine), K (Table I)

will be compared regarding their effects on survival (MTT-testing) of the mammalian cells of various tissue origin (fibroblasts, keartinocytes and macrophages).and the DNA intactness (DNA-comet assay) in treated cells.

MATERIALS AND METHODS

We compared the effect of different variants of gel composition «Benzidaflaziverdine» on the viability of mouse fibroblasts of the BALB-3T3 line, mouse macrophages of the J774.2 line, and human keratinocytes of the HaCaT line. Cell viability was assessed using MTT-reagent and genotoxic effects in treated cells were studied using DNA comet assay.

The components of the studied gel compositions which differed in the ratio and shape are presented in Table I. The applied samples included sodium alginate, nipagin and water for injections - gel base, BH in solution («Tantum Verde®») and its tablet form («T-Sept®»), drops «Proteflazid®». The untreated mammalian cells used as zero control were: murine fibroblasts of BALB-3T3 line, human keartinocytes of HaCaT line and murine macrophages of J774.2 line. Culturing of cells was performed in DMEM medium (Sigma Chem Co., USA) in the presence of decomplemented serum of cow embryos (Sigma Chem Co., USA) and 50 μg/ml gentamycin (Sigma, Chem Co., USA) in a thermostate with 5% CO_2 content at 37° C. Cells were sub-cultured every 2-3 days at 500 thousand to 1 million cells per 1 ml of culture medium [14, 15]. The number of viable cells was determined using a test with the MTT reagent according to the manufacturer's recommendations (Sigma, Chem Co., USA) [16, 17]. The principle of measuring the viability of cells is based on the ability of mitochondrial dehydrogenases of living cells to transform the unstained form the of the MTT reagent (3- (4,5-dimethylthiazol-2-yl) -2,5-dimethyl tetrazolium bromide, Sigma-Aldrich, USA) to crystalline blue formazan soluble in the dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA).

The main drug for comparison was a gel for topical treatment of the oral mucosa «Cholisal» (Jelfa S.A. Poland), whose active substances are: choline salicylate and cetalconium chloride. Doxorubicin (Kyivmedpreparat, Ukraine) was used as a prooxidant and Alfatocopherol (Technologist CJSC, Ukraine) – as an antioxidant.

To determine the cytotoxic effect of studied substances using the MTT-test, cells were seeded in 96-well plates (Greiner Bio One, Germany) at a concentration of $2x10^5$ / 100 μl in culture medium in the presence of 10% serum of cow embryos. After that, the studied substances were applied in different concentrations and kept for 72 h. 3 h before the end of the incubation time of the cells with the test substances in the medium was added 0.5% aqueous solution of MTT-reagent to a final concentration of 0.5 mg / ml. After incubation of the cells with the MTT reagent, the DMSO 100 μl was added to the wells in order to dissolve the formazan crystals formed during the reduction of the MTT-reagent by the viable cells. The concentration of formazan in wells was determined photometrically on a microphotometer «Plate Reader BioTek» 76883 (BioTek, USA) by measuring optical absorption at 490 nm. The ration of viable cells

Table I. Chemical components of gel compositions under study and preparations for comparison

Samples	Variants of the gel composition «Benzidaflaziverdine» and other tested substances
A	control (native cell culture)
B	DMSO (final concentration - 0,1%)
	Components of the gel composition (composition was added in the final concentration = 1%)
C	gel base – sodium alginate 5% (0.5 ml), nipagin (0.01 ml), water for injections (9.5 ml)
D	**Sample 1** – gel base (sodium alginate 5% (0.5 ml), nipagin (0.01 ml) water for injections (7.5 ml)), «Tantum Verde®» solution 0.15% (2 ml)
E	**Sample 2** – gel base (sodium alginate 5% (0.5 ml), nipagin (0.01 ml) water for injections (8.7 ml)), powder/tablet «T-Sept®» (0.73 g)
F	gel base (sodium alginate 5% (0.5 ml), nipagin (0.01 ml), water for injections (8.1 ml)), «Tantum Verde®» solution 0.15% (1 ml), powder/tablet «T-Sept®» (0.365 g)
G	gel base (sodium alginate 5% (0.5 ml), nipagin (0.01 ml), water for injections (8 ml)), «Proteflazid®» (1.5 ml)
H	gel base (sodium alginate 5% (0.5 ml), nipagin (0.01 ml), water for injections (6.6 ml)), «Tantum Verde®» solution 0.15% (1 ml), powder/tablet «T- Sept®» (0.365 g), «Proteflazid®» (1.5 ml)
I	**Sample 3** (patented gel composition «Benzidaflaziverdine») – gel base (sodium alginate 5% (0.5 ml), nipagin (0.01 ml, water for injections (8 ml)), powder/tablet «T-Sept®» 0.73 g), «Proteflazid®» (1.5 ml)
J	Cholisal (gel was added in the final concentration = 1%)
K	Doxorubicin (final concentration – 1 μg/ml)

Table II. DNA damage effect in murine J774.2 macrophages under their treatment (72 h) with various substances under study (% Tail DNA and Olive Tail Moment)

Variants of gel composition «Benzidaflaziverdine» and other substances											
Substances (see: Fig. 2)	A	B	C	D	E	F	G	H	I	J	K
Parameters, percentage of DNA in the Tail	4%	8%	6%	8%	5%	7%	3%	12%	6%	5%	8%
Olive Tail Moment	0.33	0.33	0.69	1.3	0.4	0.61	0.25	0.48	0.4	0.56	0.57

was expressed as a percentage relative to the control (untreated cells), which was taken as 100% [14, 15].

GENOTOXICITY TESTING

The method of DNA-comets under alkaline conditions was used [18, 19]. Cells (1 million / ml) were incubated for 24 h with test samples. The cell suspension (3×10^4) was mixed at 37 ° C with 250 μl of 0.5% fusible agarose solution (Sigma, USA) and applied in a thin layer on slides, which were pre-coated with 1% agarose solution (Sigma, USA) and dried. Cell lysis and electrophoresis were performed, as described [19]. DNA comets were stained with the Ethidium bromide (10 μg / ml) and examined under a Carl Zeiss fluorescence microscope (Germany). Assessment of DNA damage was performed using the program «CASP» 1.2.2 (CASPlab, Wroclaw, Poland), comparing the average values (%) of DNA in the tail of 250 comets for each sample [18, 19]. Calculation of the relative units of the Olive Tail Moment (OTM) for quantification of the induced DNA fragmentation (damage) was performed as: OTM = (Tail.mean - Head. mean) × (Tail % DNA)/100.

Bioethics was kept in accordance with requirements of the basic bioethical provisions of the European Convention on Human Rights and Biomedicine (from 04.04.1997) and Helsinki Declaration of the World Medical Association on the ethical principles for medical research involving human subjects (1964-2008). Protocol N9 dated by 21.12.2020 and Protocol N8 dated by 18.10.2021were approved by the Committee on BioEthics of scientific research, experimental development and scientific works at Danylo Halytskyi Lviv National Medical University.

STATISTICAL PROCESSING OF RESULTS

The experiments were performed in three parallels in each variant. Each indicator shown in the figures (the ordinate of the columns in the diagrams) corresponds to the average value of «M» calculated from the results of three measurements in one of several experiments of the same type. The error «m» of the obtained result was calculated by the value of the root square error «σ». This is represented on the illustration near each indicator by a vertical line, the length of which corresponds

to the value of «m». The computer analysis was based on Phenom II X4 975 with Windows 7 64-bit operating system (Microsoft, USA). Statistical processing of the obtained data was performed in MS Excel 2010 (Microsoft, USA). The P-value of ≤0.05 was considered as statistically significant.

RESULTS

Various modifications of gel composition «Benzidaflaziverdine» and drugs for comparison (Samples A-K: see Table I) were studied. The application of methods for assessing viability in cell populations and estimating genotoxic effects (DNA comet assay) showed that Substance C was toxic to all used cell lines. The level of its cytopathic effect is comparable to the effect of antitumor drug Doxorubicin (Substance K) at a concentration of 1 μg per 1 ml of culture medium. These results are presented in Figures 1 and 2.

Substance D was found to inhibit the viability of BALB-3T3 fibroblasts and even stronger inhibition was observed for J774.2 macrophages, while HaCaT keratinocytes were ersistant to the action of this substance. Substances E, F, G, H and J («Cholisal» – the drug for comparison) did not have a statistically significant effect on keratinocytes and fibroblasts, but inhibited the macrophage of J774.2 line. It should be noted that the inhibitory effect of «Cholisal» (Substance J) towards J774.2 macrophages was more pronounced than such effect of highly toxic anticancer drug Doxorubicin (Substance K) that was used at 1 μg per ml dose. An additional study is necessary to answer a question whether this action «Cholisal» was cytotoxic, cytostatic, or functionally suppressive.

On Figure 1, a quantitative assessment of the impact of studied substances on the viability of various pseudonormal mammalian cells *in vitro* is demonstrated. As one can see, the action of Sample 3 (I) which is a patented gel composition «Benzidaflaziverdine» containing gel-base + «Proteflazid®» + «T-Sept®», was the least inhibitory for the macrophages, with acceptable characteristics of its effect on the keratinocytes and fibroblasts. It might be suggested that this Sample 3 (I) possessed the highest regenerative potential for tissue cells as compared to Sample 2 (E), Sample 1 (D) and the «Cholisal» (J) – drug for comparison. Further studies are required to explain why the «Cholisal» is so toxic for the mammalian macrophages.

According to the results of the MTT-testing, it was found that sample D (Sample 1), which included gel base and BH in liquid form («Tantum Verde®»), was toxic for all cell lines, inhibited the viability of fibroblasts of the BALB-3T3 line and even more – the macrophages J774.2, but did not influence significantly on the keratinocytes of HaCaT line. The sample E (Sample 2), which contains the BH in the form of powder («T-Sept®»), did not have a significant effect on the keratinocytes and fibroblasts, but suppressed the viability of the macrophages of J774.2 line. It should be noted that the comparison drug «Cholisal» (J) was even stronger inhibitor of macrophages of J774.2 line than the Doxorubicin used at 1 μg per ml dose.

Since the macrophages of J774.2 line were found to be most sensitive to cytotoxic effect of the proposed samples of various gel compositions, these mammalian cells were selected for estimation of the DNA damaging effect of those compositions. In general, the most cytotoxic samples were also found to be the most genotoxic ones. The patented by us gel composition I (Sample 3) that did not demonstrate a pronounced cytotoxic effect, was also lacking the genotoxic effect in the macrophages of J774.2 line (Figure 2, Table II). This effect was measured as a percentage of DNA in the comet tails and as a value of the Olive Tail Moment (OTM).

Different variants of gel compositions, namely sample C (gel base), Sample 2 (E – gel base + «T-Sept®»), sample G (gel base + «Proteflazid®»), Sample 3 (I – («Benzidaflaziverdine» – gel-base + «Proteflazid®» + «T-Sept®») and the comparison drug «Cholisal» (J) were compared regarding their ability to cause the DNA damage in murine macrophages of J774.2 line. We did not reveal any significant genotoxic effect in these cells. The indicators of DNA damage estimated as a percentage of DNA in the tail were 6%, 5%, 3%, 6%, 5%, respectively, and 4% in control, and the values of the Olive Tail Moment (OTM) were 0.69, 0.4, 0.25, 0.4, 0.56 relative units, respectively, and 0.33 relative units in control.

The maximum amount of DNA in the comet tail (12%) was observed in cells treated with the gel composition H, which contained BH both in powder form and in liquid form in a residual amount. At the same time, the patented gel composition I («Benzidaflaziverdine») (Sample 3) caused significantly (2 times) less DNA damage (Table II). Thus, the proposed gel composition I (Sample 3), as well as the gel composition G («Proteflazid®» in combination with a gel base) demonstrated the DNA protecting action (Table II, Figures 2 and 3).

In Figure 3, quantitative evaluation of the results presented in Figure 2 is shown.

As can be seen from the results presented in Figure 3, the studied gel compositions were not genotoxic (% of DNA in Comet Tail and Olive Tail Moment) for murine macrophages of J774.2 line. Those biotolerance indicators (level of cell viability suppression and genotoxic effect) were comparable with the effect of «Cholisal» used in the experiments as a drug for comparison.

DISCUSSION

Application of the orthodontic treatment in patients with predisposition to periodontal diseases is an important step, because the use of fixed orthodontic techniques may cause stress-induced metabolic disorders in the periodontal tissues due to the links between the oxidative stress and inflammatory response. As a result, redox status may provoke a progressive loss of the periodontal tissues, in particular, bone tissue of the alveolar process [20-24].

That is why the development of novel complex medicines for treatment of patients with periodontal diseases that in addition to dental tissue protecting agents also include the biologically active agents of 2nd line of defence (supportive treatment) is of great significance in dentistry and periodontology.

The «Proteflazid®» (LLC NKV «ECOPHARM», Ukraine) is recommended for treatment of viral, bacterial and fungi infections. According to the manufacturer's instructions, it is based on flavonoids and possesses immunotropic properties, protects mucous membranes, and normalizes local immunity (levels of lactoferrin, secretory immunoglobulin A, lysozyme and C3 component of complement). This drug is used in the form of drops. It is an inducer of synthesis of endogenous α- and γ-interferons to physiologically active levels. It increases the non-specific resistance of the body to viral and bacterial infections, has antioxidant activity, does not demonstrate immunotoxic effects and does not cause the refractoriness (hyporeactivity) of the immune system.

Along with flavonoids, a number of the non-steroidal anti-inflammatory drugs (NSAIDs) is recommended for topical use in dentistry and periodontology are available on the pharmaceutical market. Although they are less effective than steroids, but they are not as toxic as the steroid hormones. In our study, we addressed our attention to the benzydamine hydrochloride (BH) that is a low-toxic non-steroidal drug with high lipophilicity. It penetrates well into the sites of inflammation, where the pH is lowered, and, accordingly, creates a therapeutic concentration there, accelerating the reparative processes. The severity of the local analgetic effect of the benzydamine provided by a direct membrane-stabilizing effect on sensitive nerve endings, exceeds similar action of most NSAIDs. In addition, BH demonstrates the ability to inhibit the adhesion of leukocytes to vascular endothelium, blocks platelet adhesion factor, improves capillary permeability, which ensures its vasoprotective effect [25]. The antibacterial action of benzydamine is caused by its rapid penetration through the membranes of microorganisms, damage of their cellular structures, disruption of the metabolic processes and cell lysis. The fungicidal action of the BH is realized via structural modifications of fungi cell wall and metabolic chains of the mycelium, thus, preventing their reproduction, against 20 strains of fungi of *Candida albicans* and *Candida tropicalis*, as well as *Aspergillus niger*. The representatives of the BH-based drugs are «T-Sept®» lozenges (ICN, Polfa, Poland) and «Tantum Verde®» mouthwash (Angelini Francesco A.C.R.A.F, S.p.A., Italy) [26, 27].

Here, we conducted a comparative study of cyto- and genotoxicity of different variants of gel compositions proposed for the treatment of the periodontal diseases in the orthodontic patients. The results of our study demonstrated that the composition G («Proteflazid®» in combination with gel base) and composition I (Sample 3 – «Benzidaflaziverdine») were the least toxic to the mammalian cells of various tissue origin. Besides, these compositions were found to possess the highest DNA protective activity that was evidenced by the lowest degree of DNA damage (normal shape of nuclei with minimal damage) compared to control (untreated cells).

Other applied gel compositions were less biocompatible and the comparison drug «Cholisal» was even more suppressive for the mammalian macrophages than composition G and composition I. Additional study is necessary to explain whether the action of «Cholisal» (comparison drug, J) is cytotoxic, cytostatic, or inhibits the functional activity of cells.

Composition I (Sample 3 – «Benzidaflaziverdine») composed of the flavonoids and non-steroidal anti-inflammatory agents of non-steroidal nature was sufficiently biotolerant. In other investigations [28, 29] using experimental models of rodents, a diverse effect of flavonoids on periodontal cells and tissues was found, including regulation of the inflammatory response and potential conservative effects in periodontal ligaments and bone tissue of the jaws.

It can be assumed that the effect of flavonoids is beneficial in combination with the nonsteroidal anti-inflammatory agent benzydamine hydrochloride on various types of periodontal cells, including gum epithelial cells, gum fibroblasts and periodontal ligament fibroblasts, and on osteoblasts. Therefore, the flavonoids in a combination with benzydamine hydrochloride in powder form are a promising clinical tool in the prevention and treatment of gingivitis and periodontitis. Gel composition proposed in our study might be recommended for use in clinical periodontology, in particular for treatment of periodontal pathology in orthodontic patients, both in preparation for orthodontic treatment and in its active period.

CONCLUSIONS

It was found that the presence of flavonoids in the gel composition «Benzidaflaziverdine» minimizes the cytotoxic

and genotoxic action of benzydamine hydrochloride, which allows to more fully realize its antimicrobial and anti-inflammatory action. We do not recommend the benzydamine hydrochloride in liquid form, in contrast to the tablet drug, for inclusion in prolonged forms of gel compositions used for the local treatment of gingivitis and periodontitis.

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Conflict of interest:
The Authors declare no conflict of interest.

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Received: 27.06.2022
Accepted: 06.08.2023

A - Work concept and design, **B** – Data collection and analysis, **C** – Responsibility for statistical analysis, **D** – Writing the article, **E** – Critical review, **F** – Final approval of the article

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