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Study of cytocompatibility of different supplements in gel compositions used for the treatment of periodontal diseases in orthodontic patients via exploration of mechanisms of their cytopathic and cytoprotective effects in cultured human and mouse cells

Oleg Hodovanyi¹, Natalya Chukhray¹, Olesia Martovlos¹, Olha Klyuchivska², Iryna Ivasechko², Rostyslav Stoika¹,²

Introduction. A search continues for effective means which may reduce the overload of harmful factors, eliminate the inflammatory process, and reduce stress on the periodontal tissues during the active period of orthodontic treatment. We developed and patented the gel composition (GC) Benzidaflaziverdine prepared based on Proteflazid® (flavonoids) and benzydamine hydrochloride (BH) T-Sept® for the local treatment of the periodontal tissues in the form of a periodontal dressing in the orthodontic patients.

The aim of this study was to evaluate the cytocompatibility of different combinations of components in gel composition based on flavonoid complex and benzydamine hydrochloride (Benzidaflaziverdine) used for the treatment of periodontal diseases in orthodontic patients. For this, mechanisms of their cytopathic and cytoprotective effects will be explored using cultured human and mouse cells.

Methods. We studied the effect of different supplements used in GC Benzidaflaziverdine on the viability of pseudomonar human keratinocytes of the HaCaT line and mouse fibroblasts of the BALB-3T3 line, and mouse macrophages of the J774.2 line. Various methods of cell survival assessment were used: MTT-assay, staining of cells with fluorescent dyes Hoechst 33342 and Propidium iodide (PI), as well as a test for the genotoxic effects on cells (DNA comet assay). The antioxidant properties of the developed GC variants were evaluated using DPPH (1,1-diphenyl-2-picrylhydrazyl), Merck (Damstadt, Germany), and DCFDA-H2 (2',7'- dichlorodihydrofluorescein diacetate).

Results. We demonstrated that the Sample containing gel base and BH in the form of a solution (Tantum Verde®) possessed weak prooxidant properties. While the Sample contained gel base, powdered BH (T-Sept®) and Sample containing gel base and powdered BH (T-Sept® and Proteflazid®) possessed pronounced antioxidant properties.

Conclusions. Tests with DPPH and DCFDA dyes were used to confirm the hypothesis regarding the cytoprotective effect of the patented gel composition Benzidaflaziverdine for local application in the form of a periodontal bandage due to the antioxidant activity of the flavonoid complex, which reaches the maximum level at the 2nd hour of exposure. This gel composition can be recommended for use in clinical periodontology for medical support of orthodontic patients before and during the active phase of orthodontic treatment.

Keywords: Flavonoid complex, Proteflazid®, benzydamine hydrochloride, gel composition, antioxidant activity, cell culture, periodontal diseases, orthodontic patients.
Lviv National Medical University.

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Introduction

In 65–98% of cases, diseases manifesting in the periodontal tissues in adults are accompanied by a loss of bone tissue of the alveolar processes of the jaws with the formation of dental-jaw deformations due to tooth migration and worsening of dental-jaw defects. In the active period of orthodontic treatment, such factors as the inflammatory process development, orthodontic forces, and occlusive trauma may negatively affect tissue barriers of the periodontal complex. In orthodontic patients with a tendency to develop gingivitis and periodontitis, these processes provoke recurrent abscesses and rapid destruction of the periodontal tissues, which worsens the course and prognosis of the pathological process [1-4].

The role of oxidative and mechanical stress is essential in the pathogenesis of periodontal tissue diseases [5-8]. During the orthodontic treatment, the applied orthodontic forces are transmitted through the tense tissue matrix to local cells of the periodontal ligament and alveolar bone. This stimulates them to release pro-inflammatory, angiogenic, and osteogenic agents [9, 10]. The negative impact of bracket systems is manifested, first of all, in soft tissues of gums as the development of hyperplasia of the papillae and swelling. Therefore, a search continues for effective means which may reduce the overload of harmful factors, eliminate the inflammatory process, and reduce stress on the periodontal tissues during the active period of the orthodontic treatment [4, 11, 12].

It is known that the main periodontopathogens induce a rapid development of microbial strains resistant to classical antibiotics. That is why our research was directed towards a search for alternative options for combating aggressive microbiota using herbal preparations that have anti-inflammatory and antimicrobial effects for the periodontal ligament and alveolar jaw bones [13-16]. Plant compounds, particularly flavonoids, are widely used along with chlorhexidine in periodontal therapy. flavonoids also demonstrated an antioxidant effect, as their phenolic structure makes it possible to interact with free radicals, reducing the intensity of lipid peroxidation (LPO), measured as malondialdehyde [16-18].

In view of the aggravated situation with Covid-19 and the relevance of this problem for dentistry, we addressed an antiviral drug – the flavonoid complex Proteflazid®. It has an immunomodulatory effect, inhibits free radical processes, prevents the accumulation of lipid peroxidation products, reduces intoxication, and promotes body recovery after infection and adaptation to adverse environmental conditions [19, 20].

To strengthen Proteflazid® properties and ensure pronounced analgesic, anti-exudative and antimicrobial effects, an anti-inflammatory non-steroidal agent for local use, which belongs to a group of indazoles – benzydamine hydrochloride (BH) – was chosen. Effective drugs based on benzydamine hydrochloride are produced as Tantum Verde® oral cavity rinsing solution and T-Sept® tablets for absorption [21, 22].

BH is consumed in the form of T-Sept® tablets (1 tablet contains 3 mg of BH) and belongs to the non-steroidal anti-inflammatory drugs (NSAIDs) for local application in the oral cavity. In terms of intensity of the action, they are inferior to steroids but possess very low toxicity and have several advantages. Non-steroidal anti-inflammatory drugs act mainly during phases of exudation and proliferation, as they function as inhibitors of the cyclooxygenase (COX) enzyme, which affects the arachidonic acid with the formation of important mediators of inflammation and pain – prostaglandins and thromboxanes, whose concentration increases based on the disease severity. The lipoxygenase metabolism pathway of the arachidonic acid leads to the lipoxygenase formation – 5-LOG (lipoxygenase). Most NSAIDs can selectively inhibit two forms of this enzyme – COX-1 and COX-2, which weakens hyperemia, swelling, and pain. NSAIDs contribute to the normalization of the microcirculation process. Via the suppression of thromboxane synthesis, reducing the hyaluronidase activity, and blocking serotonin receptors in blood vessels, they prevent the formation of microthrombi. NSAIDs uncouple oxidative phosphorylation, slowing down the formation of macroergic connections due to their effect on the adenosine triphosphate in the tissues of the inflammation focus. NSAIDs have little effect on the alteration process, although, to some extent, they can weaken the generation of toxic radicals that contribute to the development of the inflammatory process [23, 24].

We developed and patented the gel composition (GC) Benzidaflaziverdine prepared based on Proteflazid® and T-Sept® for the local treatment of periodontal tissues in the form of a periodontal dressing in orthodontic patients. This composition also included a gel base – sodium alginate, nipagin, and injection water [25].

The aim of this study was to evaluate the cytocompatibility of different combinations of components in gel composition based on flavonoid complex and benzydamine hydrochloride (Benzidaflaziverdine) used for the
treatment of periodontal diseases in orthodontic patients. For that, mechanisms of their cytopathic and cytoprotective
effects will be explored using cultured human and mouse cells.

**Materials and Methods**

We studied the effect of different variants of gel composition (GC) Benzidaflaziverdine on the viability of
pseudonormal human keratinocytes of the HaCaT line, as well as mouse fibroblasts of BALB-3T3 line and mouse
macrophages of J774.2 line. Various methods of cell survival assessment were used: MTT-assay [26-28], staining of
cells with fluorescent dyes Hoechst 33342 and Propidium iodide (PI) [29, 30], as well as a test for the genotoxic
effects on cells (DNA comet assay) [30, 31]. The antioxidant properties of the developed GC variants were evaluated using
DPPH (1,1-diphenyl-2-picrylhydrazyl) reagent, Merck (Dam-stadt, Germany) [32-34] and DCFDA-H2 (2',7'-
dichlorodihydrofluorescein diacetate), Molecular Probes™ produced for the Thermo Fisher [29, 32].

We studied GC samples that differed in the ratio and shape of the components (Table 1). The samples included
alkaline alginate, nipagin, and water for injections – base gel, benzylamine hydrochloride (BH) in the form of the
solution (Tantum Verde®, Angelini Francesco A.C.R.A.F S.p.A., Italy) and tablet (T-Sept®, ICN, Polfa, Poland),
Proteflazid® drops (LLC NKV ECOPHARM, Ukraine). Untreated cell culture was used as a zero control. The gel used
for the local treatment of the oral cavity’s mucous membrane, Cholisal (Jelfa S.A. Poland), whose active substances are
choline salicylate and cetalkonium chloride, was applied as a comparison drug. Doxorubicin (Kyivmedpreparat,
Ukraine) was used as a prooxidant, while the alpha-Tocopherol (Tehnologi ZAO, Ukraine) and the Ascorbic acid
(Darnytsia, Kyiv, Ukraine) as antioxidants, were used as comparison drugs.

**Table 1:** Chemical components of gel compositions under study and preparations for comparison

| Samples    | Varieties of the gel composition Benzidaflaziverdine and other tested substances
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>zero control – native cell culture</td>
</tr>
<tr>
<td><strong>Components of the gel composition</strong> (composition was added in the final concentration = 1%)</td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>gel base (sodium alginate 5% (0.5 ml), nipagin (0.01 ml), water for injections (7.5 ml)), Tantum Verde® solution 0.15% – BH solution (2 ml)</td>
</tr>
<tr>
<td>Sample 2</td>
<td>gel base (sodium alginate 5% (0.5 ml), nipagin (0.01 ml), water for injections (8.7 ml)), T-Sept® tablet – powdered BH (0.73 g)</td>
</tr>
<tr>
<td>Sample 3</td>
<td>(patented gel composition Benzidaflaziverdine) – gel base (sodium alginate 5% (0.5 ml), nipagin (0.01 ml, water for injections (8 ml)), T-Sept® tablet – powdered BH 0.73 g), Proteflazid® drops – flavonoid complex (1.5 ml)</td>
</tr>
<tr>
<td>Cholisol</td>
<td>comparison drug – gel was added in a final concentration of 1%</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>toxicological control – used in a final concentration of 1 μg/ml</td>
</tr>
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Cell culturing was carried out in the Dulbecco-modified Eagle’s medium (DMEM) (Sigma Chem Co., USA) in the
presence of the decomplemented fetal bovine serum (Sigma Chem Co., USA) and 50 μg/ml gentamicin (Sigma, Chem
Co., USA) in a thermostat with 5% CO2 content at 37°C and 100% humidity. Cells were subcultured every 2–3 days at
0.5–1 million cells per 1 ml of culture medium [26, 27]. The number of living cells was determined with the MTT
assay, using the manufacturer’s recommendations (Sigma, Chem Co., USA) [28]. The measurement principles are based on the ability of the mitochondrial dehydrogenases of living cells to reduce colorless forms of the MTT-reagent [3-(4,5-di-methylthiazol-2-yl) - 2,5 – dimethyl bromide tetrazolium, Sigma-Aldrich, USA)] to blue crystalline formazan which is soluble in the dimethyl sulfoxide.

Cytomorphological study of mammalian cells. To qualitatively determine the viability of cells in vitro, double staining of cultured cells with the fluorescent dye Hoechst 33342 (Sigma-Aldrich, USA, Ex/Em: 350-354/460-470 nm) and Propidium iodide (Sigma-Aldrich, USA, Ex/Em: 520-538/617-618 nm) was used. The first dye stains the cell nucleus, while the second one serves as an indicator of the permeability and damage of the cell plasma membrane. Hoechst 33342 dye was added for 30 min to cells at a final concentration of 20 μg/ml before the addition of Propidium iodide solution used in a final concentration of 2 μg/ml.

DCFDA-H2 (2,7-dichlorofluorescein diacetate, Ex/Em: ~470-510/525-575 nm) is a fluorescent dye sensitive to hydrogen peroxide (H₂O₂). It was used to measure the reactive oxygen species (ROS) content in cells treated with studied samples. To determine the generation of H₂O₂, tested samples and substances were added, after which (in 30 min) DCFDA-H2 (10 μM, 30 min) and Hoechst 33342 (final concentration 30 μg/ml) were added. After cell incubation with the applied drugs, cell luminescence was examined on a Zeiss Axiosmager A1 microscope (Carl Zeiss, Germany) and photographed with a digital camera in the microscope kit at a magnification of ~400 times in the corresponding excitation and emission fields. Image processing was performed using AxioVision software (customized by Carl Zeiss Microscopy GmbH, Königsallee 9-21, 37081 Göttingen, Germany; photography) and Image Pro Plus 7.0 (Media Cybernetics, USA; image and color composition) [29].

Genotoxicity testing. The method of DNA comets under alkaline conditions was used [26, 32]. Cells (1 million / ml) were incubated for 72 h with test samples. The cell suspension (3 × 10⁴) 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 [30, 31]. The determination principle is based on the fact that during alkaline electrophoresis, single-strand breaks in DNA turn into double-strand breaks, which allows for the assessment of generalized genotoxic effects. DNA comets were stained with the Ethidium bromide ((Sigma-Aldrich, USA, Ex/Em: ~520-538/617-618 nm, 10 μg/ml) and examined under a Carl Zeiss fluorescence microscope (Germany) [30]. DNA damage was assessed using CASP 1.2.2 software (CASPlab, Wroclaw, Poland), comparing the average values (%) of DNA in the tail of 250 comets for each sample.

The total pro-/anti-oxidant activity was measured in solutions of tested GC samples and comparison drugs (Table 1) in the presence of the DMSO introduced into the wells of a 96-well plastic dish in the amount of 10 μl per well. The DPPH substrate (2,2-Diphenyl-1-picrylhydrazylradical) was prepared as a 0.01% solution in the DMSO. The substrate was introduced into wells with tested substances immediately before the solution’s optical density measurement began using a multichannel microphotometer Plate Reader BioTek 7688 (BioTek, USA) at 490 nm. Optical density was measured in 20 min, 1, 2, and 3 h. The percentage of change in the solution’s optical density was determined based on the formula: (%) = [(A₀-A₁)]/(A₀)]×100. A solvent (DMSO) was used as a zero control [33, 34].

Bioethical rules were observed following the requirements of basic bioethical provisions of the European Convention on Human Rights and Biomedicine (from 04.04.1997) and the Helsinki Declaration of the World Medical Association on the ethical principles for medical research involving human subjects (1964-2008). Protocol No. 9, dated December 21, 2020, and Protocol No. 8, dated October 18, 2021, were approved by the Committee on Bioethics of scientific research, experimental development, and scientific works at Danylo Halytsky Lviv National Medical University.

Statistical processing of results. Experiments were performed in three parallels in each variant. Each indicator shown in the figures (the ordinate of the diagram columns) corresponds to the average value of “M” calculated based on the results of three measurements in one of several experiments of the same type. The M error of the obtained results was calculated as the root square error of the “σ” value. The computer analysis was based on Phenom II X4 975 on Windows 7 64-bit operating system (Microsoft, USA). Statistical processing of the obtained data was performed in MS Excel 2010 (Microsoft, USA).
Results

Fig. 1 (A) presents the results of assessing the survival level of cells of different histological origins under the influence of studied substances and GC samples. The cytotoxic effect of Sample 1 (Table 1) containing the liquid form of the BH Tantum Verde® and the comparison drug Cholisal towards the macrophage culture was revealed. It was comparable to the effect of the anticancer drug Doxorubicin used in a therapeutic dose. At the same time, no cytopathic effect was observed on fibroblasts and keratinocytes.

The results of the genotoxicity assessment (see Fig. 1, B) showed the presence of a DNA-damaging effect of GC acting with the liquid form of Tantum Verde® in the absence of a pronounced suppressive effect in the case of Cholisal comparison drug and the antitumor antibiotic Doxorubicin used in a therapeutic dose. Medians of the Olive tail moment (OTL) (tail DNA% versus nucleus of comet DNA%) were used for assessing the DNA damage.

Figure 1. Comparison of viability rates measured with the MTT-assay in various lines of pseudonormal mammalian cells: murine fibroblasts of BALB-3T3 line, human keratinocytes of HaCaT line, and murine macrophages of J774.2 line. A – MTT-assay, B – DNA-comet assay

Notes: Control 0 – intact native cells (Table 1); Comparison drug Cholisal gel was added in the final concentration of 1% (Table 1); Toxicological (positive) control – Doxorubicin, 1 µg/ml (Table 1) The significance of difference with the non-treated control cells: * P<0.05; *** P<0.001

Fig. 2 presents differential interference contrast images (DIC) of cells treated with Sample 3 (Table 1) and stained with fluorescent DNA-specific dye Hoechst-33342 (visualized in blue – alive cells) in combination with the fluorescent DNA/RNA-specific dye Propidium iodide (dead cells are visualized in red).
Figure 2. Fluorescence images of treated cells demonstrating the bio-tolerance effect. Hoechst 33342 & PI staining of cells treated for three days with Sample 3. Blue fluorescence (Hoechst 33342) – living cells and red fluorescence (PI) – dead cells.

Presented images visualize GC effects – Sample 3 (patented gel composition) on three types of cells – human keratinocytes of the HaCaT line, mouse fibroblasts of the BALB3T3 line, and mouse macrophages of the J774.2 line. The absence of red staining in treating J774.2 macrophage cells and HaCaT keratinocytes means the lack of a toxic effect. A certain amount of red staining in the BALB-3T3 fibroblast cell line may indicate the stimulation of proliferation simultaneously with increased cell death. This may be due to the phenomenon of contact inhibition of growth resident in pseudonormal cells in culture.
Based on the results of cytomorphological studies and MTT and DNA comet assay, the question arises about defining the potential mechanisms of observed phenomena in the studied samples’ actions. Determining the pro- and antioxidant properties of the studied variants of GC and other substances noted in Table 1 suggests the ability of studied Samples 1, 2, 3 and comparison substances to capture free radicals or serve as Hydrogen donors (Fig. 3).

According to obtained results, Sample 1 showed weak prooxidant properties (−1.6 CU of the Conventional Units (CU)) to 0.5 CU). Thus, the BH in a liquid form, as opposed to the tablet preparation, should not be recommended for inclusion in case of prolonged forms of GC.

Samples 2 and 3 demonstrate antioxidant properties that increase over time (for the 2nd sample – from 0.98 CU to 3.9 CU at the 2nd hour and 7.6 CU at the 3rd hour). The antioxidant properties of Sample 3 reach their maximum level at the 2nd hour (13.3 CU), and by the 3rd hour, they start decreasing (7.2 CU).

Samples 1, 2, 3, Cholisal, and alpha-tocopherol were used as antioxidants, and Doxorubicin was used as a prooxidant. The method of rapid measurement of the antioxidant capacity of samples using the Diphenyl-picolyl-hydrazyl reagent was applied to test the ability of studied compounds to act as free radical scavengers or hydrogen donors. The diagram in Fig. 3 presents the results of the quantitative evaluation of anti- and prooxidant properties of our developed variants of GC and the comparison substance. Based on these data, one can see that Sample 3 proved to be the most pronounced antioxidant. The maximum of its effect occurs at the 2nd hour of action. Sample 2 is a weaker antioxidant that reacts slower. Sample 1 proved to be a prooxidant, while Doxorubicin was a strong oxidant. Cholisal has shown a weak antioxidant activity comparable to that of alpha-Tocopherol.

Alpha-Tocopherol was used as an antioxidant control because the temporal dynamics of its effect were comparable to the biological effects of tested samples. The Ascorbic acid reacts at high speed with dynamics within a few seconds to a minute, and quercetin and rutin, respectively, react within several hours. Their maximum effect develops within a day.

To visualize the studied phenomena, the presence of peroxide radicals in target cells was detected under the influence of the studied samples. For this, the fluorescent dye dihydrochlorofluorescein diacetate (DCFDA-H2), specific for the hydrogen peroxide, was used (Fig. 4).
Figure 4. The cytomorphological testing results of hydrogen peroxide formation using Hoechst 33342 and DCFDA-H2 fluorescent dyes in cultured J774.2 macrophages treated with Sample 1, Sample 2 and Sample 3 on the 3rd day of treatment. Blue fluorescence (Hoechst 33342) – the nucleus of living cells, green fluorescence (DCFDA-H2) – generated H₂O₂.

The images in these figures demonstrate the biological effect of three samples of our best design – Sample 1, 2, and 3 (Table 1) on macrophages treated for three days. The nucleus of living cells is visualized in blue fluorescence, while green fluorescence indicates the free radical concentration. It should be noted that in our experiments, Samples 2 and Sample 3 acted as antioxidants. The appearance of more intensive green fluorescence may suggest the activation of macrophages which observed in the case of the action of Sample 1.
Discussion

Summarizing the results obtained regarding samples developed by us, as well as the comparison drug Holisal, it was found that they do not affect dense tissues represented by keratinocyte and fibroblast lines. In contrast, macrophages were sensitive to the suppressive effect of Cholisal and Samples 1 and 2. It can be assumed that Proteflazid® stops the suppressive effect of other sample components on cells of the macrophage line. Summarizing the obtained results, it can be stated that Sample 1 (gel base, BH in the form of a solution (Tantum Verde®)) demonstrated weak prooxidant properties. While Samples 2 (gel base, powdered BH (T-Sept®)) and 3 (gel base, powdered BH (T-Sept®) and Proteflazid®) demonstrated pronounced antioxidant properties. The evaluation of pro- and antioxidant properties of various modifications of the gel composition (samples 1, 2, 3) with a time sweep using the DPPH reagent allowed for establishing that the prolongation of the periodontal dressing effect in the oral cavity for 2 hours is optimal. Thus, T-Sept® has pronounced analgesic and anti-exudative properties, as well as an active anti-inflammatory effect. With local application in the form of periodontal dressing with a prolonged effect, benzydamine accumulates in inflamed tissues, where effective concentrations are precisely achieved due to its ability to penetrate through the mucous membrane [35, 36].

Including the direct-acting flavonoid antiviral drug Proteflazid® in the GC Benzidaflaziverdine we developed has a local and general effect, increasing the non-specific body resistance to viral and bacterial infections, which allows ensuring an increased antioxidant activity of the periodontal application. This composition, in particular, inhibits a course of free radical-dependent processes, preventing the accumulation of lipid peroxidation products, strengthening the antioxidant status of cells, and, thus, reducing intoxication.

According to literary sources [19, 20, 37-41], Proteflazid® has immunotropic properties, protects mucous membranes, normalizes indicators of local immunity (lactoferrin, secretory immunoglobulin A, lysozyme and C3 component of the complement). This drug induces synthesis of the endogenous α- and γ-interferons to a physiologically functional level, which increases the body’s non-specific resistance to viral and bacterial infections.

Conducted clinical studies showed that under the conditions of daily intake based on age-related doses and application schemes, Proteflazid® does not have an immunosuppressive effect [37-41]. It also does not cause the immune system’s refractoriness (hyporeactivity). There was no suppression of synthesis of α- and γ-interferons, which makes it possible to use the drug for a long time if necessary. Proteflazid® is a modulator of apoptosis that enhances the effect of apoptosis-inducing substances and activates caspase 9 [38-41]. This may contribute to eliminating virus-damaged cells and the primary prevention of chronic diseases, latent viral infections, disease relapses, and prolonging remission.

In conclusions: Tests with DPPH and DCFDA dyes were used to confirm the hypothesis regarding the cytoprotective effect of the patented gel composition Benzidaflaziverdine for local application in the form of a periodontal bandage due to the antioxidant activity of the flavonoid complex, which reaches the maximum level at the 2nd hour of exposure. This gel composition can be recommended for use in clinical periodontology for medical support of orthodontic patients before and during the active phase of orthodontic treatment.

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