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Evaluation of the effectiveness and safety of a new dental gel in experimental *in vitro* model systems

Ocena skuteczności i bezpieczeństwa nowego żelu dentystycznego w eksperymentalnych systemach modelowych *in vitro*

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żel dentystyczny, hodowla komórkowa, chlorheksydyna, żywotność komórek, reaktywne formy tlenu (ROS)

Summary

Introduction. *Toxicological studies today are one of the important stages of the biological assessment of the potential toxicity of drugs.*

Aim of the study. *To evaluate the biological activity of the new dental gel in experimental model systems using cell lines, and to conduct a comparative analysis with known medicinal products.*

Material and methods. *Cell culture, flow cytometry, and statistical analysis methods were used.*

Results. *The similarity of the effect of the studied sample (dental gel) on the viability of normal (human fibroblasts) and tumour cells (KB cell line), as well as its active substances and the comparison drug, was shown. The dental*

Streszczenie

Wstęp. *Badania toksykologiczne są dziś jednym z ważnych etapów biologicznej oceny potencjalnej toksyczności leków.*

Cel pracy. *Ocena aktywności biologicznej nowego żelu dentystycznego w eksperymentalnych układach modelowych z wykorzystaniem linii komórkowych oraz przeprowadzenie analizy porównawczej ze znanymi produktami leczniczymi.*

Materiał i metody. *Zastosowano hodowlę komórkową, cytometrię przepływową i metody analizy statystycznej.*

Wyniki. *Wykazano podobny wpływ badanej próbki (żelu dentystycznego) i jej substancji czynnych oraz leku porównawczego na żywotność komórek prawidłowych (fibroblasty ludzkie) i nowotworowych (linia komórkowa KB). Żel den-*

gel showed a lower cytotoxic effect compared to chlorhexidine. According to the results of the assessment of the level of ROS production in tumour cells after their exposure to the drugs, the similarity of the test sample effect with the comparison drug chlorhexidine was shown only in the largest of the tested doses – 3%. When evaluating the indicators of cell death due to the effect of the test sample, a statistically significant increase in the percentage of cells at the stages of early and late apoptosis compared to the control was shown only at a higher concentration.

Conclusions. *The established similarity of the action of the test sample with chlorhexidine is promising for further preclinical and clinical studies of this dental gel. In addition, the results regarding the toxicity of the test sample against tumour cells show the potential for further studies of the dental gel as an anti-tumour agent in the future.*

tystyczny wykazywał mniejsze działanie cytotoksyczne w porównaniu z chlorheksydyną. Zgodnie z wynikami oceny poziomu wytwarzania ROS w komórkach nowotworowych po ich ekspozycji na leki, podobieństwo efektu próbki badanej do leku porównawczego chlorheksydyny wykazano jedynie w największej z badanych dawek – 3%. Oceniając wskaźniki śmierci komórek pod wpływem badanej próbki, statystycznie istotny wzrost odsetka komórek na etapach wczesnej i późnej apoptozy w porównaniu do kontroli wykazano jedynie przy wyższym stężeniu.

Wnioski. *Ustalone podobieństwo działania badanej próbki z chlorheksydyną jest obiecujące dla dalszych badań przedklinicznych i klinicznych tego żelu dentystycznego. Ponadto wyniki dotyczące toksyczności badanej próbki wobec komórek nowotworowych wskazują potencjał do dalszych badań żelu dentystycznego jako środka przeciwnowotworowego w przyszłości.*

Introduction

It is known that conducting toxicological studies today is mandatory and one of the extremely important stages of the biological assessment of the potential toxicity of drugs and medical products, as they allow scientists to detect the possible negative impact of the test substance on the body at the initial stages of the study and to prevent side effects. Such studies include both experiments in the *in vitro* system and testing of the toxicity and safety of the drug *in vivo*. At the same time, the use of cell lines in preclinical studies as pharmacological models has many advantages: the possibility of controlling the physicochemical environment (temperature, oxygen and carbon dioxide content, pH), biochemical and physiological conditions, which, in turn, contributes to the repeatability and reproducibility of results, economy of reagents and the term of research.[1-5] Cell culture quite often complements or even replaces existing models using experimental animals, especially at the current stage, when the use of 3D cultures and Organ-on-a-chip approaches is developing.[6-8] The use of cell/tissue culture is often a more convenient and cheaper way to evaluate drugs (forms) and medical devices compared to traditional experimental models using animals. At the same time, cell culture with sufficient reliability can represent the processes that occur in a certain type of cells of the body, because when conducting an experiment, you can choose an adequate and organ-specific model for studying the drug and conduct the necessary number of repetitions to obtain statistically significant results.[9]

It should be added that today the problem of reducing the number of animals in experimental research is extremely acute. In particular, the European Commission recognizes that animal welfare remains a serious problem for European citizens, and the role of the European Union

(EU) in the gradual rejection of the use of animals for drug testing is a leading one, which is confirmed by the complete ban on testing cosmetics on animals (in the EU since 2013).[10] In addition, the European Commission plans to launch a new roadmap with a set of legislative and non-legislative actions to further reduce animal testing, with the aim of eventually moving towards an animal-free regulatory framework under chemicals legislation. That is why the expansion and adaptation of *in vitro* and *ex vivo* toxicological tests, including dental products, is relevant and in demand. In this regard, we chose experimental models using cell culture to evaluate the effectiveness and safety of the dental gel. It should be added that cell model systems also allow revealing and/or investigating in more detail the mechanisms of action of drugs (forms), which is extremely important in its further preclinical and clinical evaluation.

Currently, various oral hygiene products used in dental practice have been developed.[11-13] At the same time, chemical agents in oral rinses should be effective in changing the microbiota by selectively eliminating pathogens without negatively disturbing the normal flora.[14] The oral cavity contains up to 800 types of microbes, which are commensal microbiota and can be dysbiotically disturbed. Certain types of microbiota are dysregulated for certain reasons and increase the risk of oral diseases, in particular dental caries and/or periodontal disease, which are the most common non-infectious diseases worldwide.[15] Chemical plaque control using antibacterial mouthwashes can be an alternative to mechanical methods when these are ineffective. Oral antiseptics contain a mixture of various substances, among which the most common are: iodine compounds, phenolic agents, alcohols, cationic surfactants derived from bisbiguanides, bispyridinamines and quaternary ammonium derivatives, and various mixtures of organic compounds.[16] Although there is a wide variety of antiseptic mouthwashes and other similar agents on the market that have been shown to be clinically useful in the treatment of oral diseases, a decrease in patient tolerance and acceptability has been noted due to the presence of certain side effects such as: unpleasant taste and staining of teeth, irritation of the mucous membrane in some patients in the presence of alcohol, and are also unacceptable due to the association between denatured alcohol and the progression of oral cancer.[16] The long-term use of antiseptics causes concern about the possibility of undesirable changes in the composition of the microflora of the oral cavity and its modification, which can lead to the multiplication of pathobionts. Therefore, the development of new dental products that will be both effective and safe is always a relevant and popular concern in dental practice.

Material and Methods

Cell lines The studies were conducted on cell cultures of epidermoid carcinoma of the human oral cavity - KB cell line and human diploid fibroblasts. The cell lines were obtained from the collection of the Bank of Cell Lines from Human and Animal Tissues of the R. E. Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology of the NAS of Ukraine.

Sample characteristics

Test preparation (dental gel) – a mixture of "Fitodent" tincture (15%), choline salicylate (8%), lidocaine hydrochloride (1.5%) and water for injections (75.5%), pH=7.0;

Tincture "Fitodent" (PJSC "Khimpharmzavod Chervona Zirka", Ukraine) – for the experiment, a 15% aqueous solution of "Fitodent" was used, pH=7.0;

Choline salicylate 80% (Siegfried Evionnas SA, Switzerland) - an 8% aqueous solution of

choline salicylate was used for the experiment, pH=7.0;

Lidocaine hydrochloride (PJSC "Halychpharm", Ukraine) – for the experiment, an aqueous 1.5% solution of lidocaine hydrochloride was used, pH=7.0;

Chlorhexidine (PJSC Viola Pharmaceutical Factory, Ukraine);

Water for injections (Lekhim, Ukraine) – negative control.

Cell culture

Cells were maintained in complete DMEM medium with 4 mmol/l L-glutamine (BioWest, France), 10% fetal bovine serum (FBS) (BioWest, France) without antibiotic at 37°C in a humidified atmosphere with 5% CO_2.

The studied drugs were diluted in a complete nutrient medium to working concentrations.

Determination of cell viability by colorimetric method

Cells were plated into the wells of 96-well plates (SPL, Korea) in the complete DMEM medium at a *density of* $1x10^4$/well. Cells were cultured in a humidified atmosphere at 5% CO_2 and 37^0C for 24 hours. After a day, the studied gel, other research agents and negative control were added to the corresponding wells of the plates to the final concentration: 20%, 10%, 5%, 2.5%, 1.2%, 0.6%, 0.3 %. Cells were cultured in a humidified atmosphere at 5% CO_2 and 37^0C for 24 hours.

After the end of the incubation, the number of live cells was counted visually (direct microscopy method) and using the colorimetric method, staining live cells with crystal violet or the MTT test.[17]

The absorbance was measured by Labsystems Multiskan PLUS spectrophotometer at 540 nm. All experiments were performed in triplicate and the results were expressed as the number of viable cells in the experimental group relative to control (%):

$$X = \frac{A540\ (experiment)}{A540\ (control)} \times 100\%$$

The degree of cell viability after 24h of incubation for the tested samples and negative controls was compared with intact cells.

Determination of the production level of reactive oxygen species in cells.

To estimate the production of reactive oxygen species (ROS)[17] in the cells of the KB cell line after their treatment with the test preparation (dental gel), the cells were planted on Petri dishes (d=35 mm), in which sterile coverslips were placed. Cells were planted in the complete DMEM medium at a *density of* $1x10^5$ cells/dish and cultivated in a humidified atmosphere at 5% CO_2 and 37^0C for 24 hours.

Next, the test drug (dental gel) or chlorhexidine was added to the corresponding cups to final concentrations of 3% and 0.5%. Cells were incubated under standard conditions for another 24 hours. After 24 hours, nutrient medium was removed from all dishes and the cells were washed twice with phosphate-buffered saline (PBS, (BioWest, France)). 1 ml of PBS and DCFH dye were added to the cups to a final concentration of 50 mmol. Cells were incubated for 20 minutes at 37^0C and 5% CO_2. The fluorescent probe 2',7'-dichlorodihydrofluorescein (DCFH) readily enters cells, where it is oxidized to the fluorescent product 2',7'-dichlorofluorescein (DCF) by several ROS, so it is not specific for any particular ROS. Next, cells were washed twice with PBS and coverslips with cells were placed on glass slides. The level of fluorescence in the studied cells was immediately analysed using a microscope with a UV lamp (wavelength 490 nm).[18]

The level of ROS production in cells was assessed by the method of semiquantitative analysis (in points). At the same time, the

negative control (unstained cells) was accepted as a minimum of 0.0 points, while the stained cells with the brightest glow were given a maximum of 100.0 points, which corresponded to the luminescence intensity of cells treated with hydrogen peroxide (positive control).[19]

Determination of indicators of cell death (apoptosis, necrosis) by flow cytometry.

The percentage of live, apoptotic and necrotic cells of the KB cell line after exposure to the test drug (dental gel) was determined using annexin V, conjugated with the fluorescent dye FITC, and propidium iodide (PI) using the appropriate kit from Dojindo (Japan). Annexin V binds to the surface of a cell that is in the initial stages of apoptosis, and PI stains only those cells in which the permeability of the cell membrane is disturbed, which occurs in the late stages of apoptosis and during necrosis.[20]

KB cells were planted in the wells of a 12-well plate in complete DMEM nutrient medium at a density of $1x10^5$ cells/well and cultivated under standard conditions for 24 hours. Next, the test drug (dental gel) or chlorhexidine was added to the corresponding wells of the plate to final concentrations of 3% and 0.5%. After 18 hours of incubation of the cells with the studied agents, the cells were removed and counted. After washing the cells with FSB, they were stained according to the kit manufacturer's protocol. Cells were resuspended in staining buffer at a concentration of $1.0x10^6$ cells/ml, 0.1 ml was taken for each sample in a tube for cytofluorimetry and 5 µl of Annexin V-FITC solution was added, suspended and incubated at room temperature in the dark for 15 minutes, after which 0.4 ml of staining buffer was added to each sample, resuspended, and 50 µl of PI solution (10 µg/ml) was added, incubated for 1 min, and immediately analysed on a DxFlex flow cytometer (Beckman Coulter) with a 488 nm blue laser. Annexin V-FITC staining was analysed using the FITC channel (light filter 525/40 nm BP), and PI staining was analysed using the ECD channel (light filter 610/20 nm BP). The gain index (gain) for the FITC channel was 2 and for the ECD channel – 10. For each experimental group, the study was carried out in three repetitions. The gating strategy during the cytofluorimetric study was to gate the cells as wide as possible with cutting off only cellular debris on the FSC-A/SSC-A dot raft and to exclude doublets from the analysis using the FSC-A/FSC-H dot raft. Apoptosis/necrosis parameters were assessed using a four-quadrant type gate on FITC-A/ECD-A dot. Depending on the sample, 30,000-46,000 events were analysed in the study. Analysis of the obtained data was carried out using the CytExpert for DxFlex program.

Statistical analysis

IC50 indicators were determined by the method of non-linear regression analysis. Calculations of the average value of the studied indicators (M) and standard deviation (SD) were performed using the Excel software package. Statistical analysis of the obtained data was also performed using the Excel program using descriptive statistics and the Student's t-test.

Results and Discussion

At the first stage, the effect of the test sample (dental gel) on human epidermoid carcinoma cells of the oral cavity - KB cell line and normal human fibroblasts was evaluated. At the same time, viability was analysed based on the number of living cells and their respiratory activity (mitochondrial dehydrogenase activity) after their incubation in the presence of the studied test sample, as well as its active substances (phytodent tincture, lidocaine hydrochloride, and choline salicylate). The study of the test drug was carried out in comparison with water for injections (negative control) and chlorhexidine

as a comparison drug, since it is widely used in dentistry, belongs to antiseptic and disinfectant substances, has similar properties and indications for use in dentistry, in particular in stomatitis, gingivitis, periodontitis, alveolitis (according to the manufacturer's instructions).

A wide range of the drugs concentrations was investigated – from 20.0% to 0.3% of the content in the culture medium, which allowed analysing and evaluating their cytotoxic concentrations and establishing the IC50 (concentration that causes the death of 50% of cells). It was shown that dental gel (test drug) in a dose of 5%, in addition to a decrease in the total number of cells compared to the control, caused an increase in the number of cells with vacuoles and morphological signs of apoptotic cells, while in a dose of 10%, the morphology of the cells did not differ significantly from the control population. At the same time, the number of vacuolated cells with signs of apoptotic changes after the action of 5% chlorhexidine was significantly higher (compared to the test sample): the cells acquired a rounded shape with reduced adhesion to the substrate, with a large number of vacuoles, with a changed nuclear-cytoplasmic ratio, the location of the nucleus and a violation of the integrity of the membranes, which indicates a significant toxic effect of the drug. High doses of choline salicylate (10%), which is a component of the test sample, also caused significant toxic effects on cells, which was also noted in morphological changes: a violation of the integrity of membranes with an increase in the number of vacuoles in the cell and indistinct boundaries of the cell membrane and cytoplasm in general. Cells after exposure to choline salicylate, in addition to a significant decrease in the total number relative to the control, acquired a rounded shape with reduced adhesion to the substrate and signs of apoptotic and, in some cases, necrotic cells. While phytodent (in the studied doses) practically did not affect the morphology of the cells of the KB line, and lidocaine in a dose of 10% caused a decrease in the total number of cells and an increase in the relative (compared to the control) number of vacuolated cells and cells of a slightly elongated shape, which may indicate slight changes in the adhesion of the specified cells.

It was noted that after the effect of the test sample on normal fibroblasts in doses of 5 and 2.5%, not only a decrease in the total number was observed, but also a certain change in their shape with a decrease in adhesion to the substrate and an increase in the number of cells with vacuoles and signs of apoptotic changes (in a dose of 5%), while fitodent in a dose of 10% practically did not affect either the number of cells or their morphology. At the same time, chlorhexidine in a dose of 5% and even 2.5% caused more significant changes in the number of cells and their morphology compared to the test sample (dental gel): a significantly smaller number of living cells and a larger number of cells with signs of apoptotic and necrotic death were noted, with broken integrity of membranes and a large number of vacuoles, which indicates a significant toxic effect of the drug in the tested doses on cells (fibroblasts).

As a result of evaluating the effect of the test sample on normal (fibroblasts) and malignantly transformed cells (as an adequate model for conducting research on cell lines of the oral cavity) in comparison with the components of the complex test sample (lidocaine, choline salicylate, phytodent) and chlorhexidine relative to the negative control (water), it was shown that the effect of the drugs on normal and tumour cells had a similar effect (relative to cell viability, namely the number of living cells).

It was shown that in the highest tested concentration (20%) all the studied drugs had a significant (statistically significant difference, $p<0.01$) toxic effect (up to 1-3% of living cells were observed after exposure of cells to the

T a b l e 1. Viability of cells of the KB cell line (the results of staining with crystal violet)

% content of test agents	Lidocaine	Choline salicylate	Chlorhexidine	Phytodent	Test sample	Negative control (water)
	Number of live cells, % # (M±m)					
20.0	1.2±0.8**	0.9±0.4**	0.8±0.1**	71.9±4.5	0.6±0.2**	83.7±3.3
10.0	39.7±1.8**	1.7±0.4**	0.9±0.2**	93.4±3.5	1.0±0.6**	94.9±2.3
5.0	67.3±2.3**	57.4±0.9**	1.3±0.4**	101.4±1.6	17.6±1.9**	100.5±1.0
2.5	100.4±1.2	80.1±5.0**	51.1±3.9**	100.7±1.3	69.8±5.4**	99.7±1.1
1.25	99.2±1.6	99.0±0.9	64.5±1.7**	101.5±1.5	83.4±1.8**	100.9±1.3
0.6	100.2±1.0	100.8±0.9	77.7±2.8**	100.3±1.3	97.4±0.5	100.6±2.0
0.3	101.3±0.4	100.5±0.5	82.3±0.3**	100.9±1.3	101.1±1.1	100.2±1.1
IC50	9.3±0.8	5.3±0.1	2.9±0.2	–	3.1±0.3	–

Note: *p<0.05, **p<0.01 – statistically significant deviation compared to the corresponding indicator of the control group (negative control – water).
Note: #calculated relative to cell control (without studied substances or negative control) – 100%.

T a b l e 2. Viability of cells of the KB cell line relative to respiratory/mitochondrial activity (assessment based on the results of the MTT test)

% content of test agents	Lidocaine	Choline salicylate	Chlorhexidine	Phytodent	Test sample	Negative control (water)
	Metabolic activity of cells, % # (M±m)					
20.0	2.0±0.1**	1.0±0.3**	0.9±0.6**	83.2±1.7	1.0±0.6**	85.0±2.0
10.0	84.7±4.7	6.4±0.3**	0.7±0.2**	87.4±2.4	2.6±0.9**	88.6±2.7
5.0	101.0±1.2	94.0±3.6	8.2±2.6**	94.2±0.8	18.6±4.3**	94.8±1.9
2.5	100.1±0.5	96.9±2.7	73.3±1.6**	100.3±1.8	82.3±1.6**	100.1±2.3
1.25	100.2±1.2	101.7±0.7	89.8±1.6**	100.7±0.8	100.2±0.7	101.7±1.4
0.6	100.2±0.7	100.4±0.7	98.8±2.1	99.4±1.7	101.5±0.5	101.7±0.9
0.3	100.7±1.3	100.5±0.5	100.3±1.8	101.3±1.3	101.0±1.4	100.4±1.1
IC50	12.1±0.2	7.2±0.6	3.1±0.1	–	3.6±0.2	–

Note: *p<0.05, **p<0.01 – statistically significant deviation compared to the corresponding indicator of the control group (negative control – water).
Note: #calculated relative to cell control (without studied substances or negative control) – 100%.

Table 3. Viability of fibroblasts (the results of staining with crystal violet)

% content of test agents	Lidocaine	Choline salicylate	Chlorhexidine	Phytodent	Test sample	Negative control (water)
	Number of live cells, % # (M±m)					
20.0	1.2±0.7**	2.7±0.7**	0.5±0.1**	52.9±2.6**	1.0±0.4**	92.0±2.0
10.0	52.1±3.6**	41.5±2.2**	0.3±0.1**	78.3±3.1**	2.8±1.9**	99.0±1.6
5.0	79.2±3.9**	54.6±1.0**	0.8±0.2**	99.9±1.3	29.9±1.7**	99.6±1.7
2.5	89.2±2.1**	65.2±1.9**	52.1±4.4**	100.8±1.2	66.6±2.2**	99.4±0.8
1.25	99.9±1.2	80.8±1.0**	81.7±2.3**	99.7±1.6	83.9±1.1**	100.2±0.3
0.6	99.8±1.0	92.1±1.3*	93.2±0.3*	100.7±2.6	89.4±0.6**	101.3±2.4
0.3	99.8±1.6	95.6±1.2*	97.01±1.9	100.7±0.7	91.7±0.7**	99.7±1.3
IC50	11.0±2.5	5.8±0.5	2.5±0.2	≥20.0	3.8±0.2	–

Note: *p<0.05, **p<0.01 – statistically significant deviation compared to the corresponding indicator of the control group (negative control – water).
Note: #calculated relative to cell control (without studied substances or negative control) – 100%.

drugs), except for phytodent: in the latter from 52.9% to 83.2% of living cells were recorded in this concentration, depending on the cell line and the method of their staining. At the same time, even with a concentration of 5% of phytodent, there was no statistically significant difference in the number of living cells compared to the negative control. While the number of live cells (according to crystal violet staining) after exposure to the test sample up to 0.3% (in the variant with fibroblasts) and up to 1.25% (in the variant with KB cells) was statistically significantly lower relative to the negative control (p<0.01) (Tables 1 and 3): by 93-96% at a concentration of 10%; by 83% (KB cells) and 70% (fibroblasts) at a concentration of 5%; by 30% (KB cells) and 32.8% (fibroblasts) at a concentration of 2.5%; at a concentration of 1.25% (the final concentration of the drug in the culture medium) – the number of live cells was 16% less compared to the negative control indicator, and at 0.6 and 0.3 – from 3 to 10% (Tables 1 and 3). That is, starting with a concentration of the test sample of 2.5%, the drug had a fairly moderate toxic effect on the investigated cell lines, and with 0.6%, it had an insignificant effect. It should be noted that after exposure to the components (lidocaine and choline salicylate) of the complex dental gel (test sample) with cells in concentrations of 10 and 5%, a significant and statistically significant decrease in the number of living cells was also noted (p<0.01) (Table 1 and 3): by 93% (KB in the choline salicylate version) and 55% (KB in the lidocaine version) and by 47 – 57% (fibroblasts) at 10% concentration of the drug; by 33-43% (KB cells) and 45% (fibroblasts in the of choline salicylate version) and 20% (fibroblasts in the lidocaine version). That is, it can be assumed that choline salicylate and lidocaine can have a toxic effect (as evidenced by a decrease in the number of living cells). It should be added that the analysis of the respiratory activity (according to the MTT test) of KB cells after their exposure to the drugs showed similar results (Table 2), with

some differences in certain concentrations: IC50 for the studied drugs were slightly lower relative to the negative control compared to such in the test with crystal violet staining (Table 1). The comparison of the effect of the test sample with chlorhexidine turned out to be extremely interesting: both drugs were characterized by a high toxic effect on the examined normal and malignantly transformed cells at high concentrations (20%, 10% and 5%) (Tables 1–3). At the same time, chlorhexidine caused even more significant cytotoxic/cytostatic effects relative to malignantly transformed cells: after exposure of cells with the test sample, 16% – 19% more live cells (KB cells) were observed at 5%, 2.5% and 1.25% relative to chlorhexidine (Table 1). It should be noted that the lower toxicity of the test sample (dental gel) compared to chlorhexidine was also observed when the drugs were exposed to normal fibroblasts, while this difference in concentration of 5% was more significant: 29% more live cells after exposure to the test sample in comparison with the number of cells after the action of chlorhexidine (Table 3). This similarity of the action of the test sample with chlorhexidine is promising for further preclinical and clinical studies of this new dental gel, because despite the toxic properties of the comparison drug in high concentrations in *in vitro* studies, it is widely used in clinical practice for various pathological processes. In addition, in the literature there are data on the antitumour effect of chlorhexidine in model systems,[21,22] which may indicate the potential use of the new dental gel (test sample) as an antitumour agent in the future.

To detail the mechanism of action of the test sample (dental gel), a comparative study of the effect of drugs on cellular processes characterizing the pathways of cell death was conducted.

Evaluation of the effect of the studied new dental gel on the level of production of reactive oxygen species (ROS) by cells of the KB cell line. As is known, ROS are constantly produced in all aerobic organisms in response to various endogenous and exogenous factors, which in physiological concentrations are involved in such processes as signal transmission in the cell and protection against microorganisms, but the excessive formation of free radicals or their insufficient inactivation leads to a violation of cell structure and metabolic processes. An increase in the processes of peroxidation indicates a violation of the body's protective reactions at the cellular level and homeostasis in general, and in a state of oxidative stress under the action of ROS, both lipids and proteins, and primarily plasma membrane proteins, are subject to peroxidation.

For a more detailed analysis of the potential mechanism of action of the test sample in comparison with chlorhexidine, a study of the level of ROS production by cells of the KB line was conducted. According to the results of the assessment of the level of ROS production in the cells of the KB line, the similarity of the effect of the test sample with the comparison drug (chlorhexidine) in a higher concentration – 3% was shown: the level of ROS production was estimated at 100 points after the action of chlorhexidine and 92.5 after the action of the test sample (dental gel). At the same time, at a lower concentration (0.5%), exposure of cells of the KB line to the test sample led to a slight increase in the level of ROS production (30.0 points), in contrast to chlorhexidine, where the level of ROS production remained high (85.0 points) (Fig. 1, Table 4).

To calculate the points, the number of cells (%) in the field of view with a certain luminescence intensity was taken into account. The luminescence level of control unstained DCFH cells was taken as 0 points. 100 points corresponded to the maximum level of luminescence, which was determined by

Table 4. The level of ROS production in KB cells after their exposure to drugs

Group	Level of ROS production, points
Negative control. unstained cells	0.0 ± 0.0
Control. stained cells	30.0 ± 6.0
Chlorhexidine 3%	100.0 ± 0.0
Chlorhexidine 0.5%	85.0 ± 5.0
Test drug 3%	92.5±2.5
Test drug 0.5%	30.0 ± 2.0

the level of this indicator in cells treated with hydrogen peroxide (positive control).

Determination of indicators of death of cells of the KB cell line after the action of the test agent (dental gel). It is known that cell death occurs by apoptosis, autophagocytosis and/or necrosis, each of which has its own signs and characteristics. Understanding the mechanisms of cell death is an extremely important fact at the preclinical stage of the development of new drugs and medical products. The assessment of the death rate of tumour cells of the KB line after the action of the test agent (dental gel) and the comparison drug chlorhexidine was carried out using the simultaneous staining of cells with annexin V conjugate with FITC and propidium iodide.

As a result of the conducted studies, it was shown that the test sample (dental gel) causes similar effects to the comparison drug (chlorhexidine) when evaluating the indicators of cell death, but in the case of chlorhexidine, the effects of the drug were more pronounced. A higher concentration of chlorhexidine preparations and the test sample (3%) caused an increase in the % of cells in early apoptosis by 15 and 8%, respectively ($p<0.01$), while at a concentration of 0.5% there was a slight but statistically significant increase in the % of

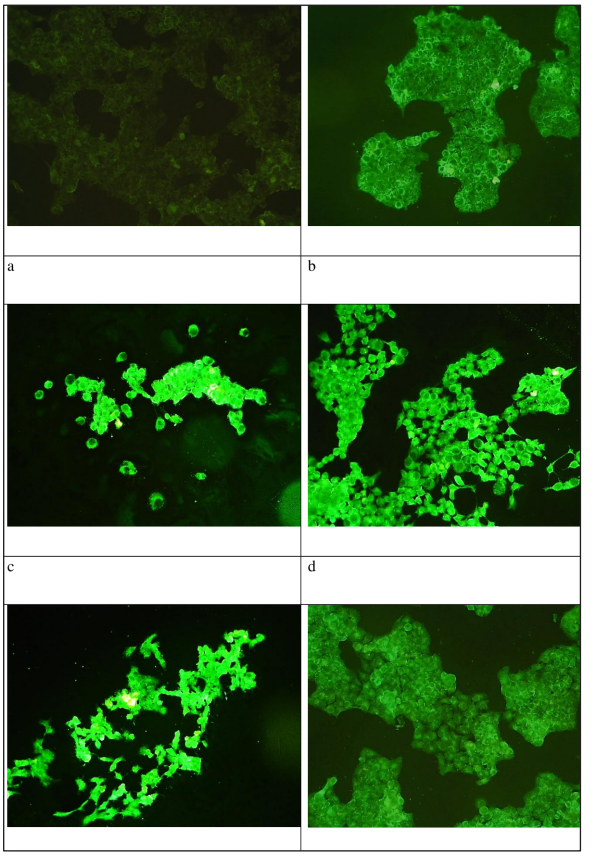

Fig. 1. Cells of the KB cell line after their exposure to the test sample (dental gel) and chlorhexidine. Staining with the fluorescent probe 2',7'-DCFH (objective x 20): a) Negative control, unstained cells; b) Control, stained cells; c) Chlorhexidine 3%; d) Chlorhexidine 0.5%; e) Test drug 3%; f) Test drug 0.5%.

tumour cells in early apoptosis relative to the control was observed only when exposed to chlorhexidine (Table 5). The same trend was observed with regard to the % of cells in late apoptosis: a slight but statistically significant increase in the % of cells was recorded under the influence of the test sample by 3.4%, and under the influence of chlorhexidine – by 6.6% compared to the control during the exposure of cells to a 3% concentration of the drug (Table 5) while the effect of the drug in a concentration of 0.5% caused a slight (less than 3%), but statistically significant increase in the % of cells in late apoptosis only in the case of chlorhexidine.

T a b l e 5. Live cells, cells at the stage of early and late apoptosis and necrosis after the action of the test agent and the comparison drug, %

	Live cells (%), M±m	Early apoptosis (%), M±m	Late apoptosis (%)	Necrosis (%)
Control	85.02±0.41	5.33±0.17	7.88±0.25	1.75±0.08
Chlorhexidine. 0.5%	80.89±0.29**	6.77±0.11*	10.14±0.17**	2.19±0.21
Chlorhexidine. 3.0%	63.01±1.27**	20.80±1.1**	14.50±0.48**	1.67±0.25
Test agent (Dental gel). 0.5%	85.71±0.08	4.86±0.35	7.82±0.31	1.59±0.20
Test agent (Dental gel). 3.0%	73.65±0.51**	13.74±0.32**	11.28±0.60**	1.32±0.17

Note: *p<0.05, **p<0.01 - statistically significant deviation compared to the corresponding indicator of the control group.

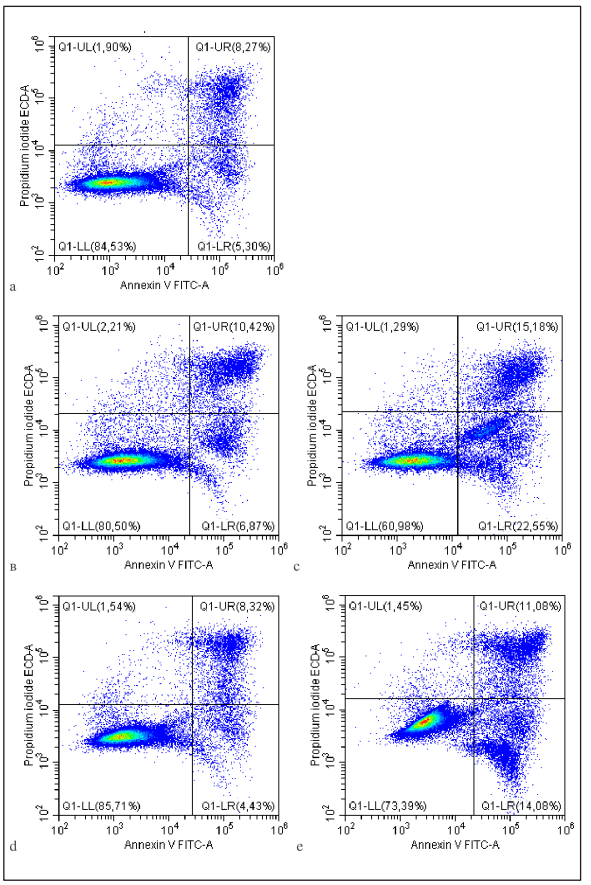

Fig. 2. Representative plots of the KB cell death parameters after treatment of the dental gel and chlorhexidine. a) KB control cells; в) KB + chlorhexidine 0.5%; c) KB + chlorhexidine 3%; d) KB + dental gel 0,5%; e) KB + dental gel 3%.

When evaluating the percentage of cells in necrosis, no statistically significant differences were noted between the studied groups (Table 5, Figure 2).

Conclusion

As a result of the evaluation of the effect of the test sample (Ddental gel) on human fibroblasts and cells of epidermoid carcinoma of the human oral cavity of the KB cell line, as well as its active substances (lidocaine hydrochloride, choline salicylate, phytodent) and the comparison drug (chlorhexidine) relative to the negative control, similarity was shown in their effects on the viability of normal and tumour cells. A study of the effect of a 24-hour exposure of the active substances of the dental gel on the viability of KB cells and fibroblasts showed that the phytodent drug had the least, and choline salicylate the greatest cytotoxic effect. The dental gel showed a lower cytotoxic effect compared to the comparison drug – chlorhexidine. According to the results of the assessment of the level of ROS production in tumour cells after their exposure to the drugs, the similarity of the effect of the test sample

with the comparison drug Chlorhexidine was shown only in the largest of the tested doses – 3%, while at a drug concentration of 0.5%, the test sample caused 2.8 times less increase in the level of ROS production by cells compared to chlorhexidine. When evaluating the indicators of cell death due to the effect of the test sample, a statistically significant increase in the percentage of cells at the stages of early and late apoptosis compared to the control was shown only at a higher concentration of the drug (3%). Thus, in our opinion, the established similarity of the action of the test sample with chlorhexidine is promising for further preclinical and clinical studies of this new dental gel, because despite the toxic properties of the comparison drug in high concentrations in *in vitro* studies, it is widely used in clinical practice in various pathological conditions processes. In addition, the results regarding the toxicity of the test sample against tumour cells show the potential for further studies of the new dental gel as an antitumour agent in the future.

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