DOI:10.14753/SE.2022.2727

INVESTIGATION OF THE CELL PHYSIOLOGICAL EFFECTS OF SUBSTANCES USED IN DENTISTRY

PhD thesis

Zsófia Ágnes Kőhidai

Molecular Medicine Doctoral School
Semmelweis University

Supervisor: Prof. Csaba Dobó Nagy, DMD, Ph.D

Official reviewers: László Köles, MD, Ph.D

Rita Szabó, Ph.D

Head of the Complex Examination Committee: Prof. István Gera, Ph.D, C.Sc

Members of the Complex Examination Committee:
Gábor Gerber, DMD,Ph.D, C.Sc

External member: Prof. Zoltán Rakonczay, D.Sc

Budapest
2021

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List of Abbreviations

CHX – Chlorhexidine

CI– Cell Index

ClO_2 – Chlorine dioxide

CPC – Cetylpyridinium chloride

delta CI– normalized data of CI over the time

GIC – Glass Ionomer Cement

GPCR – G-protein coupled receptor

HFF1 – Human foreskin fibroblasts

HGEP – Human Gingival Epithelial Progenitor cell

HGF – Human Gingival Fibroblast

H_2O_2 – Hydrogen peroxide

IC50 – Half-maximal inhibitory concentration

IDDM – Insulin dependent diabetes mellitus

PDL – Periodontal Ligament stem cell

PEI – Polyethyleneimine

PEI- Ag – Polyethyleneimine-Silver complex

PLA – Polylactic acid

Abbreviations used to present level of significance:

x – $p < 0.05$; y – $p < 0.01$; z – $p < 0.001$

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Introduction

A wide variety of materials are used in dentistry. In line with today's trends, the materials used should be aesthetically pleasing as well as they should also have adequate biocompatibility. In the development of many dental materials, the primary points of consideration are (i) the fight against pathogenic bacteria, (ii) adequate mechanical properties and (iii) long service life in the oral cavity, however, the side-effects developed in the patient's body is often relegated to the background.

The prokaryotic flora of the oral cavity and the patient's own eukaryotic cells are fundamentally different targets from a cell biological and pathological point of view. Due to their chemical nature, the surface membrane and the cytoplasmic components (e.g. GPCR and signaling pathways) are capable of drug-specific perturbations of the cell. (1)

In the course of my PhD work, the focus of my research was the characterization of three different groups of substances (mouthwashes, glass ionomer cements, polyethyleneimine complexes) used or in the process of being used clinically in diverse fields of dentistry (prosthetic dentistry, oral medicine and preventive dentistry). The measured indices are based on the cell physiological responsiveness of model-cells composing tissue elements in the oral cavity.

1.1 Mouthwashes

Mouthwashes are the most commonly encountered by patients at home, so it is especially important to know their effects due to the uncontrolled conditions in which they are applied. These substances may also contain bactericidal ingredients thus inhibiting the re-formation of the biofilm (2, 3, 4).

Although mouthwashes had already applied by the ancient Egyptians, Romans, and Greeks, the first true antiseptic rinse aid did not enter stores until 1893. It was the Odol mouthwash that is still in circulation today, developed by Karl August Lingner (5). Another rinsing agent with a historical background still in use today is Listerine. When Listerine first appeared it was not used as a mouthwash but as an antiseptic in the First World War. After the war, rebranding it as a mouthwash brought unexpected success to the company (6).

Although the primary function of mouthwash for most people is to eliminate bad breath (7, 8), mouthwashes are also involved in reducing the pathogenic flora of the oral cavity due to the antibacterial effect of the active ingredients in them, thus contributing to achieve better oral hygiene conditions (9, 10).

1.1.1 Chlorhexidine

Chlorhexidine (CHX) is the most commonly used ingredient in commercially available mouthwashes (Fig. 1). It has a broad-spectrum antimicrobial effect, effective against Gram-positive and negative bacteria as well as aerobic and anaerobic bacteria. It has very long-lasting effectiveness up to 8-12 hours. This substantivity is very favourable against the re-forming of bacterial biofilm (11, 12).

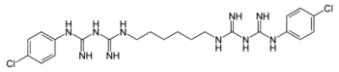
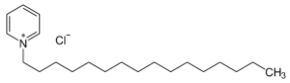
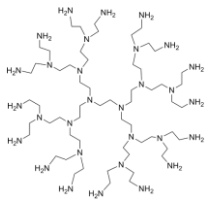
Chemical structure	Compound	Reference
	chlohexidine (CHX)	(13)
	hydrogen peroxide (H_2O_2)	(14)
	chlorine dioxide (ClO_2)	(15)
	cetyl pyridinium chloride (CPC)	(16)
	polyethyleneimine polymer (PEI)	(17)

Figure 1 Chemical structures of reference compounds mouthwashes and PEI

Using mouthwash in healthy patients can only be treated as a supplementary action to the mechanical cleaning of the teeth (18). It's the ability to reduce the formation of new biofilm following mechanical brushing is very welcome, especially in patients unable to effectively clean their teeth (physical or mental disabilities, immunocompromised patients, patients with braces, prosthetic wearers). It can also prevent peri-mucositis and peri-implantitis around dental implants (19, 20). Jenkins et al. conclude that the anti-

plaque activity of CHX is more due to the formulation (concentrations and processes) than to the concentration of CHX used (12). In patients who use CHX for a long time, some side effects can occur. The most common side effect is the aesthetically not pleasing staining of the teeth. Dark, brownish stains appear on the surface of the teeth, but can also appear on the tongue and even on restorations. The other side effect is taste loss. This, however, can easily be resolved with some water rinsing after the use of the mouthwash or choosing a mouthwash with less CHX or none at all (21, 22, 23).

1.1.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2, Fig. 1) is one of the oldest disinfection compound in use (14). In dentistry, it has been used for tooth whitening and for its antiseptic nature thanks to its oxidising powers. Its antimicrobial effects is weaker than that of the previously mentioned CHX or even cetylpyridinium chloride (CPC), it is, however, still a broad-spectrum antimicrobial compound. It is not effective against bacteria with catalase activity. When H_2O_2 is used it needs higher concentrations and longer exposure times than CHX or CPC (24, 25).

In higher concentrations of H_2O_2, it has an immediate toxic effect (with a wide-range damaging effect on lipids, DNA and proteins), while in lower concentrations it can induce apoptosis through the mitochondrial pathway (26). In recent years higher concentration of H_2O_2 usage is not advised as it damages the 6uta t6a sprocess of wound healing in in vitro (e.g. scarring-assay) (27). In dentistry it has been used for tooth whitening for decades 6uta t a relatively high concentration (maximum: 6%) (28, 29). This compound is also used for root canal cleaning during endodontic treatments (30, 31).

1.1.3 Chlorine dioxide (ClO_2)

Chlorine dioxide (ClO_2, Fig. 1) has a size-selective antimicrobial effect not only on bacteria but also on viruses (15). Because of the special target mechanism, the microbe has the ability not to develop resistance against the ClO_2. This chemical attacks the cell membrane and cytoplasmic proteins through amino acids (Tyr, Cys, Trp, Met and Gln) but can also react with cations such as Mn^{2+} and Fe^{2+}. This ability to only react with the

substances listed above makes it possible for the ClO_2 to be also effective in mixedenvironment (32, 33).

A new way of production (which results in a super-pure ClO_2) made it possible for the ClO_2 to be used as a highly active disinfectant agent (34). This new, high purity ClO_2's degradation takes only a few minutes, thus not being dangerous for eukaryotic cells. Bacterial cells and viruses being much smaller than eukaryotic cells, are therefore in danger of their antiseptic effects. The essence of ClO_2's mechanism of action is that the critical exposure time increases with the square of the characteristic size of the target cell (32). The ClO_2 action on different target cells have a size selective character which means that the small particles (bacteria) are killed by the active released Cl^- ion, while the bigger eucaryotic target cells are resistent to this mechanism of killing (Fig. 2 a and b).

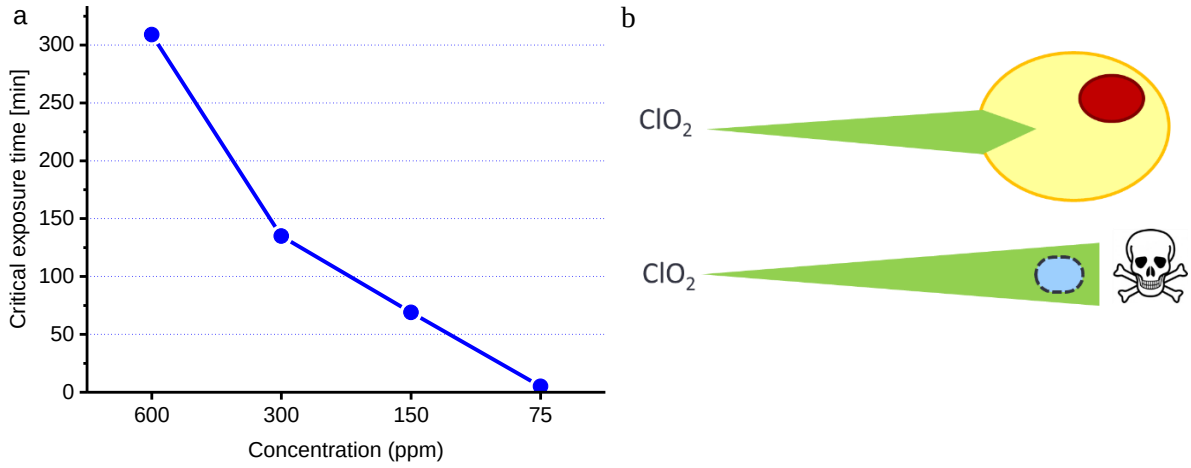


Figure 2 a and b - Size-selective effect of ClO_2 a – diverse dinamics of time-concentration dependent degradation elicited by ClO_2. b – schematic representation of target cell specificity of ClO_2 activity.

1.1.4 Cetylpyridinium chloride

Cetylpyridinium chloride (CPC, Fig. 1) is a broad-spectrum antimicrobial compound used frequently in dentistry (16). It is often used as an active ingredient in mouthwashes on its own or in combination with CHX.

Compared to CHX's long-lasting effectivity (8-12 hours) (35) CPC is only effective up to 3-5 hours. Other than preventing the formation of new bacterial biofilm it can prevent pathogen bacteria activated release of pro-inflammatory agents (e.g. IL-1β, IL-8, TNF-α),

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thus reducing bleeding of the gingiva (36, 37). CPC's side effects are less prone to happen and are quicker to disappear with the discontinuation of use than those of CHX. In dentistry, CHX on its own – in higher concentrations – is advised to be used only for a short period of time as an acute cure (up to a maximum of 2 weeks), while CPC with its less effectiveness could be used every day for longer periods (38, 39).

1.2 Glass ionomer cements

Glass ionomer cements (GIC) are aqueous solutions of acidic polymers used in fillings, or for final prosthetics fixing in (Fig. 1). Their aesthetics lag behind composite fillings, but they are highly biocompatible materials and can release fluoride ions during bonding, thus reducing the chances of secondary caries (40, 41, 42).

In recent decades, there have been tremendous changes in the making of dental fillings. Although, amalgam appeared in the 7th century, however, it only became widespread in the 1800s (43).. The preparation of the amalgam was withdrawn from dental usage due to the polluting and health-damaging properties of mercury which is a component of the amalgam. It is important to emphasize that mercury itself in amalgam fillings is not harmful to the health (44), since the mercury in the amalgam is in bound state. The removal of an amalgam filling should be performed with a rubber dam isolation and with proper exhaustion. Removal of existing clinically asymptomatic amalgam fillings is not necessary as long as it does not cause an aesthetic problem to the patient (45).

The alternative filling materials to amalgam are glass ionomer cements. In addition to the high biocompatibility and better aesthetics of glass ionomer cements, it also has better bonding to the tooth. An important property is that the formation of secondary caries is reduced during its maturation due to fluoride release (46). In the development of glass ionomer cements, the most important objective is to find the perfect balance between the biocompatibility, the mechanical and the physical properties of the cement. Examination of the new formulations revealed that due to the new atomic bonds and changing interfacial configurations, the fracture toughness (K_{Ic}) of cements, unfortunately, decreases, which is an essential property when making long-term fillings (47).

To examine potential new GICs we used a standardised method (48). For these experiments, we used commercially available GICs (Fuji Equia and Fuji Triage, GC Europe, Leuven, Belgium). Fuji Equia is used for posterior restorations, while Fuji Triage is used to reduce tooth-sensitivity (49). A ring mould developed by us was used to ensure

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the standard size and shape of the GICs as well as to make visualisation of cell and GICs interaction under inverted microscope possible (48).

1.3 Polyethyleneimine

Polyethyleneimine (PEI) is a highly branched polymer with many free amino residues (17) (Fig. 1). Due to these positively charged free residues, PEI greatly enhances cell adhesion. This polymer also has antibacterial and antifungal effects and by applying nano silver particles, this effect of PEI can be increased (50).

Oral administration has been actively addressed by other research groups in the past. They aimed to enrich temporary cements and composite fillers with PEI (50, 51). Considerable results were obtained where the synthesized substances had significant bactericidal/bacteriostatic properties without a notable change in physicochemical properties. In addition to the enrichment of temporary glass ionomer cements and composites in the oral cavity, PEI is also being examined as an additional layer on the mucosal contact surface of acrylate-based dentures made for prosthetic purposes. Some research aimed to develop an antibacterial and antifungal coating material and technology that will make it possible to PEI the material in a dental practice or dental laboratory.

Unfortunately, PEI's toxic effects are not only targeted to the pathogenic flora but also the cells of the tissues of the human body. These toxic effects can occur in several ways. An immediate necrotic effect resulting from positive charges was observed. These positive charges are able to bind very strongly to the surface membrane of the cell and elicit a destructive effect. The dynamics of the other mechanism are slower, resulting in delayed cell death due to apoptotic effects. After binding of the PEI-DNA complex to the cell surface, it reaches the nucleus by signal-induced endocytosis, where the associated gene sequence of DNA can be incorporated. (52). If PEI containing large amounts of free bonds is internalized, the cell is killed by damage to the mitochondria.

The other main component of the complex formed and included in our studies is polylactate (PLA). This substance is able to reduce the toxicity of the complex by binding to the previously mentioned free amino residues. In addition, with the help of PLA, it will be possible to bind the PEI-PLA complex to the mucosal surface of the denture. This additional layer on the mucosal side of the denture enables us to have a new way of treating inflammatory diseases (denture stomatitis) caused by protheses. These inflammations are caused by the growth of bacterial and fungal flora. This (denture stomatitis = Candida-associated denture-induced stomatitis = denture-associated

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erythematous stomatitis), is a mild inflammatory disease (main manifestation: erythema on the mucosal surface covered by dentures), can in many cases be completely asymptomatic for the patient. This inflammation's incidence in denture wearers is 15-70%, and the rare removal and improper cleaning of dentures promote its development (54). In women and with advancing age, the development of the disease may become more frequent. Diabetes mellitus (IDDM) can further increase the risk of developing this disease (55). The roughness of the mucosal surface of dentures provides a favorable environment for the growth of *Candida albicans* and thus the development of the disease.
Another aim of research with the PEI complex is to develop a new material that allows the development of a material that can be applied to the mucosal surface of dentures and is thus able to cure inflammatory diseases that develop here. By binding PLA to the complex, the toxic effects are reduced on the tissues of the body, while its effectiveness against the pathogenic flora is not reduced as PLA is a completely biodegradable substance for the body (53).
. With the help of the designed new material (PEI-PLA) applied – in addition to its antifungal properties - a more even surface can be created, thus reducing the pathological growth of *Candida albicans* (56). The antibacterial effect of silver has been known for centuries. Research is also underway in adhesive cements in dentistry and in pediatric dentistry to exploit this ability of silver (57, 58). PEI-PLA's protection against pathogenic flora can be enhanced by the addition of nano silver particles resulting in PEI-PLA-Ag complex. One of the main objectives of my PhD work was to examine the significance of silver in PEI-PLA complexes.

1.4 Model cells

In the case of most dental materials, it is important to know whether the effects of the applied dental materials (mouthwashes etc.) are focused only on the pathogenic flora or significant disadvantageous effects are also elicited on the eukaryotic cells of the oral cavity (e.g. gingival epithelium or stem cells). In the case of restorations and protheses used in dentistry, from a dental point of view, aesthetics and long-term effectiveness are the most important. In some cases of indirect (e.g. crows, bridges, inlays, onlays) or direct restorations (e.g. G.V. Black - Caries Classification – Class II, III, IV, V) (59) contact is made not only with tooth tissues but also with other living oral tissues.
In the experiments presented in my PhD work, special groups of model cells were investigated, which allow us to study the effects of different groups of substances used in

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dental practice. During this work 3 main groups of model cells – epithelial cells, fibroblasts and stem cells – were chosen. In these model cells, the effect of the chemicals used is not negligible since their anatomical localization, histological characteristics and their high sensitivity to chemicals. In our work human gingival epithelial progenitor cell line (HGEP) represented the epithelial cells of the oral cavity; periodontal ligament stem cells (PDL) were used as a representative of a stem cell target of several interventions of dental care; while human gingival fibroblasts (HGF) and human foreskin fibroblasts (HFF1) were used as human fibroblasts with different histological origin.

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2 Objectives

The main goal of my investigations was to contribute with novel data for better understanding of the biological and clinical effects of three groups of substances (mouthwashes, glass ionomers and polyethyleneimine complex) performed on primary and secondary model cells.

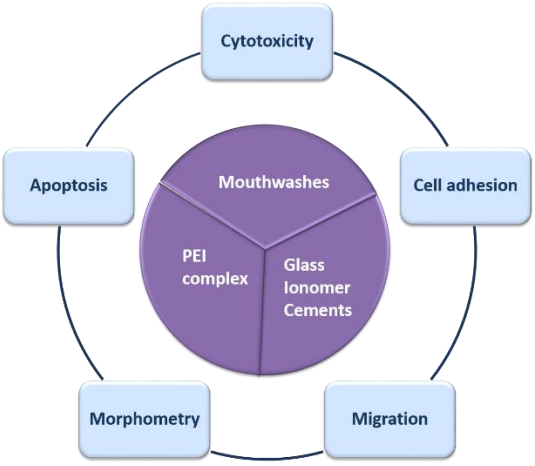

Figure 3 The main groups of dental materials investigated in the present PhD thesis and their cell physiological responses measured

Our dental material-specific aims were as follows:

Mouthwashes

1 How do rinsing agents with different chemical compositions and their active ingredients affect the viability and proliferation of human gingival epithelial cells (HGEP)?

2 Could apoptotic mechanisms contribute to cell number decrease observed in viability changes?

3 Do the tested substances/mouthwashes cause cell morphological deviations detectable by computer-assisted morphometry?

4 Which of the tested reference compounds have the least cytotoxic effect?

5 Are the additional components responsible for some cytotoxic effects?

Glass ionomers

6 Development of a novel, cost-effective, easy to perform and standardized method to investigate the cytotoxicity of GICs.

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7 Is there any difference in cytotoxicity of commercially available GICs?

8 Does the two model fibroblast cell lines (HGF and HFF1) express diversity to treatments with glass ionomer cements in their cell physiological responsiveness (viability and cell adhesion)?

Polyethyleneimine (PEI)

9 Does the PEI or PEI-Ag extracts show size stabillity over the time?

10 Does the presence of PEI affect the adhesion, proliferation, and apoptotic processes of periodontal ligament (PDL) and epithelial (HGEP) cells?

11 Does the application of the PEI-Ag complex result in differences in the studied physiological processes: apoptosis, cell adhesion, cell proliferation/cytotoxicity, cell morphology, cell migration?

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3 Results

The cell physiological responses elicited by the dental substances were monitored by impedimetry. The responses are explained using the impedimetric curve profile, depending on the phase the cells are in when the treatments were applied (Fig. 4). In the case of a cell adhesion study, the cells and the test compound are loaded into the system simultaneously and the different effects can be determined on the basis of the slopes of the curves, obtained in a short time. In the case of cytotoxicity / proliferative effects, cells that have been loaded previously and are in the plateau phase of growth are treated with the test compounds and the registered signal indicates the cytotoxic or proliferative effects.

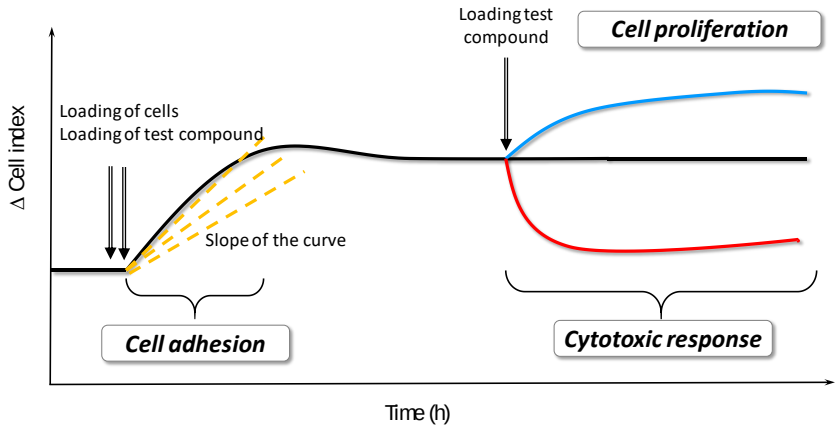

Figure 4 Understanding of impedimetric strategy to measure cell adhesion and cytotoxicity/proliferation

3.1 Mouthwashes

Model-cells: HGEP human gingival epithelium cells

Techniques applied: cytotoxicity – impedimetry (xCELLigence SP); apoptosis – Annexin V staining (BD FACSCalibur); morphometry – automatic cell imaging system (JuLI FL, NanoEntek); Zeiss Axiovert A1, Biomorph 1.1; statistics – RTCA2.0, OriginPro 8.0

(levels of significance: x – $p < 0.05$; y – $p < 0.01$; z – $p < 0.001$)

3.1.1 Cytotoxicity

In the pilot experiments, some high concentrations of mouthwashes and their active ingredients proved to be unusable in the cell physiology assays due to their precipitation in the solvent . In case of the reference compound CHX these concentrations were 1 and

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2 %. In some of the commercially available mouthwashes precipitation happend in 0.4 and 0.2 %v/v concentrations (Gum Paroex, PerioAid 0,12%, PerioAid Maintenance, Vitis Gingival, Vitis Orthodontic). Listerine Fluoride Plus and Listerine Cool Mint 0.4 %v/v precipitated. No precipitation was detected in H_2O_2, ClO_2, ClO_2 and Dentaid Xeros. Therefore these precipitating concentrations were omitted from evaluation.

In general, analysis of the obtained impedimetric curves shows that fundamentally different curve-characteristics were observed for the 4 basic compounds (3.1.1.1.)). In the case of commercially available mouthwashes, except for the significant cytotoxic effects elicited in high concentrations, the effects did not mostly deviate from the control during the first 20 hours of the study. After 20 hours of incubation, depending on the nature of each substance, a cell proliferation increasing effect could be recorded at different concentrations.

3.1.1.1. Reference compounds

3.1.1.1.1 Hydrogen peroxide

The results of proliferation (Fig. 5a) of the HGEP cells treated with the applied concentrations were immediate. The maximal concentrations applied had a prompt effect resulting in decreased proliferation of the cells. This is visible from the decreased impedance signal and the signals run low throughout the experiment in the case of concentrations 6% and 3%. In the case of 0.3%, an increase in the impedimetric signal happened at the beginning of the experiment but then dropped around 10h. In contrast, the 0.03% followed the above-described profile with the exception that it had a gradual increase for the rest of the experiment. However, it stayed well below the lines of the control and the 0.3%. These decreases mentioned above (6-0.03%) suggest a cytotoxic effect of the H_2O_2. The 0.003% was the lowest concentration tested and its impedimetric signal remained close to the control line implying that it was neither cytotoxic nor does it influence the proliferation (or adhesion) of the HGEP cells. At the very beginning of the experiment, we see signal peaks but these are artefacts caused by inserting and/or removing the E-plates.

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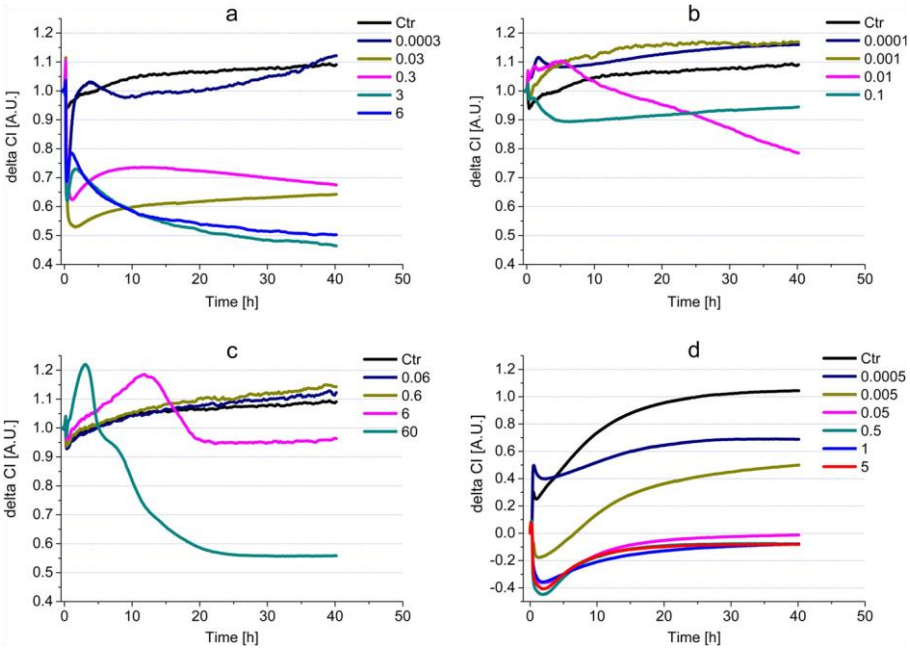

Figure 5 Impedimetric analysis of cytotoxicity elicited by the most common active ingredients (a - H_2O_2, b - CHX, c - ClO_2, d – CPC; delta CI – cell index)

The IC50 values (referring to the concentration dependence effect of the treatment) show how the cells loss of viability developed in the first 24 hours (0.027%) without significant change in the 48 hours (0.028%) (Table 2).

Table 2 Comparison of IC50 values and therapeutic concentrations of main active ingredients and commercially available mouthwashes

Active ingredients	**Maximal non-toxic**	**IC50 (24h)**	**IC50 (48h)**	**Unit**	**Therapeutic conc.**
CHX	0.001	0.01	0.009	%	0.20%
H_2O_2	0.0003	0.027	0.028	%	3%
ClO_2	0.6	20.40	20.51	ppm	25ppm
CPC	*	0.003	0.003	%	0.05%
Commercially available mouthwashes					
Gum Paroex	0.0002	0.002	0.0015	%v/v	

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PerioAid 0.12%	0.0002	0.04	0.005	%v/v	
PerioAid Maintenance	0.0002	0.004	0.001	%v/v	
Vitis Gingival	0.0002	0.01	0.001	%v/v	
Vitis Orthodontic	0.0002	0.01	0.005	%v/v	
Dentaid Xeros	0.02	0.069	0.063	%v/v	
Listerine Fluoride plus	0.00002	0.005	0.002	%v/v	
Listerine Cool mint	0.002	0.01	0.01	%v/v	

** In the case of CPC all the measured concentrations had a significantly lower delta CI than the control, implying that the concentrations measured in our experiment were all toxic to the model cells.*

3.1.1.1.2 Chlorhexidine

Chlorhexidine (CHX) is the most commonly used component in mouthwashes. The most concentrated solution (0.1%) had an immediate decrease in the impedimetric signal which for the time of the experiment remained persistent implying a strong cytotoxic effect (Fig. 5b). The 0.01% solution caused a transient and increased impedimetric signal (even surpassing the control line) but after the 10th hour turned into a consistent decrease. Around the 25th hour of the experiment, the lines of 0.01% and 0.1% are crossing which implies that in the long run (>25 hours) 0.01% concentration CHX can express a stronger cytotoxic effect than 0.1% CHX. The lowest concentrations (0.001% and 0.0001%) of CHX surpassed the control line significantly for whole experiment.

The concentrations used in the experiment were more diluted than those which are used in commercially available mouthwashes or the concentrations used for therapeutic purposes in dental practices. The 24h IC50 value (0.01%) shows that the CHX had a strong cytotoxic effect on the epithelial model cells, this effect did not have a significant change for the rest of the experiment (IC50 for 48h was 0.009%) (Table 2).

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3.1.1.1.3 ClO_2

The most concentrated solution in our experiment was 60 ppm ClO_2 which is outside the reported (60) therapeutic range of ClO_2 (Fig. 5c). This 60 ppm ClO_2 had a rapid increase of impedimetric signal in the first hours of the experiment (2-3h), but this increase turned into a deep dive and remained toxic for the rest of the experiment. The 6 ppm ClO_2 had an almost identical effect to the above-mentioned concentration with the difference that the 6 ppm solution had an elongated increase and decrease (reaching its peak at ~13 hours). This decrease continued in a plateau (starting from the 20th hour) which was close but still lower than the control line. The 0.6 ppm and 0.06 ppm solutions had similar impedimetric signals to the control line. A slight increase (21st hour) was detectable in both treatments which resulted in a higher, nevertheless not significantly different signal to the control.

The IC50s for 24h and 48h had similar values (IC50 24h -20.40 ppm, IC50 48h – 50.51 ppm) suggesting that the 24 hour incubation was enough to achieve the maximal decrease in cell viability (Table 2).

3.1.1.1.4 CPC

Cetylpyridinium chloride (CPC) is mostly found in mouthwashes as an active ingredient in combination with CHX; however, CPC can also be found as the main active ingredient on its own, too. The four highest tested concentrations (5%, 1%, 0.5% and 0.05%) had very similar profiles of the impedimetric curves (Fig. 5d) which means that after the initial depressed impedimetric values (probably caused by loading the E-plate into the controller unit of xCELLigence) these concentrations of CPC elicited a steadily increasing impedimetric value up to 10h followed by a plateau phase. It is important to mention that these delta CI values (normalized data of CI over the time) compared to the control still remained very low which means that these high concentrations of CPC proved to be toxic on the epithelial target cells. The delta CI values of the 0.005% CPC curve was higher and had an increase just like the previously mentioned impedimetric curves of CPC. However, the impedimetric curve of 0.005% CPC, and its plateau had a higher delta CI value than the four more concentrated CPCs (5, 1, 0.5, 0.05%). Impedimetric profile of the 0.0005% CPC was the closest to the control among the tested CPCs.

The calculated IC50 for 24h and 48h were 0.003% which shows that CPC reaches its maximal toxicity at 24h (Table 2).

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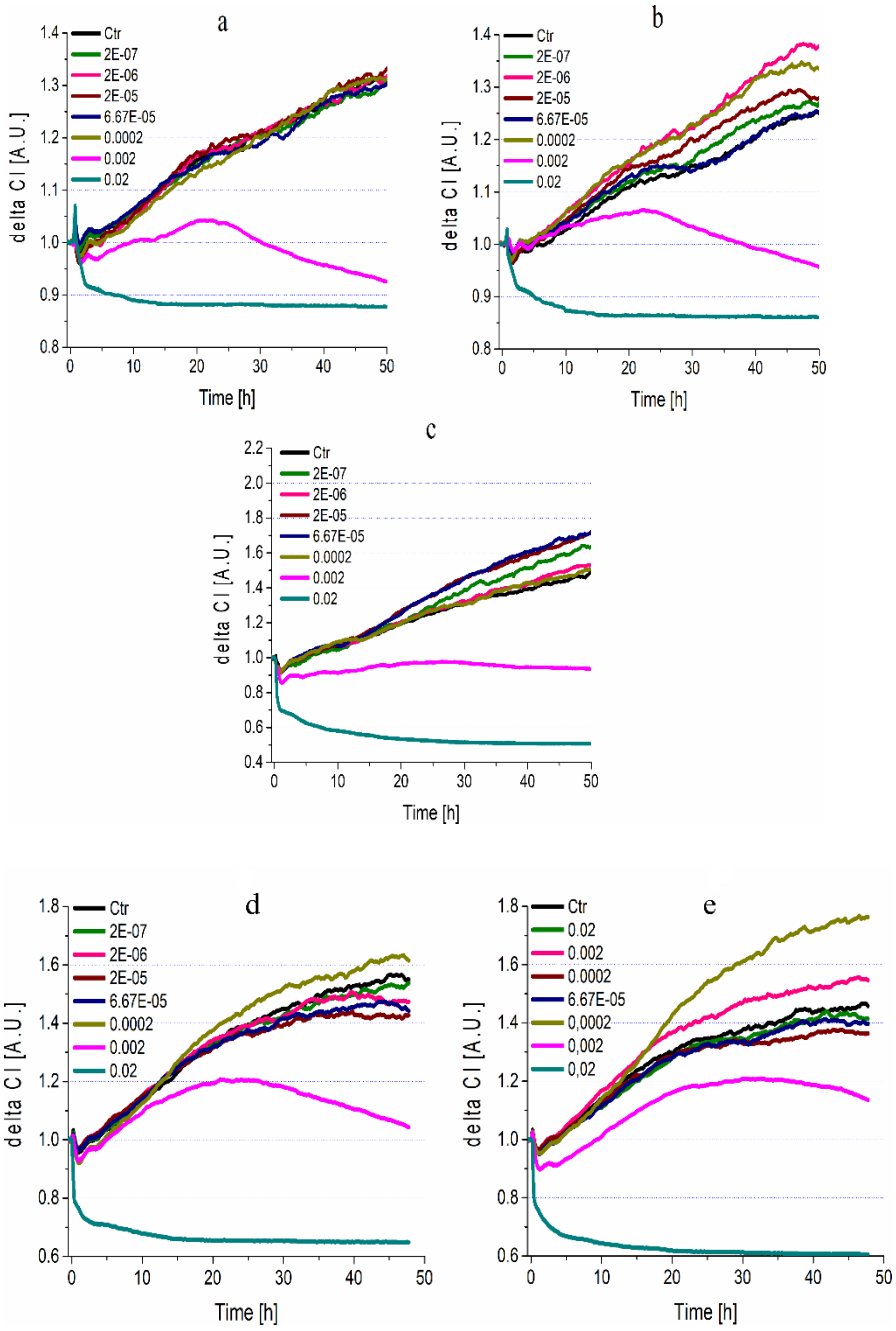
3.1.1.2. Commercially available mouthwashes

3.1.1.2.1 Gum Paroex

The delta CI values of the most diluted solutions (0.0002 - 2E-07 %v/v) of Gum Paroex stayed very close to the control values throughout the experiment. This implies that these solutions had a neutral effect on the adhesion and proliferation of the HGEP cells (Fig. 6a). On the other side of the dilution scale, a more concentrated solution (0.002 %v/v) resulted in a slight and steady increase of impedance signal (staying below the control line) up until 20h, when the impedance signal turned into a decrease. A significantly toxic nature was recorded from 30h. The most concentrated solution (0.02 %v/v) remained toxic throughout the whole experiment.

The IC50 (24h, 48h) values showed very similar results (IC50 24h – 0.002 %v/v; IC50 48h – 0.0015 %v/v) (Table 2).



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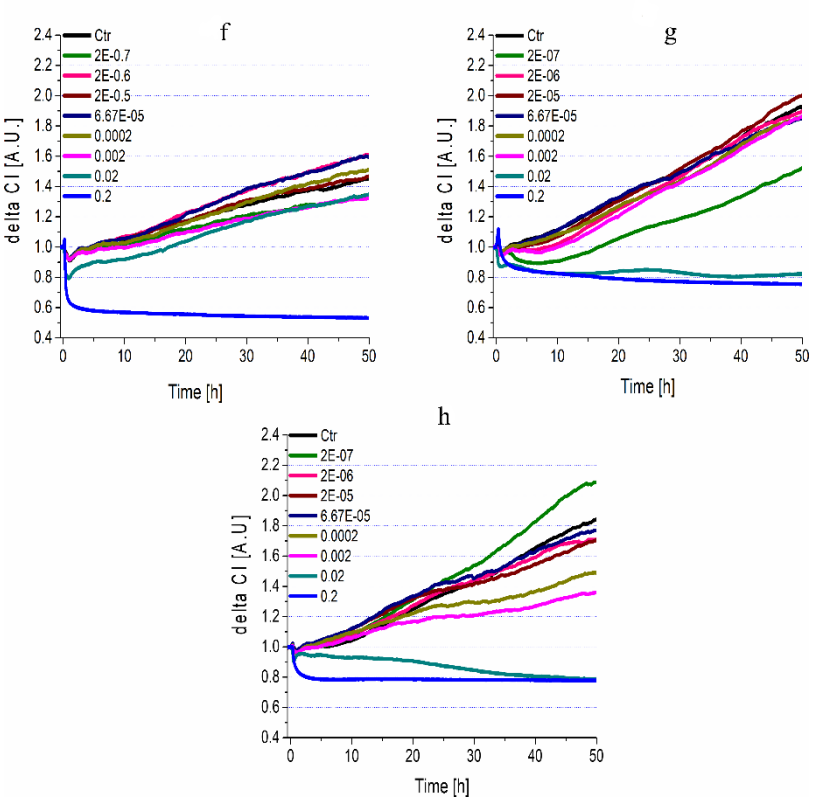

Figure 6 Impedimetric analysis (xCELLigence SP) of cytotoxicity elicited by commercially available mouthwashes on HGEP cells. (a - Gum Paroex; b - PerioAid 0.12; c – PerioAid Maintenance d – Vitis Gingival; e – Vitis Orthodontic f – Dentaid Xeros; g – Listerine Cool Mint; h – Listerine Fluoride Plus)

3.1.1.2.2 Perio Aid 0.12%

Perio Aid 0.12% had very similar effects on the HGEP cells as Gum Paroex had between 0-20h period of time (Fig. 6b). From 25h, some of the most diluted solutions (2E-06, 2E-05, 0.0002 %v/v) had even greater impedance signals than the control had and this trend was preserved for the rest of the experiment. These elevated values suggest that the solutions mentioned above were proliferation inducer on the cells. In contrast, the most concentrated solution (0.02 %v/v) had a very toxic, immediate and maintained effect. The 0.002 %v/v solution had a similar curve to the same dilution of Gum Paroex. Although the impedimetric curve showed a cytotoxic course covering the entire duration of the measurement, a gradual increase was observed with a peak at 23h, followed by a decrease until the end of the measurement.

Comparison of IC50 24h 0.04 %v/v and 48h 0.005 %v/v shows a 1-fold change which refers to a strong and time-dependent, early cytotoxic activity of the Perio Aid 0.12%.

DOI:10.14753/SE.2022.2727
The IC50 values for Perio Aid 0.12% were also significantly higher than the ones for Gum Paroex were (IC50 24h - 0.002 %v/v and IC50 48h - 0.0015 %v/v) (Table 2).

3.1.1.2.3 Perio Aid Maintenance

Incubations with the two most concentrated dilutions (0.02 and 0.002 %v/v) resulted in immediate and long-lasting cytotoxic effects. Here different levels of cytotoxicity were recorded, where 0.02 %v/v was significantly more toxic than 0.002 %v/v. In the case of lower concentrations (2E-07–0.0002 %v/v) of Perio Aid Maintenance the profile of impedimetric curves was not different from the control in the first 20 hours of the experiment. After 20 hours of incubations, the significantly increased curves indicate that the lower concentrations of this product (6.67E-05, 2E-05, 2E-07 %v/v) have also proliferation promoting effects lasting for the rest of the experiment (Fig. 6c).

The corresponding IC50 values show that the longer treatment meant a lower threshold for toxic effects. The IC50 value for 24h was 0.004 %v/v compared to the 48h - 0.001 %v/v or the 72h - 0.0009 %v/v. The 72h IC50 value also meant that this mouthwash still had some long-lasting toxic effect on the HGEP cells (Table 2).

3.1.1.2.4 Vitis Gingival

Similarly to the above-referred mouthwashes, the highest concentration (0.02 %v/v) of Vitis Gingival was the only dilution in which strong cytotoxic character developed from the beginning of the impedimetric measurement and it was detectable until the end of the experiment (Fig. 6d). In the case of 0.002 %v/v a depressed impedimetric curve was also assayed with a cytotoxic nature, but showing a slight peak at 20h of incubation. The more diluted samples of Vitis Gingival (2E-07 %v/v – 0.0002 %v/v) had similar impedance values to the control in the 0-20h part of the assays, nevertheless, in the 25-50h frame of time 2E-05 %v/v Vitis Gingival proved to be also cytotoxic, while 0.0002 %v/v concentration resulted in a gradual increase of proliferation inducer efficiency.

In the case of Vitis Gingival, the obtained IC50 values (Table 2) clearly show that cytotoxicity is greater at 48h compared to 24h values (IC50 24h - 0.01 %v/v, 48h - 0.001 %v/v).

3.1.1.2.5 Vitis Orthodontic

The most concentrated solution of Vitis Orthodontic (Fig. 6e) also had strong toxic effects on the epithelial model cells. The 0.002 %v/v reached its plateau at around 20h.

The course of the curve remained essentially unchanged for the rest of the experiment. From 20h 0.0002 %v/v and 2E-06 %v/v treatments resulted in a consistent increased impedance signal. The 0.0002 %v/v induced proliferation most substantially from the tested Vitis Orthodontic treatments. The other treatments (2E-07 %v/v, 2E-05 %v/v and 6.67E-05 %v/v) resulted a similar tendency to the control line.

The IC50 values (24 h - 0.01 %v/v; 48 h - 0.005 %v/v) show an increased cytotoxicity over the time. However, the highest non-toxic concentration was observed at 0.0002 %v/v for both 24h and 48h measurements (Table 2)

3.1.1.2.6 Dentaid Xeros

Dentaid Xeros (Fig. 6f) does not have CHX nor CPC as an ingredient. Thus we were able to examine the solution with an even higher concentration (0.2%v/v) than the previously mentioned most concentrated treatments (0.02%v/v, 0.002%v/v) (Table 1). The 0.2 %v/v treatment showed a very cytotoxic nature. In contrast to the other mouthwashes, the 0.02 %v/v had a weak but steady increase in impedance signal and remained close to the control line. The 0.02 %v/v surpassed (from 50h) the other two solutions with moderate toxic treatments (0.002 %v/v and 2E-07 %v/v). Treatments with 6.67E-05 %v/v and 2E-06 %v/v had the ability to increase the proliferation of the HGEP cells.

The IC50 values for 24, 48 and 72 h did not show a significant change (IC50 24h – 0.069 %v/v; IC50 48h – 0.063 %v/v; IC50 72h – 0.065 %v/v) (Table 2).

3.1.1.2.7 Listerine Cool Mint

Two most concentrated (0.2 %v/v and 0.02 %v/v) doses of Listerine Cool Mint elicited prompt and long-lasting (0-50h) cytotoxic effects measured by impedimetry (Fig. 6g). A similarly cytotoxic character was measured in the case of 2E-07 %v/v, nevertheless, a negative peak of this course was registered at 7h incubation which was followed by a gradual increase in the rest of the still cytotoxic course. Two concentrations (2E-06 and 0.002 %v/v) proved to be also toxic, however, this effect was detectable only between 7-20 h. This mouthwash expressed also proliferative character, but this was only a weak effect at later timepoints (45-55h).

IC50 values (IC50 24h – 0.01 %v/V) of Listerine Cool Mint were consistent with the identical values for CHX, Vitis Gingival and Vitis Orthodontic (Table 2). During the 0-72h course, the initial cytotoxicity became greater by 72h (IC50 24h - 0.01 %v/v; 48h - 0.01 %v/v; 72h - 0.009 %v/v).

3.1.1.2.8 Listerine Fluoride Plus

In the case of the tested mouthwashes, uniquely, Listerine Fluoride Plus was found to have a very wide range of cytotoxicity (Fig. 6h). However, this cytotoxic effect varied (strong, moderate and weak cytotoxicity) depending on the incubation time. The 0.2 and 0.02 %v/v concentrations elicited the strongest cytotoxicity throughout the experiment (0-55h). Moderate and continuous cytotoxicity was also observed from 20h in 0.002 and 0.0002 %v/v treatments. The weakest, however, still toxic characters were observed in treatments with 2E-06, 2E-05, 6.67E-05 %v/v. The only treatment eliciting proliferative character was the 2E-07 %v/v mouthwash.

IC50 values of 48 and 72 hour values (IC50 24h - 0.005 %v/v; 48h - 0.002 %v/v; 72h - 0.002 %v/v) (Table 2) show weak but still intensifying differences in cytotoxicity compared to the 24h value.

3.1.2 Apoptotic effects

The changes (i) in living cell numbers (cytotoxicity - measured by the decrease of impedimetric signals) and (ii) cell morphology (more rounded cells) are the consequences of the cell deaths caused by the concentration-dependent effects of mouthwashes. Samples tested after the treatments also show a decrease in living cell numbers (Fig. 7a).

The control results of these 24h treatments indicate that there is a significant decrease in the proportion of living cells treated with H_2O_2, CHX and high-concentration of ClO_2, as well as in treatments with Perio Aid Maintenance and Gum Paroex.

In the case of a mouthwash not containing CHX (Vitis Orthodontic) and the lowest concentration of ClO_2 (0.06 ppm) this proportion was similar to the control value, and despite PerioAid 0,12% contained 0.12% CHX, showed similar proportions.

The molecular-level understanding of the effects described above raises more possibilities i.e. induction of apoptosis, increased membrane permeability, inhibition of intracellular target mechanisms.

The most likely cause of these values is early apoptosis, which can be detected by the use of an Annexin V assay. This type of programmed cell death might be behind the cell deaths caused by 60 ppm dilution of ClO_2 (119.46%) and the examined dilution of Gum Paroex (146.49%) (Fig. 7b).

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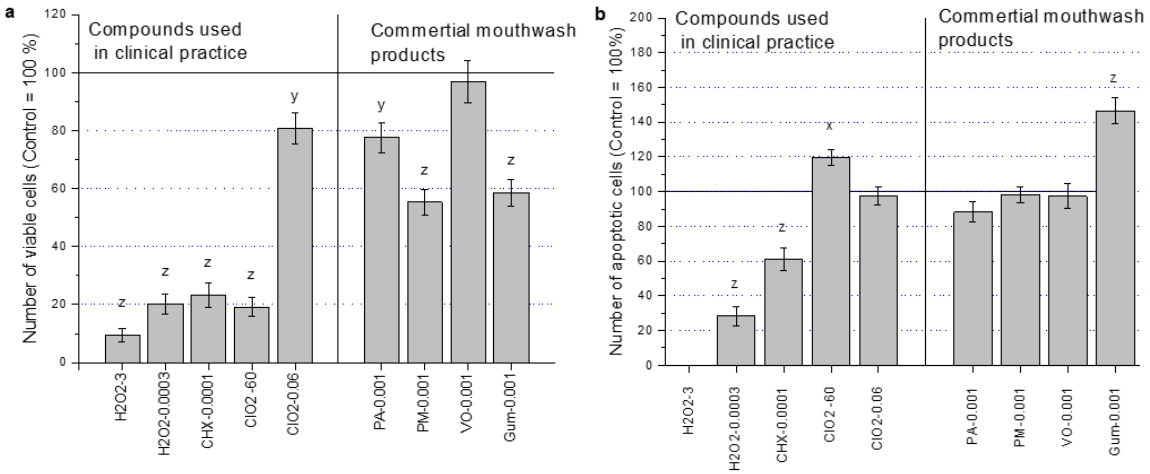

Figure 7 Viability (a) and Annexin V positivity (b) of HGEP cells treated by H_2O_2, CHX, ClO_2 and commercially available mouthwashes (PA – PerioAid, PM – PerioAid Maintenance, VO – Vitis Orthodontic, Gum – Gum Paroex)

3.1.3 Morphology and morphometry analysis

The changes in cells caused by the mouthwashes not only influence their viability but also influenced the morphological characters of the surviving and living cells. Changes in morphology were visible at 0.1% and 0.0001% CHX and 3% and 0.0003% H_2O_2. The 60 ppm (0.006%) and the 0.06 ppm (0.000006%) ClO_2 induced the smallest changes to the control (Fig. 8). The 0.05 %v/v CPC elicited toxic effects resulting in more rounded cells.

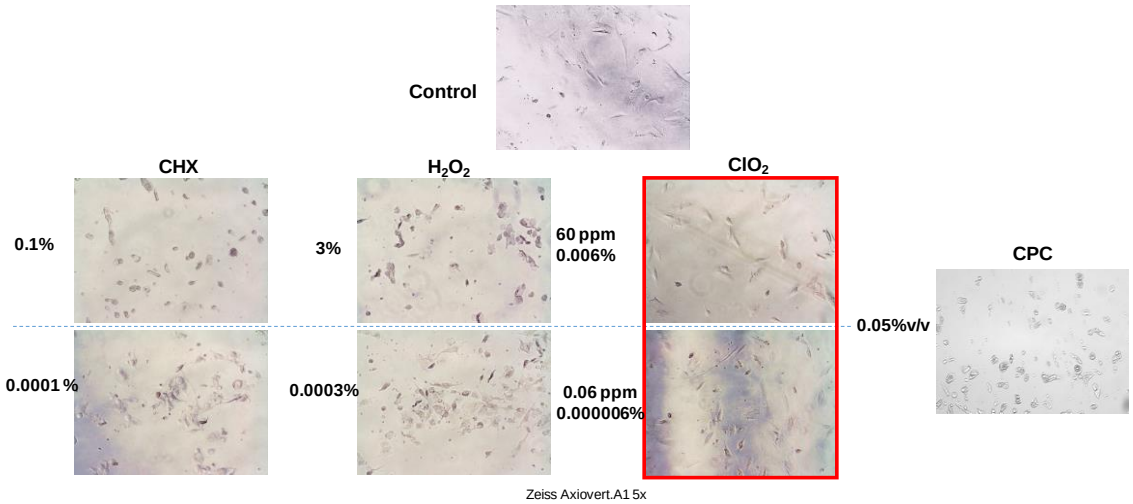

Figure 8 Morphological changes in the HGEP cells in case of CHX, H_2O_2, ClO_2, CPC treatment (Zeiss Axiovert A1 invert microscope 50x)

DOI:10.14753/SE.2022.2727

These changes were also detectable with a computer-based morphometric evaluation of indices ‘Area’ and ‘Perimeter’ (Fig. 9). The only concentration which did not cause a characteristic change in the cell morphology was the 0.06 ppm ClO_2. This concentration was neutral to the cells as it caused no significant change in the morphology of the cells. If a cell becomes rounded, or its size decreases it is considered to be the result of some internal regulatory change of mechanism(s).

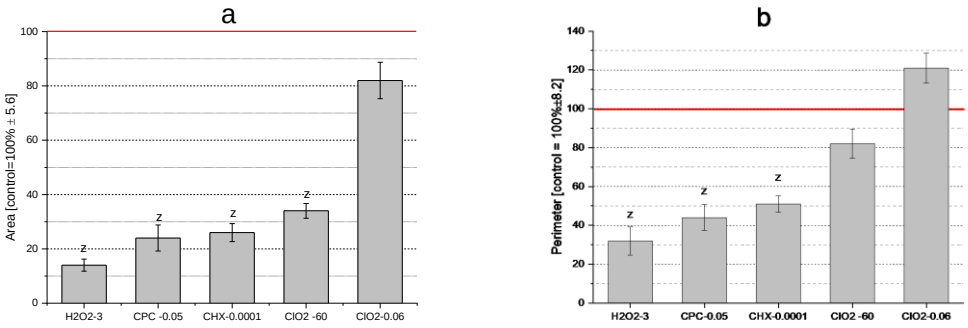

Figure 9 Morphometric changes measured by ’Area’ (a) and ’Perimeter’ (b) values in the case of treatments with CHX, H_2O_2, ClO_2 on HGEP cells. (JuLI-FL NanoEntek and Biomorph 1.1 program)

Even though the cells became more elongated because of treatments with the mouthwashes, it was more of a shrinking effect, than a characteristic change in their shape. This change was also visible from the lower numbers of the ’Area’ value (Fig. 9a). The cells’ surface ruffling characteristic can be indicated by the ’Perimeter’ value. Ruffled cell surface was detected in case of treatment with ClO_2; since some compounds (CHX and H_2O_2) caused the cells to become more rounded (Fig. 9b). Unfortunately, separate subpopulations could not be identified by Biomorph 1.1.’s cluster analysis.

In cells treated with mouthwashes containing CHX (Gum Paroex, Perio Aid 0.12%, Perio Aid Maintenance), the data showed similar morphological changes seen in treatments with CHX on its own. In the case of the mouthwash free of CHX (Vitis Orthodontic), the cell morphology did not differ from the control cells (Fig. 10).

DOI:10.14753/SE.2022.2727

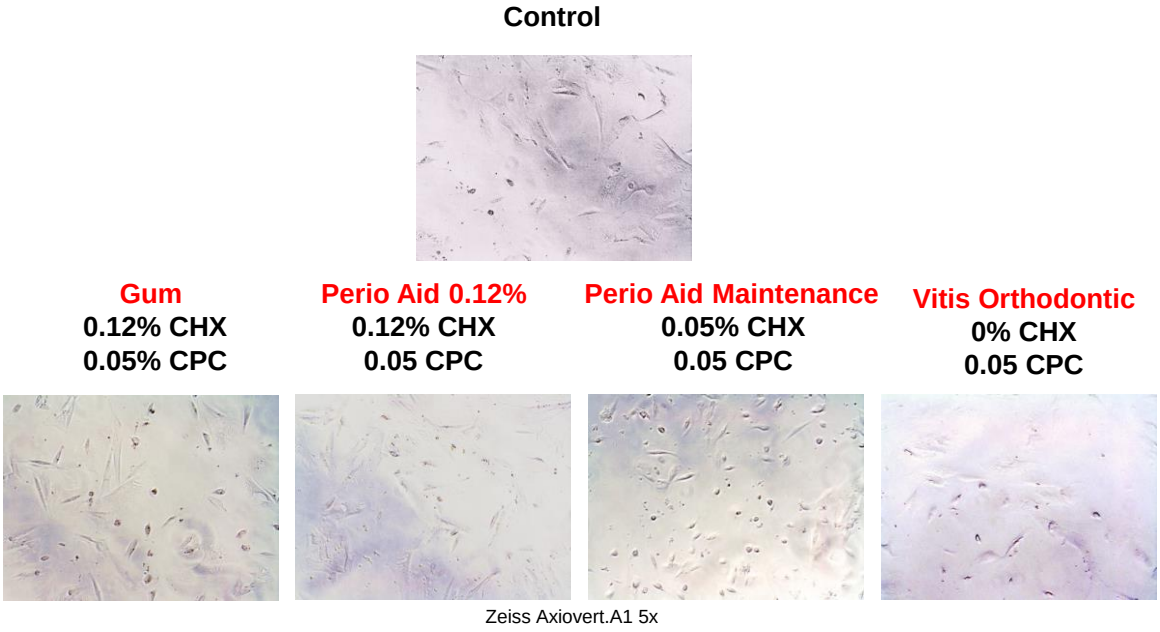

Figure 10 Morphological changes in the HGEP cells in the case of treatments with commercially available mouthwashes (GUM – Gum paroex, Perio Aid 0,12%, Perio Aid Maintenance, Vitis Orthodontic) (Zeiss Axiovert A1 invert microscope 50x)

The mouthwashes caused a significant change in the model cells morphometric values (Fig. 11a and 11b). As mentioned above, the roundness of the cells was measured by their 'Area' value. Perio Aid Maintenence, Gum Paroex, Perio Aid 0.12% (all containing CHX) significantly reduced their 'Area' values. Even though, the 'Area' value of Vitis Orthodontic (this mouthwash does not have CHX as one of its ingredients) did not change significantly. The 'Perimeter' values suffered a significant decrease in every mouthwash tested (Fig. 11a and b).

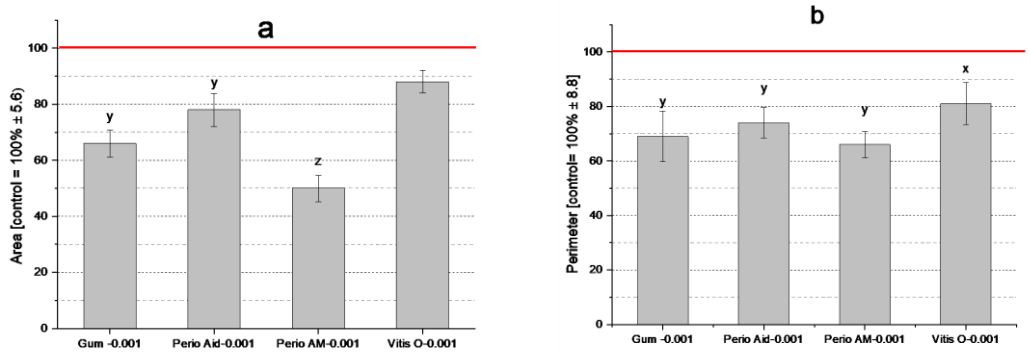

Figure 11a and b Morphometric changes measured by 'Area' (a) and 'Perimeter' (b) values in the case of treatments with some of the commercially available mouthwashes

(GUM – Gum paroex, Perio Aid 0.12%, Perio Aid Maintenance, Vitis Orthodontic)

(JuLI-FL NanoEntek and Biomorph 1.1 program)

3.2 Glass Ionomer cements

Model-cells: HGF, HFF1 fibroblast cells

Techniques applied: development of new mould; preparation of extracts; cytotoxicity/cell adhesion - impedimetry (xCELLigence SP) and AlamarBlue assay; statistics – RTCA2.0, OriginPro 8.0

(levels of significance: x – $p < 0.05$; y – $p < 0.01$; z – $p < 0.001$)

3.2.1 Design and application of novel stainless steel ring mould

As it is shown in Fig. 12, the technical design of the mould and the tool for making the cement rings have been completed. The size of the cement rings made with the new mould was within the required quality limits (Mass = 0.263 + 0.05 g), the rings were used in the chambers of the 24-wells culture plates.

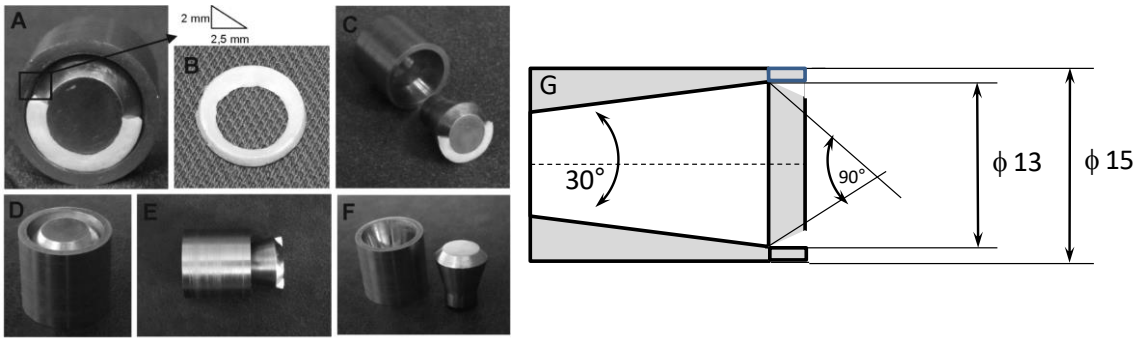

Figure 12 Open stainless steel ring mould designed in order to achieve cement rings with high-level accuracy (size and contact surface). A: the outer and inner parts of the mold placed one on top of the other, indicating the surfaces forming the cement ring; B: a cast cement ring; C, D, E, F: the external and internal elements of the mold from different points of view; G: technical drawing of the mold

With the help of the new method, the GIC rings were made with a minimum error rate. Using the rings made in this way, it is possible to make extracts under sterile conditions in the wells of the plastic plates (and also to monitor the living cells on the surface of the rings by continuous microscopic control). We measured cell physiological responses with extraction experiments where the preincubation/extraction time was 24 h.

DOI:10.14753/SE.2022.2727

3.2.2 Cytotoxicity

3.2.2.1 Human gingiva fibroblast cells (HGF)

On HGF cells (Fig. 13) the high concentrations of Fuji Equia glass ionomer cement (GIC) had (i) weak and transient (20 %v/v – 2-12h) or (ii) intense and continuous (100 %v/v – 2-30h) toxic effects. The 20 %v/v treatment resulted in a biphasic curve as the 20-30h period turned to be a proliferation inducer. The treatment with 4 %v/v was also a proliferation inducer in the 15-30h frame. In the case of Fuji Triage GIC, 100 %v/v was the only to elicit continuous (2-30h) and moderate toxic effect. The 20 %v/v treatment – similarly to treatment with Fuji Equia – caused a weak proliferative effect from 20h. Fuji Equia 100 %v/v had a more intense toxic effect compared to Fuji Triage 100 %v/v.

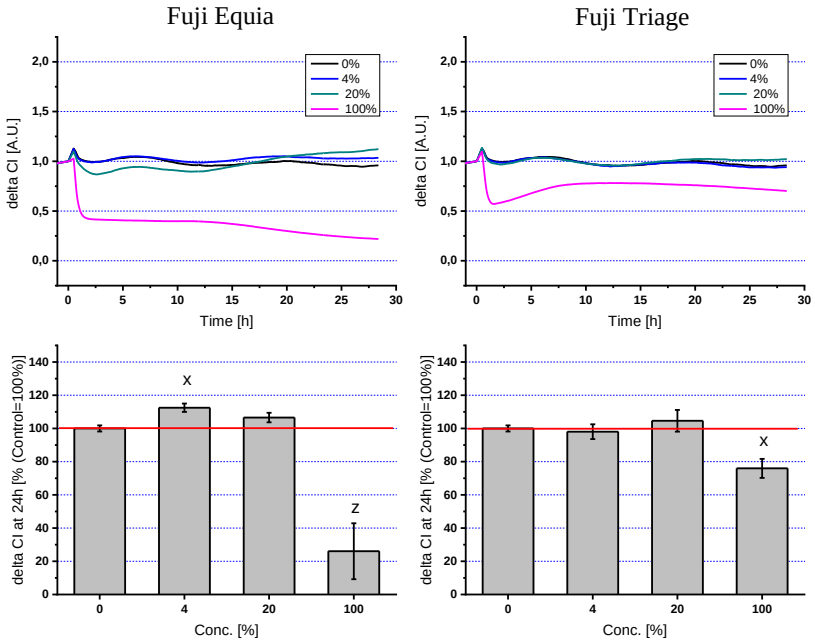

Figure 13 Impedimetric analysis (xCELLigence SP) of cytotoxicity elicited by Fuji Equia and Fuji Triage glass ionomer cements HGF cells

The cell viability and proliferation ability were further examined with colorimetry, AlamarBlue assay (Fig. 14). Results show a significant difference between the 100 %v/v Fuji Equa's and the 100 %v/v Fuji Triage's effects on HGF cells. Fuji Triage was less cytotoxic than Fuji Equia was.

DOI:10.14753/SE.2022.2727

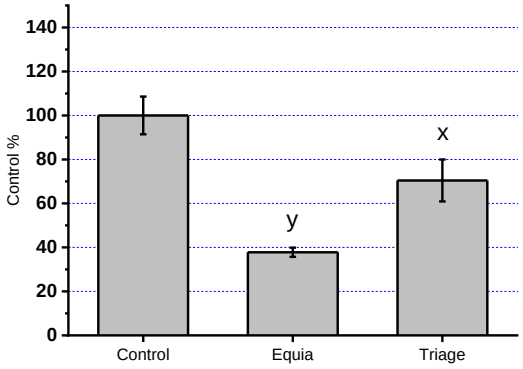

Figure 14 Colorimetric evaluation(AlamarBlue assay) of cytotoxicity induced by glass ionomer cements in HGF cells

3.2.2.2 Human foreskin fibroblast cells (HFF1)

The obtained results of cytotoxicity induced by GICs on HFF1 cells (Fig. 15) were different to the effects on HGF cells described above. The 100 %v/v Fuji Equia had an intense and continuous (2-30h) cytotoxic effect; the 20 %v/v elicited a moderate and gradually (2-30h) decreasing cytotoxic character; while the 4 %v/v concentration induced only a weak and biphasic response (cytotoxic: 2-8h; proliferative: 12-30h). The 100 %v/v Fuji Triage proved to have a moderate cytotoxic character which had a gradual decrease over time (2-30h); the 20 and 4 %v/v concentrations had weak and declining cytotoxicity in the first period of the experiment (2-8h). The Fuji Triage's overall effects on the cells were less pronounced (both cytotoxic and proliferative) than the recorded effects of Fuji Equia.

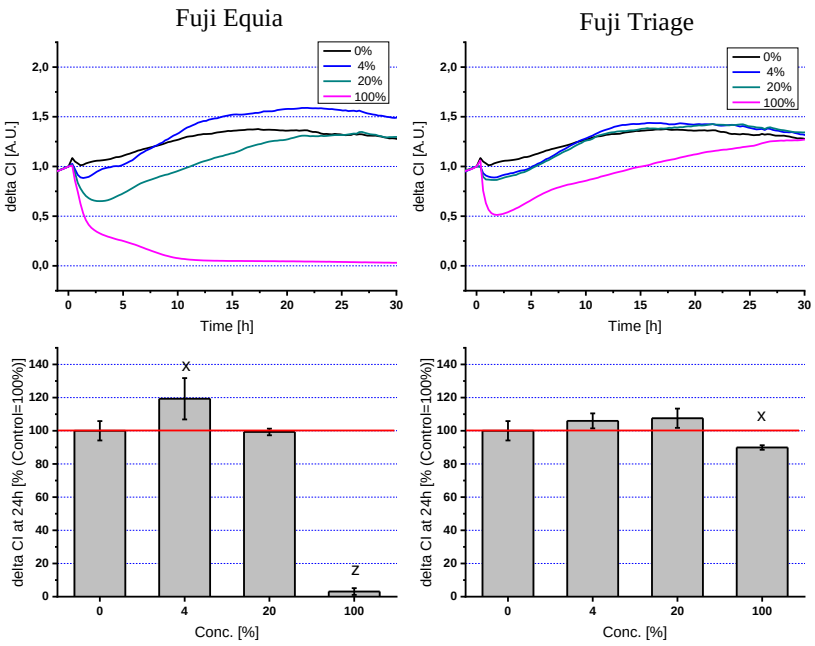

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Figure 15 Impedimetric analysis (xCELLigence SP) of cytotoxicity elicited by Fuji Equia and Fuji Triage glass ionomer cements on HFF1 cells

Alamar Blue assay showed the same results presented at HGF cells. The 100 %v/v Fuji Triage was less toxic for the cells compared to the 100 %v/v Fuji Equia (Fig. 16).

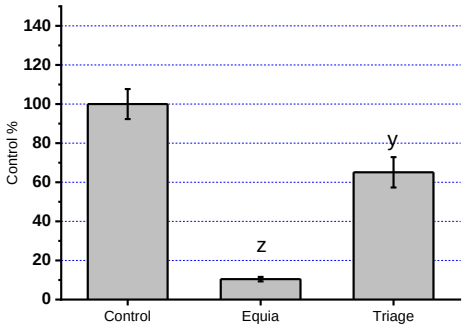

Figure 16 Colorimetric (AlamarBlue assay) evaluation of cytotoxicity induced by glass ionomer cements in HFF1 cells

3.2.3 Cell adhesion

(Adhesion was measured with dilutions not cytotoxic in 3.2.2. experiments)

3.2.3.1 Human gingiva fibroblast cells (HGF)

Impedimetry assay of HGF cell adhesion for Fuji Equia showed that only the lowest concentration (1%) had an adhesion-enhancing effect. In contrast, for Fuji Triage, a weak increase at all the three concentrations were detectable, of which, only 1% and 20% were found to be significant Fig. 17.

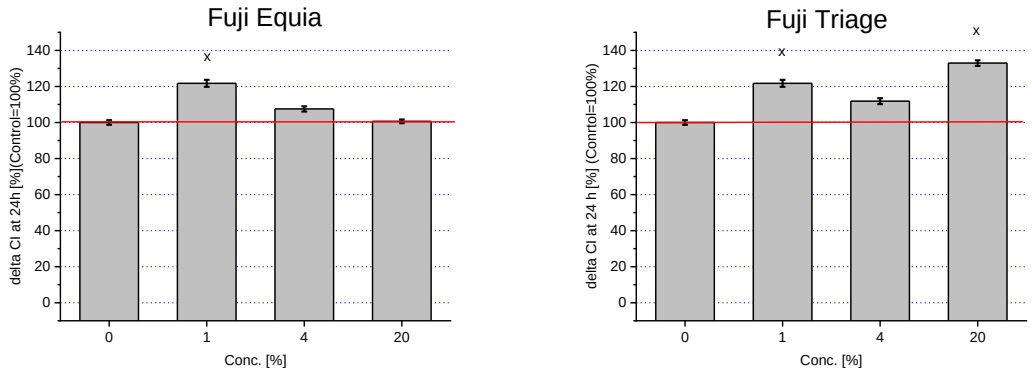

Figure 17 Cell adhesion in HGF cells induced by Fuji Equia and Fuji Triage extracts

3.2.3.2 Human foreskin fibroblast cells (HFF1)

DOI:10.14753/SE.2022.2727

For HFF1 cells, GIC extracts had a negative effect on cell adhesion. In the case of Fuji Equia, a significant reduction was measured at 20%. In the case of Triage, the extracts did not affect cell adhesion (Fig. 18).

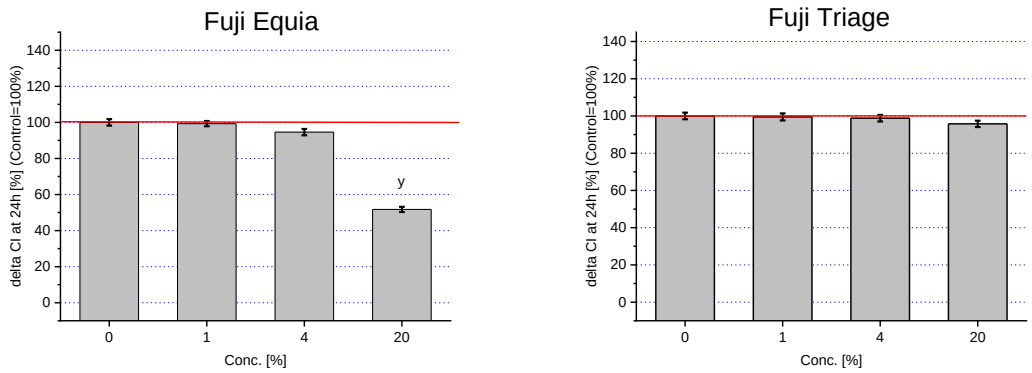

Figure 18 Cell adhesion in HFF1 cells induced by Fuji Equia and Fuji Triage extracts

3.3 Polyethyleneimine (PEI)

Model-cells: HGEP gingiva epithelium and PDL stem cells

Techniques applied: preparation of extracts; Multi-Angle Dynamic Light Scattering MADL (Zetasizer Nano) characterization of nanoparticles; cytotoxicity - impedimetry (xCELLigence SP); apoptosis - Annexin V staining; morphometry and migration and motility assays - holographic microscopy (HoloMonitor M4); statistics – RTCA2.0, Holostudio™ M4 2.6.2, OriginPro 8.0

(levels of significance: x – $p < 0.05$; y – $p < 0.01$; z – $p < 0.001$)

The cell physiological experiments were performed by nanoparticles extracted from PEI and PEI-Ag membranes. To acquire these nanoparticles (size range 10–13 nm) the PEI and PEI-Ag membranes were extracted in an FBS-free medium for 1, 5 and 10 days (Fig. 19). There was a significant difference between the particles released from the silver-containing (PEI-Ag) and non-silver-containing (PEI) membranes in an impedimetric analysis, the higher impedance signal was generated by the silver-containing complexes (Fig. 20).

DOI:10.14753/SE.2022.2727

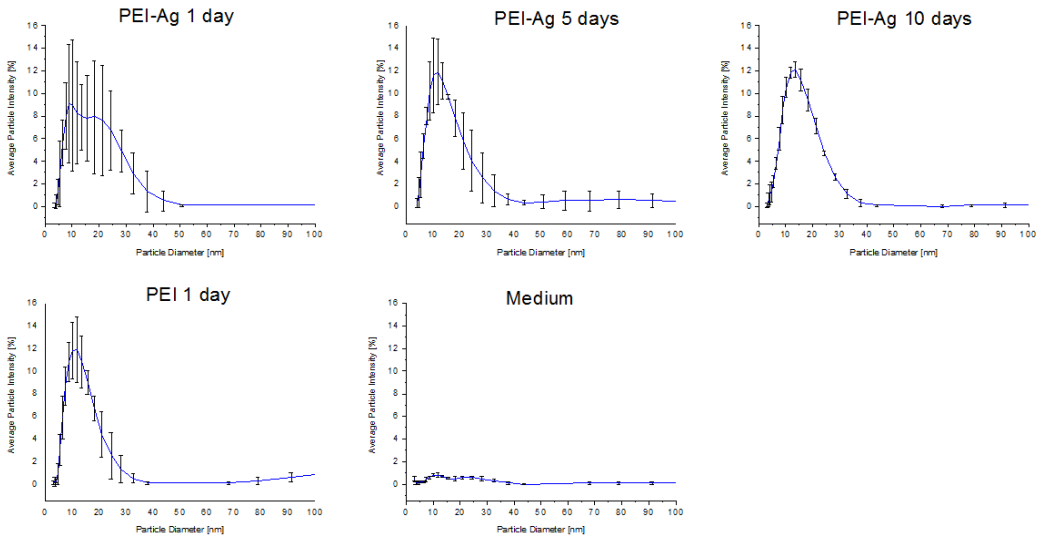

Figure 19 Multi-Angle Dynamic Light Scattering MADL (Zetasizer Nano) characterization of nanoparticles released from PEI and PEI-Ag membranes

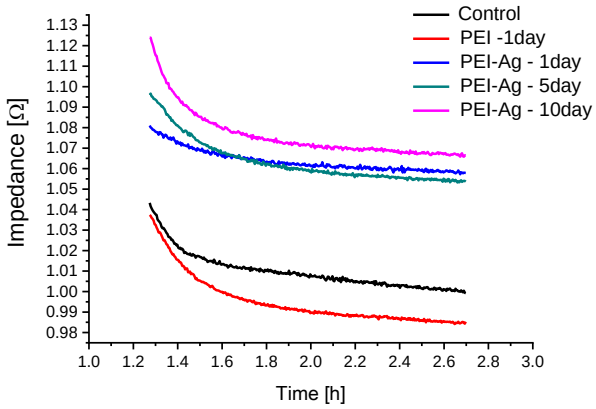

Figure 20 Impedimetric analysis (xCELLigence SP) of the nanoparticles released from PEI and PEI-Ag membranes

Note: in the case of the 10 day extracts of PEI and PEI-Ag - due to the long time of extraction -, the volume of the available extract was limited, therefore it was not applied to every combination.

3.3.1 Cell adhesion (0-24h tume interval of impedimetric measurement)

3.3.1.1 Cell adhesion – PDL cells

For particles released from the PEI-Ag membranes, only the 1 day 1/1000 dilution extract resulted in a similar adhesion signal to the control (Fig. 21). The other dilutions of the 1 day extracts had a significant adhesion blocker effect. Every dilution of the other extracts

DOI:10.14753/SE.2022.2727

(5 and 10 days) elicited a significant concentration-dependent inhibitory effect on adhesion.

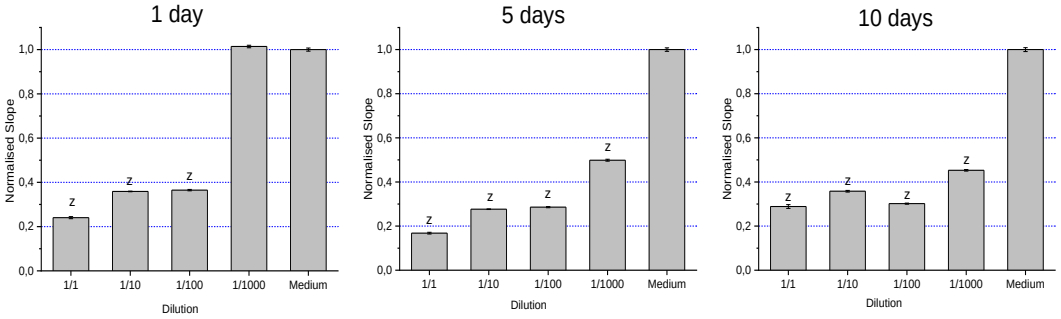

Figure 21 Impedimetric analysis (xCELLigence SP) of cell adhesion of PDL stem cells elicited by extracts of PEI-Ag membranes incubated in FBS-free medium for 1, 5 or 10 days. (Slope: The ascend of the line fitted to the impedimetric measurement for the period of 0 to 20 h.)

For the silver-free complexes of PEI (Fig. 22), none of the extraction-times or the dilutions had an adhesion inducer effect. Adhesion was inhibited in a concentration-dependent manner.

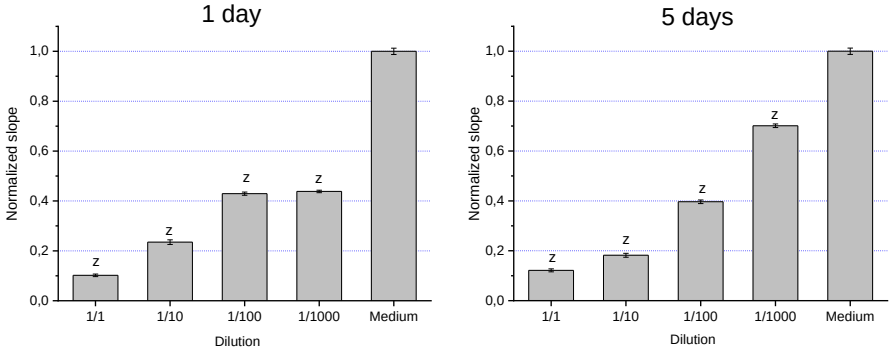

Figure 22 Impedimetric analysis (xCELLigence SP) of cell adhesion of PDL stem cells elicited by extracts of PEI membranes incubated in FBS-free medium for 1 or 5 days

3.3.1.2 Cell adhesion – HGEP cells

In the case of gingival epithelial cells, the undiluted (1/1), as well as the relatively concentrated (1/10) extracts of PEI-Ag (in both 1 and 5 days), proved to have an intense (1/1) or moderate (1/10) adhesion blocker character. In contrast, 1/100 dilutions not only

DOI:10.14753/SE.2022.2727

lost their adhesion inhibitory effect but also became significant adhesion enhancers. This ability was also detected for the 1/1000 dilution but to a moderate extent (Fig. 23).

Investigations of PEI effects on the cell adhesion of HGEP showed similar results to PEI-Ag, however, differences were also detected (Fig. 24). A similar intense adhesion blocker character was detectable in 1/1 and 1/10 dilutions of both extracts (1 day and 5 days). PEI also had an adhesion-increasing effect, which was detectable at 1/1000 dilution. Between the two opposite characters (adhesion inhibitor and promoter) in the case of PEI, the neutral effect of 1/100 dilution represents a gradual transition.

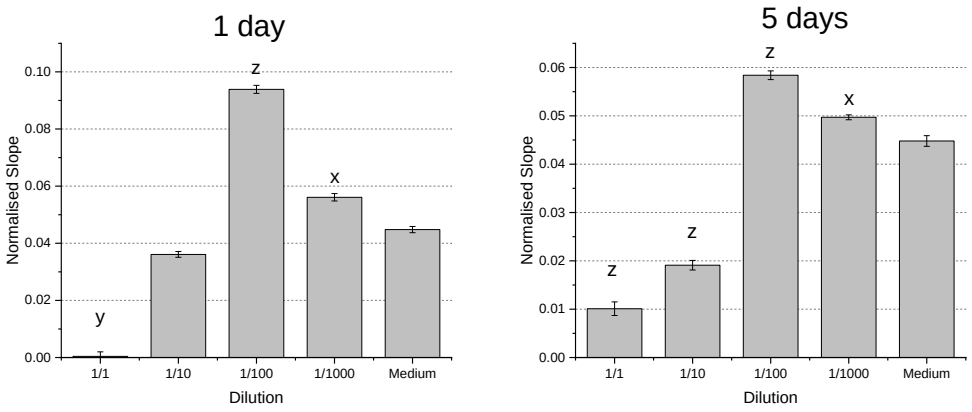

Figure 23 Impedimetric analysis (xCELLigence SP) of cell adhesion of HGEP cells modulated by extracts of PEI-Ag membranes incubated in FBS-free medium for 1 or 5 days

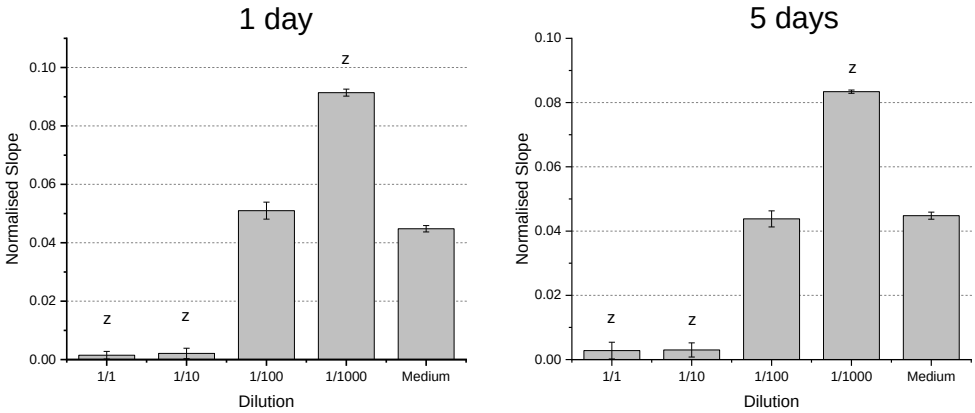

Figure 24 Impedimetric analysis (xCELLigence SP) of cell adhesion of HGEP cells elicited by extracts of PEI membranes incubated in FBS-free medium for 1 or 5 days

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3.3.2 Cytotoxicity (0-72h tume interval of impedimetric measurement)

3.3.2.1. Cytotoxicity – PDL cells

The nanoparticles extracted from the PEI-Ag complex with 1, 5 and 10 day procedure (Fig. 25) were mostly cytotoxic (1/1 and 1/10 dilutions) compared to the control. The 1/100 and 1/1000 dilutions were proliferation promoter or neutral in all series of the extracts.

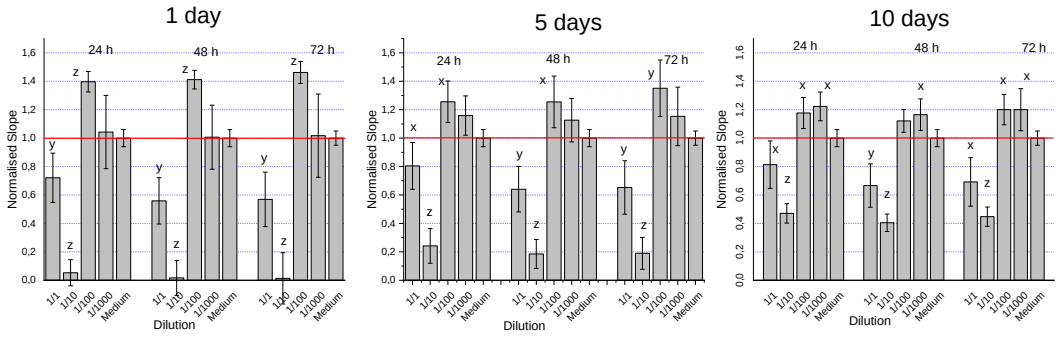

Figure 25 Impedimetric analysis (xCELLigence SP) of cytotoxicity of PDL stem cells elicited by extracts of PEI-Ag membranes incubated in FBS-free medium for 1, 5 or 10 days

In contrast to PEI-Ag, in the case of the silver-free extracts of PEI (Fig. 26), only the 1/1000 dilutions of the 1 or 5 day extracts proved not to be cytotoxic, all the other dilutions had a significantly strong cytotoxic character on PDL cells. The 1/1000 dilutions of both 1 and 5 days extracts elicited significant proliferative effects.

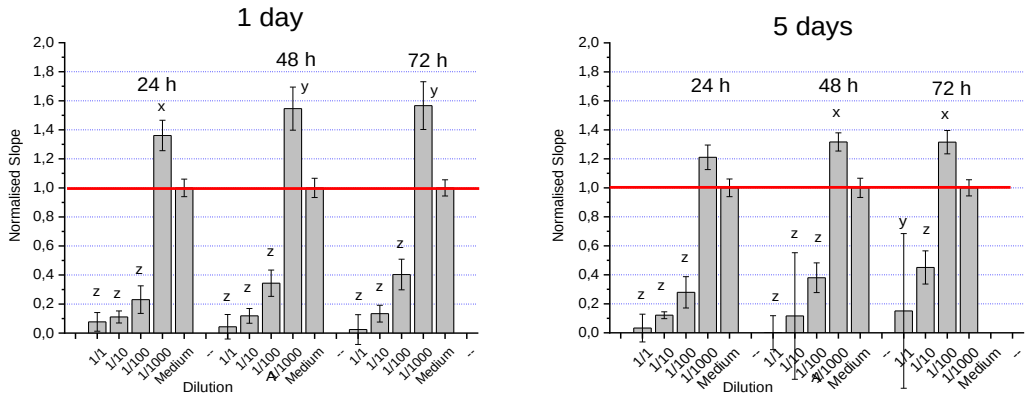

Figure 26 Impedimetric analysis (xCELLigence SP) of cytotoxicity of PDL stem cells elicited by extracts of PEI membranes incubated in FBS-free medium for 1 or 5 days

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3.3.2.2 Cytotoxicity – HGEP cells

PEI-Ag was also cytotoxic on HGEP cells (Fig. 27) in a concentration-dependent manner. The 1 day extract's 1/1 and 1/10 dilutions showed a gradually increasing cytotoxicity at 24, 48 and 72h. The 1/100 dilution developed and sustained a mild cytotoxic nature at 48 and 72h. The above described growing cytotoxicity in 1/1, 1/10 and also 1/100 dilutions were also detectable in the 5 days extracts with the difference that it was more pronounced. The 5 day extract of 1/1000 dilution at 24h had a significant and strong proliferation inducer effect.

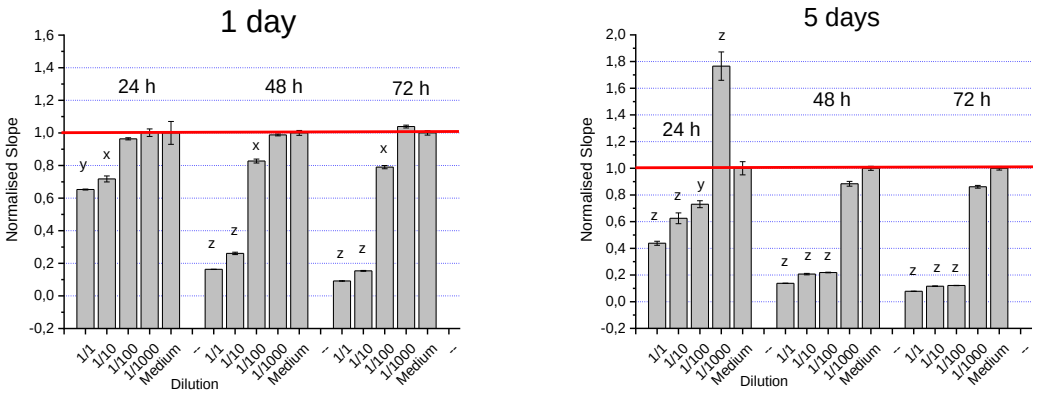

Figure 27 Impedimetric analysis (xCELLigence SP) of cytotoxicity elicited by extracts of PEI-Ag membranes incubated in FBS-free medium for 1 or 5 days on HGEP cells

The dilutions 1/1, 1/10, 1/100 of 1 day and 5 day extracts had a growing (24h<48h<72h) and significant cytotoxic effect on the HGEP cells (Fig. 28). The 1/1000 dilution of 1 and 5 days extracts induced proliferation but it only became significant after 72h treatment by the 5 days extracts.

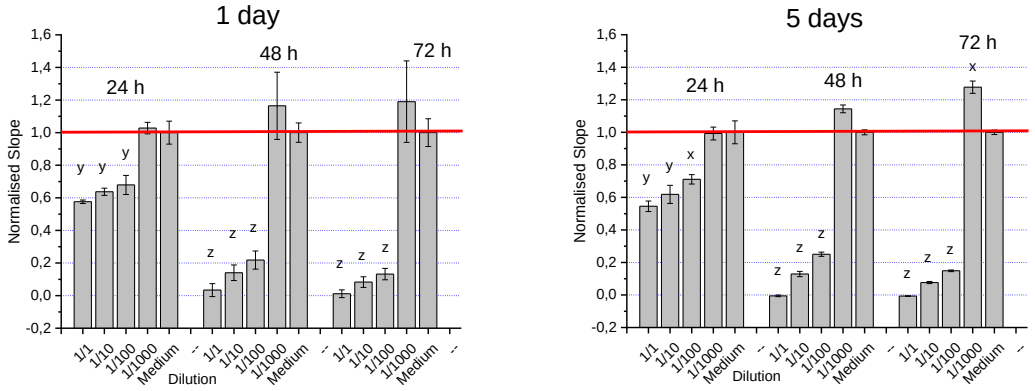

Figure 28 Impedimetric analysis (xCELLigence SP) of cytotoxicity elicited by extracts of PEI membranes dissolved in FBS-free medium for 1 or 5 days on HGEP cells

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3.3.3 Apoptosis

The results presented in this chapter are based on the rearrangement of phosphatidyl serine of the surface membrane detected by Annexin V-assay at the 24 hour. In the lated time points (48 and 72h) there was no significant change comparing to the first 24h treatments with 1, 5 and 10 extracts of nanoparticles.

3.3.3.1 Apoptosis – PDL cells

Apoptotic mechanisms may also contribute to the cytotoxic character of nanoparticles elicited by PEI or PEI-Ag. In PDL stem cells the 1/10 dilution of PEI-Ag extract was the only apoptosis inducer of our samples (Fig. 29). However, in the case of PEI nanoparticles, apoptosis was already visible even in 1/100 dilution. PEI induced early apoptosis in a wider concentration range than PEI-Ag. Thus, it is presumable that the apoptotic effect of PEI can be reduced by the incorporation of silver into the complex.

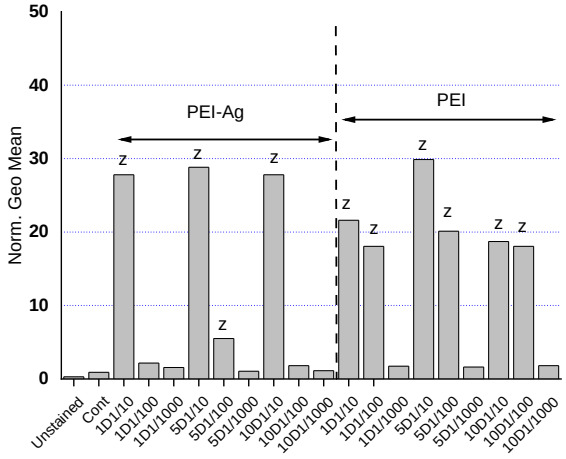

Figure 29 Annexin V positivity in PDL cells as a marker of apoptosis induced by nanoparticles released from PEI-Ag and PEI membranes

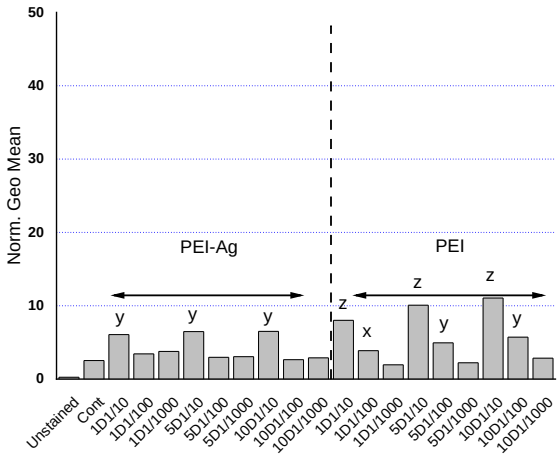

Figure 30 Annexin V positivity in HGEP cells as a marker of apoptosis induced by nanoparticles released from PEI-Ag and PEI membranes

3.3.3.2 Apoptosis – HGEP cells

In HGEP cells the extracts of PEI or PEI-Ag induced apoptosis in a smaller amplitude than in PDL cells (PDL: 1-28 Norm Geo Mean; vs. HGEP: 2-11 Norm Geo Mean) (Fig. 30). In the case of PEI-Ag, only the lowest dilution levels (1/10) induced apoptosis significantly. Apoptosis induced with PEI-Ag dilutions 1/100 and 1/1000 were weak. The treatments with compounds not containing silver (PEI) resulted in an apoptotic response

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that was more dependent on the degree of dilution. The 1/10 dilutions had the most intense and significant apoptotic behaviour. In contrast to PEI-Ag, 1/100 dilution of PEI proved to be apoptotic also, nevertheless, only on a moderate level.

3.3.4 Morphometry

In experiments of morphology and migrations (3.3.4 and 3.3.5) – in contrast to the previously reported experiments where the time-course of treatments was long-lasting (24-48-72h), due to the cell physiological characteristics of the response measured, the time frame of the experiment was only 0-400 s. (In morphometry/migration experiments the dilution which induced apoptosis significantly- 1/10 - was tested.)

The morphometric changes caused by the treatment with the nanoparticles released from PEI or PEI-Ag membranes were detected with a holographic microscope (HoloMonitor). This cell-friendly method gave a 3D image of the cells and allowed us to follow the cells' morphometric changes as they happened. Using this facility made it possible to measure series of morphometric characteristics including 'Area', 'Optical thickness' and 'Optical volume' of the cells.

Treatment with PEI-Ag (1/10) resulted in decreased 'Area' compared to the control except for treatments with nanoparticles released from the PEI-Ag membranes after 10 days. (Fig. 31a). This 10 day extract had a moderate peak of 'Area' values at 160 s. The 'Optical thickness' showed a different result: the profile of the curves showed a different course as the 1 day extract had a negative and continuous effect from 150 s, while the 5 days extract had a constant 'Optical thickness' reducer character throughout the whole experiment. The longest extracting time (10 days) resulted in the extraction of currently unknown substances which caused an increase in the 'Optical thickness' value (Fig. 31b). From the two morphometry indices described above ('Area' and 'Optical thickness'), the HoloMonitor holographic microscope also calculated the values of the volume-specific to each cell ('Optical volume' - Fig. 31c). In our case, this calculation resulted in a decreased volume in treatments with 1 and 5 days PEI-Ag extracts, although there was also a difference between the 3 extracts. The comparable rate of volume reduction was registered as follows: 5 days> 1 day > 10 days PEI-Ag sample. The 'Optical volume' reducer effect of the 10 days extract was not detectable in the time frame 140-220 s.

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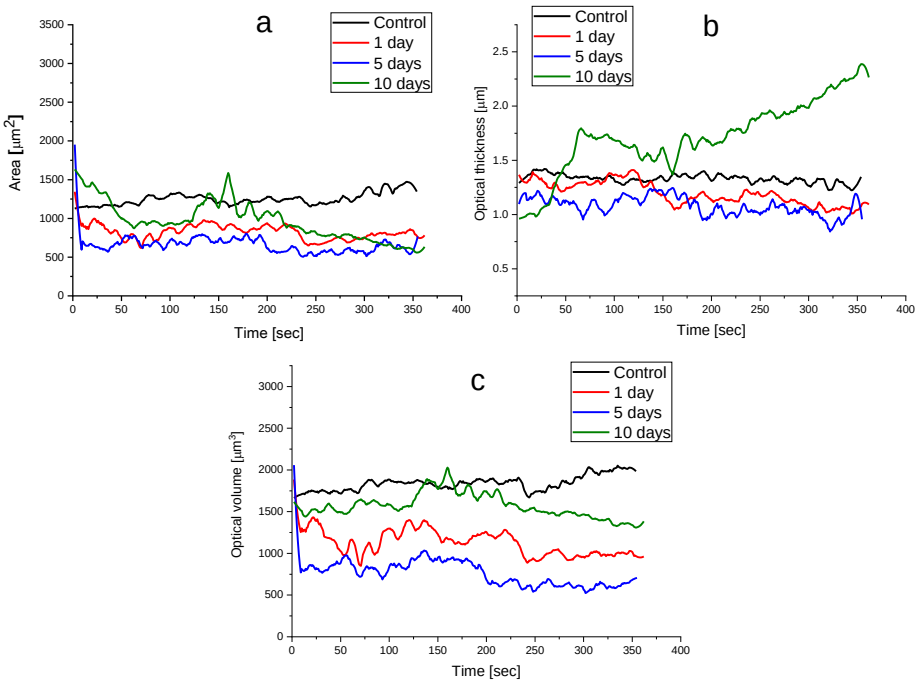

Figure 31 Morphometric changes of PDL cells caused by treatment with nanoparticles released from PEI-Ag membranes (1/10), extracted for 1, 5 and 10 days (a - Area; b - Optical thickness; c – Optical volume)

3.3.5 Cell migration and motility

To measure the migratory behavior of cells, HoloMonitor introduced the measurement of two variables, 'Migration' and 'Motility'. In the case of 'Migration' HoloMonitor calculates the distance between the starting- and the end-points of the movement of the cells. In contrast,' Motility' measures the length of the path travelled by the cells, thus characterizing the movement. As mentioned in the brief explanation above, the method used by holographic microscopy collects a multitude of individual data describing cells over time,but also allows for individual tracking of cells to perform a so-called tracking analysis. With the help of these parameters described above, our experiments measuring the migrational activity of the cells clearly showed that each of the extracts, obtained by extracting the PEI-Ag membranes for different periods, had a drastic inhibitory effect on the movement of the cells (Figs. 32, 33b). Although the distance between the starting and end-points of migratory cells was not long, the path of their journey (motility) was increased with the treatments by 1 and 5 days extracts of PEI-Ag (Fig. 33a).

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These treatments (1 and 5 days extracts) resulted in a state of motion resembling the vibration-like motion of the cells, which is very similar to micromotion described in the literature by Giaever (61). It should be noted that the difference in the effect of the three studied extracts on ’Motility’ is very similar to the result obtained for the 'Optical volume'. For both examined parameters the extracts obtained by shorter extraction times (1 and 5 days) were more effective (5 days > 1 day > 10 days extracts) while the 10 days extract proved to have milder orrather neutral effect in respect to changes in morphology (e.g. ’Area’ and ’Optical Volume) or cell motility.

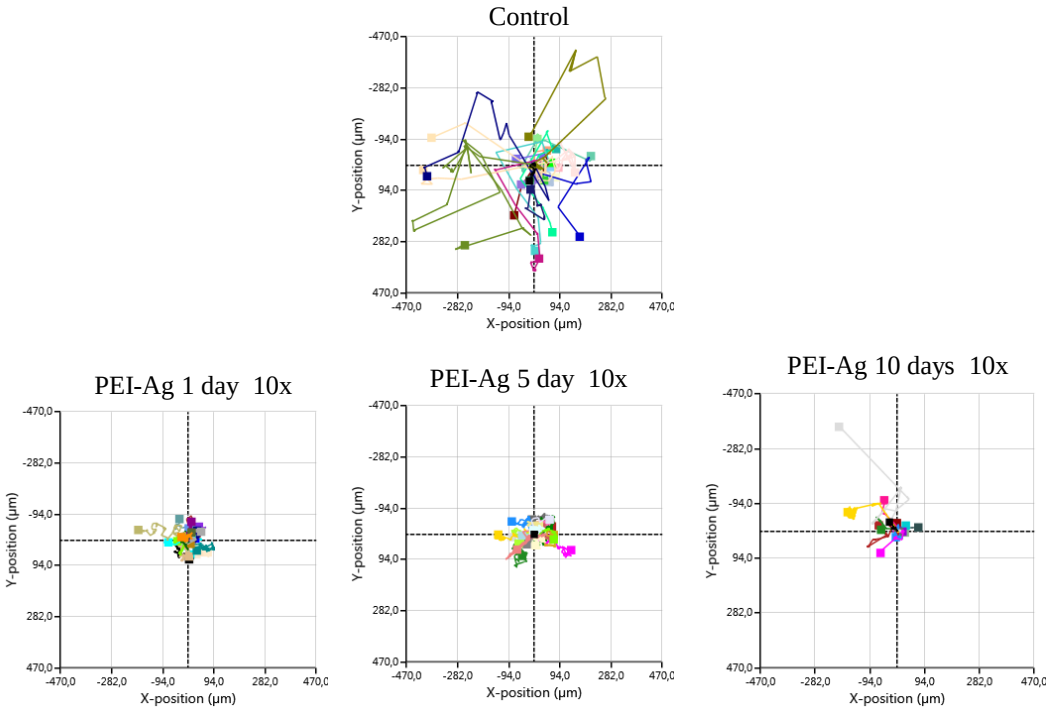

Figure 32 Migration of PDL cells after treatments of PEI-Ag membranes soaked for 1, 5 and 10 days (HoloMonitor 4 screenshot)

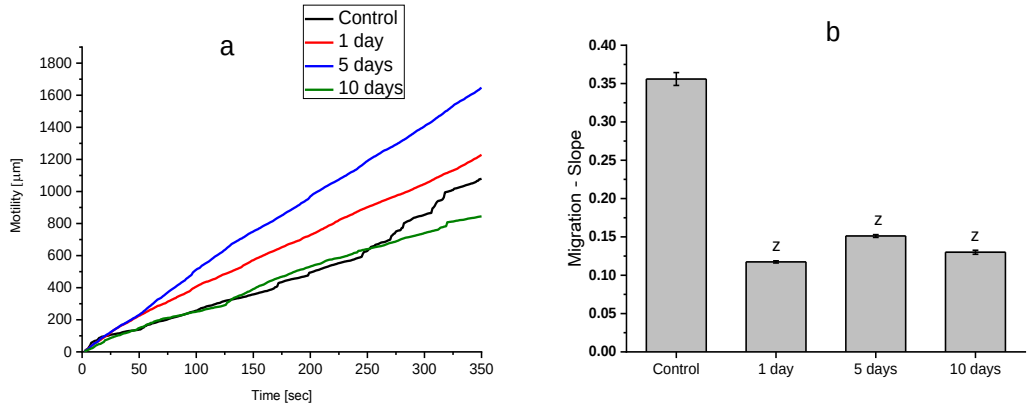

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Figure 33 Comparison of motility (a) and migration (b) of PDL cells caused by treatments with PEI-Ag membranes soaked for 1, 5 and 10 days.

DOI:10.14753/SE.2022.2727

4 Discussion

In dentistry, as it is highlighted in the *Introduction*, several substances are used where the effect on the oral cells is crucial. The evaluation of the effect on the normal and pathogenic flora (especially from the dentist's point of view) is very important, but in our present study, the effect on eukaryotic tissue elements was the main objective. The different groups of substances tested had diverse effects on the model cells. Accordingly, the reduction in both cell adhesion and cell number (cytotoxicity) were considered as pathological responses. In addition, other characteristic effects could also be registered (see apoptosis, morphometry, migration). In the following, we attempt to interpret the results obtained in the study of each group of substances and to discuss to what extent these effects may have influence on the future applicability of each of the tested dental substances.

Below we attempt to answer the issues listed in chapter *Objectives*, based on the results of our experiments.

4.1 Mouthwashes

Mouthwashes are more effective if used as an adjunct to mechanical cleaning (brushing) since they have a very important role in eliminating and/or decreasing the number of pathogen microorganisms found in the oral cavity. With the use of mouthwashes, we can also prevent the formation of a new plaque on the surface of teeth as they have a protective function due to the wide range of their ingredients, so they possess diverse biological effects (Table 3).

Table 3 Composition of the main ingredients in commercially available mouthwashes

Oral rinse – Commercial name	**Main ingredient of the product**				
	Chlorhexidine (CHX) (%)	**Cetylpyridinium chloride (CPC) (%)**	**NaF (ppm)**	**Xylitol (%)**	**Other**
Gum Paroex	0.12	0.05			vitamin E
PerioAid 0.12%	0.12	0.05		1	
PerioAid Maintenance	0.05	0.05		1	
Vitis Gingival		0.05		1	provitamin B5, zinc lactate
Vitis		0.05	226	1	vitamin E, allantoin, Aloe

Orthodontic					Vera
Dentaid Xeros			226	3.30	allantoin, betaine
Listerine Fluoride Plus			450	1	thymol, menthol, methyl salicylate, eucalyptol
Listerine Cool mint					thymol, menthol, methyl salicylate, eucalyptol

Cytotoxicity

In the case of reference molecules (H_2O_2, CHX, ClO_2, CPC), which have been known to be cytotoxic to pathogen bacteria (which is in correspondence with their clinical usage), intense cytotoxic effects were observed in many cases on human gingival epithelial cells throughout experiments (Table 4-A). This intense cytotoxic response is not advantageous for human target cells especially when inflammation is present. A number of effects on cell physiology and molecular mechanisms can be assumed in the background of the observed effects. (i) The cell viability reducing effects of H_2O_2 were reported on human periodontal ligament (hPDL) cells as well as the induction of heme oxygenase-1 and RANKL expression (62). Inhibition of cell cycle and induction of apoptosis in human gingiva fibroblast cells were also detected (63) which shows that H_2O_2 has characteristic target mechanisms of cell physiological activity on dental models. (ii) The most common main ingredient of mouthwashes is CHX which also has a protein synthesis inhibitor nature on hPDL cells while changes in mitochondrial membrane potential are also elicited by CHX. In other application of this ingredient, mobilization of intracellular Ca^{2+} and changes in ROS level was also reported as components of apoptotic and necrotic processes in osteoblast, endothelium and fibroblast cells (64). (iii) In the case of ClO_2, for human gingival epithel LD50 values - which are similar to H_2O_2 -, have been reported in the literature (ClO_2 - 0.16 M vs. H_2O_2 - 0.11 M). The mechanism of action is based on the inhibition of the cell cycle (G0/G1 arrest) in human gingival fibroblasts (62). Due to the gaseous nature of free Cl_2, it is able to penetrate tissues and to react quickly with peptides containing Cys, Met, Tyr, Trp. (iv) The best known cellular effect of CPC is the one that results in membrane destruction. Low concentrations of CPC disturbs the osmoregulation of cells. CPCs hexadecane tail is inserted into the phospholipid bilayer of the surface membrane while also exchanging pyridine to Ca^{2+} ions. Hydrophilic domains appear and the membrane fluidity decreases which results in damaged membrane function. High concentrations of CPC disintegrates membranes which are followed by cytoplasmic

DOI:10.14753/SE.2022.2727

content leakage (65). Detecting cytotoxic effects and their intensity, provided important information for us in mapping adverse side effects (while keeping in mind that in some cases an increase in proliferation is not advantageous) Table 4 and Table 5.

Table 4 Summary review of cytotoxic responses elicited by reference compounds (A) and commercially available mouthwashes(B) in HGEP cells

A	**Cytotoxicity (Intense, long-lasting effect - marked red) (*Apoptosis detected)**				
	Concentration	**Efficiency**	**Course**		
	%	Intense – I Moderate – M Weak – W	Full (h)	Early (h)	Late (h)
H_2O_2	6	**I**	**0-40**	-	-
	3	**I**	**0-40**	-	-
	0.3	**I**	**0-40**	-	-
	0.03	**I**	**0-40**	-	-
CHX	0.1	M	0-40	-	-
	0.01	**I**	-	-	**20-40**
ClO_2*	60 ppm	**I**	**10-40**	-	-
	6 ppm	W	-	-	20-40
CPC	5	**I**	**0-40**	-	-
	1	**I**	**0-40**	-	-
	0.5	**I**	**0-40**	-	-
	0.05	**I**	**0-40**	-	-
	0.005	M	0-40	-	-
	0.0005	M	-	-	15-40

B	**Cytotoxicity (Intense, long-lasting effect - marked red) (*Apoptosis detected)**				
	Concentration	**Efficiency**	**Course**		
	%v/v	Intense – I Moderate – M Weak – W	Full (h)	Early (h)	Late (h)
Gum Paroex*	0.02	**I**	**0-55**	-	-
	0.002	M	0-55	-	-
PerioAid 0.12	0.02	**I**	**0-55**	-	-

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	0.002	M	0-55	-	-
PerioAid Maintenance	0.02	**I**	**0-55**	-	-
	0.002	M	0-55	-	-
Vitis Gingival	0.02	**I**	**0-55**	-	-
	0.002	M	0-55	-	-
Vitis Orthodontic	0.02	**I**	**0-55**	-	-
	0.002	W	0-55	-	-
Dentaid Xeros	0.2	**I**	**0-55**	-	-
	0.02	W	0-55	-	-
Listerine Cool Mint	0.2	**I**	**0-55**	-	-
	0.02	**I**	**0-55**	-	-
	0.002	W	-	7-20	-
	2E-06	W	-	7-20	-
	2E-07	M	0-55	-	-
Listerine Fluoride Plus	0.2	**I**	**0-55**	-	-
	0.02	**I**	**0-55**	-	-
	0.002	M	-	-	20-55
	0.0002	M	-	-	20-55
	6.67E-05	W	-	-	20-55
	2E-05	W	-	-	20-55
	2E-06	W	-	-	20-55

Table 5 Summary review of proliferations elicited by mouthwashes in HGEP cells

	Proliferation inducing effect (Intense, long-lasting effect - marked blue)				
	Concentration	**Efficiency**	**Course**		
	%v/v	Intense – I Moderate – M Weak – W	Full (h)	Early (h)	Late (h)
PerioAid 0.12	0.0002	**W-I**	**-**	**-**	**20-55**
	2E-05	W-M	-	-	25-55
	2E-06	**W-I**	**-**	**-**	**20-55**
PerioAid Maintenance	6.67E-05	W-M	-	-	20-55
	2E-05	W-M	-	-	20-55
	2E-07	W	-	-	20-55
Vitis Gingival	0.0002	W	-	-	25-55
Vitis Orthodontic	0.0002	**W-I**	**-**	**-**	**20-55**
	2E-06	W	-	-	20-55

Dentaid Xeros	6.67E-05	W	-	-	30-55
	2E-06	W	-	-	30-55
Listerine Cool Mint	2E-05	W	-	-	45-55
Listerine Fluoride Plus	2E-07	**W-I**	**-**	**-**	**35-55**

The application of impedimetry as a novel technique to measure cytotoxicity is a dedicated method to evaluate test compounds, as its real-time data acquisition supports an accurate assessment of concentration- and time-course assay.

The investigated reference compounds (H_2O_2, CHX, ClO_2, CPC) proved to elicit intense and long-lasting cytotoxic effects where H_2O_2 and CPC had a wide range of effectiveness (Table 4-A). Concentration dependence of the intense responses induced by CHX and ClO_2 was narrower and CHX was the only reference compound in which cytotoxicity developed only in the later phase of the assays. The characteristic IC50 values of these compounds did not change significantly with the passing of the time in relation to 24-48h (see Table 2). Evaluation of our data pointed out that HGEP cells express high sensibility to the reference compounds while the fight against the pathogen flora requires higher concentrations (see Table 2 - Therapeutic concentrations) to be effective.

Commercially available mouthwashes elicited intense cytotoxicity, however, their efficiency and range of active course were rather diverse and the amplitude of effect was in good correlation with the unique composition of the products. The products where the CHX and/or CPC are present as significant components (Gum Paroex, PerioAid 0.12, Perio Aid Maintenance, Vitis Gingival, Vitis Orthodontic) the cytotoxic character was expressed in lower concentrations (0.02 – 0.002 %v/v) and in full courses of incubation times (0-55h). This clinically non-advantageous character was recorded even in the cases when some additional ingredients (e.g. NaF, allantoin, xylitol, vitamins) were present. In contrast, those products where the CHX and /or CPC ingredients were not present (Dentaid Xeros, Listerine Cool Mint, Listerine Fluoride Plus) but a list of selected additives enriched the mouthwash, the intense cytotoxic effects were elicited only in higher concentration range (0.2-0.02 %v/v). (Lower concentrations could elicit effects only in shifted time scales.) The responsible elements in these cases could be the NaF in DentAid Xerose and Listerine Fluoride Plus and the alcoholic component of the two Listerines.

The registered proliferative responses were rather sporadic, only PerioAid 0.12, Vitis Orthodontic and Listerine Fluoride Plus had intense proliferative nature in HGEP cell

DOI:10.14753/SE.2022.2727

cultures. In these cases, the gradual growth of intensity was registered which reached the real proliferative character of the curves only in the late phase of time-courses (Table 5).

Significance of additional components

In the cases discussed above, the presumed biological effects of the additional ingredients (cytotoxicity and/or proliferation) have been raised several times. Many ingredients listed in the commercially available mouthwashes are described in the literature as having anti-proliferative (antitumor) effects. These additional components include thymol, NaF, allantoin, Vitamin E, and Aloe Vera. As shown in the table below (Table 6), a significant proportion of these substances are able to exert an inhibitory effect on cell division through various mechanisms, they can also cause target cell-specific cytotoxic or proliferative effects. Based on these facts, we assume that in the background of our results, these ingredients may have an important role in the effects on the model cells. (Assessing which of these and to what extent is involved in the development of cellular responses is beyond the scope of the present PhD work, however, our future goals include the exploration of the effect of these target cell-specific substances.)

Table 6 Cell physiological effects elicited by additional ingredients of commercially available mouthwashes

	Cytotoxic	Ref.	Proliferative	Ref.	Other	Ref.
Thymol	anticancer	66			antiapoptotic	67
	antioxidant	68				
NaF	G2/M cell cycle arrest		proliferation inducer	69	migration inducer	69
Allantoin	proliferation inhibitor	70	proliferation inducer	70	wound healing promotion	71
Vitamin E derivatives	subG0 cell cycle arrest	72			antiapoptotic	73
Aloe Vera					wound healing	74
Menthol	proliferation inhibitor	75			motility	75
Methyl salicylate	proliferation inhibitor	76				
Eukalyptol	oxidative DNA damage	77				

DOI:10.14753/SE.2022.2727

Based on our results, the determination of the concentration-dependent effect of each substance provided an opportunity to give an accurate characterization of its cytotoxic / cell viability nature by determining the maximum non-toxic concentrations and IC50 values. Knowing these two values (or their quotients) can be very important for clinical applications, especially for substances such as mouthwashes, which are not only used in dental practice (see CHX or H_2O_2) but became part of everyday life due to the need of the maintenance of oral hygiene. As shown in the figure below, the proximity and distance of the maximum 'Non-toxic concentration' and the IC50 value are not indifferent to the intact preservation of certain tissue elements in the mouth (Figure 34).

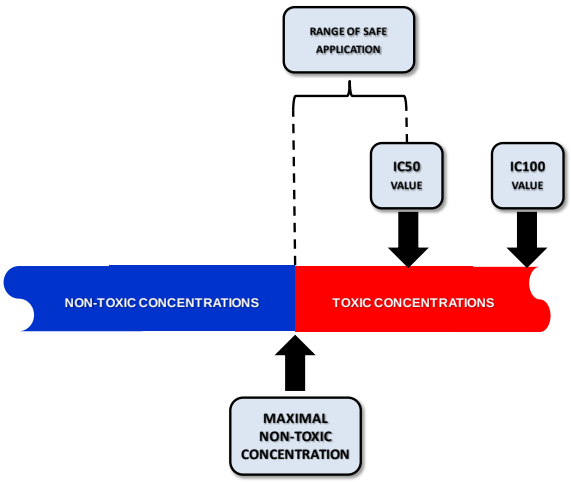

Figure 34 Interpretation of safe applicability taking into account IC50 and maximum non-toxic concentrations

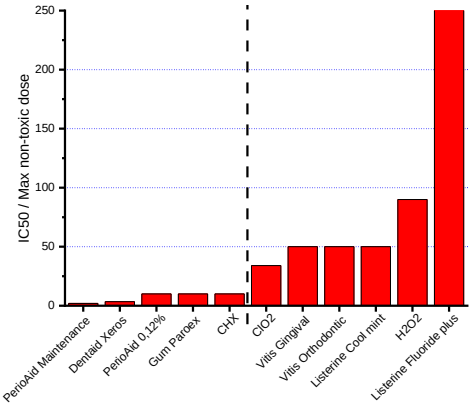

Figure 35 Characterization of safe applicability taking into account IC50 / Maximum non-toxic concentration ratios (= SI) based on viability index values for HGEP epithelial cells

This is especially important if a bacterial flora scavenger is otherwise used which has a detectable effect on other cells of the oral cavity (e.g., gingival epithelium, normal cell population of crevicular fluid, PDL cells, etc.). The materials studied in the present work fall into this category, and our studies have clearly shown that they are able to affect the viability of the gingival epithelium in a concentration-dependent manner. Therefore, we also characterized the effect of each substance by constructing the ratio (IC50 value / Maximum non-toxic concentration) using the two variables described above. Our

evaluation indicates that in this correlation the high numerical values of the ratio characterize safe compounds in practice, while low values represent a need for a more cautious usage (Fig. 35). The most ideal substances are the ones where the IC50 and the Maximal non-toxic values are the closest to each other (see Safety Index =SI) (78). In our case, the substance meeting the above-mentioned conditions is ClO_2 (SI=34). (In the case of PerioAid Maintenance the IC50 value is much smaller than the Maximal non-toxic value resulting in a low rate - SI=2, while on the other end of the spectrum with Listerine Fluoride Plus IC50 value is much greater than the Maximal non-toxic value – SI=250.)

In addition to the direct cytotoxic effects referred to by the literature (e.g. cell cycle arrest, antioxidant effect, oxidative DNA damage), the cell number-reducing effects of our tested substances suggest that early apoptosis induction may also occur. Our studies showed that this mechanism was detectable only in 60 ppm ClO_2 and 0.001 %v/v Gum Paroex among the tested substances and concentrations (Fig. 7b). Analysis of the results shows that in terms of viability (Fig. 7a), the 60 ppm ClO_2 shows a significantly better value than the other reference substances. (A comparison of the two groups, reference and commercially available mouthwashes, also showed that the 81% viability value of ClO_2 corresponds to the values of commercially available mouthwash products.) For apoptosis results, the mean of the reference substances tested (61.2%) is below the value of commercially available mouthwashes (108.7%). In the case of Gum Paroex, which has a strong apoptotic effect (148%), the proapoptotic nature of vitamin E derivatives seems to be responsible among its many ingredients (see Table 3) (73). Nevertheless, the low viability-reducing effect seems to balance the apoptotic character of HGEP cells, especially when compared to the other reference substances. (Of course, we cannot rule out the triggering/activation of mechanisms that were not analyzed in our study but may cause cell death, such as necrosis, necroptosis, anoikis etc.) Figure 36 shows the most significant epithelial cell viability reducing effects based on the study of representative compounds used in dentistry.

DOI:10.14753/SE.2022.2727

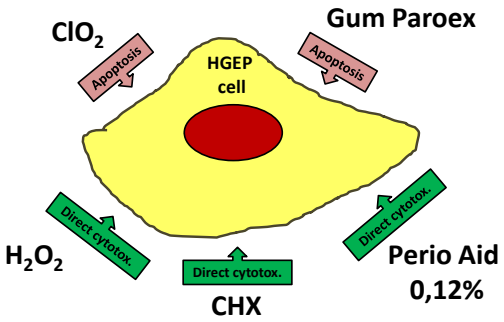

Fig. 36 Based on our measurements, the most effective compounds acting via direct cytotoxicity or apoptosis induction in HGEP epithelial cells.

Taking into account that the combined aspects of beneficial therapeutic effects and patient safety (based on our present studies) the reference compounds/mouthwashes have (i) a weak .and (ii) short-term cytotoxic effect. (Possibly even a slight proliferation-enhancing effect, on patients' cells can be considered adequate.) The (iii) apoptosis-inducing effect of the tested compounds is small.

In our experiments, we observed the effects on cell morphology in reference substances as well as in mouthwashes containing reference substances. As shown in the *Results* section, ’Area’ and ’Perimeter’ values proved to be sensitive variables for cell morphology. However, of the reference materials, ClO_2 caused the slightest change, and mouthwashes that included both CHX and CPC caused a significant morphological change. Data found in the literature suggest that the two reference components (CHX, CPC) also affect cell morphology by altering (i) the permeability of the cell surface membrane; (ii) the cell adhesion and (iii) certain elements of the cytoskeletal system. These mechanisms mentioned above, individually or with each other, may be able to show a reduced value for ’Area’ and ’Perimeter’.

It is clear from the answers to our questions in Objectives 1, 2, 3that each of the reference compounds have a cytotoxic effect, on which their clinical application is also based on. However, the duration of application can be significantly different in practice as well, and the maximum incubation time of 0-40 hours used in our experiments significantly exceeds the time used in dental practice. However, real-time impedance measurements show that high concentrations are able to exert a significantly earlier cytotoxic effects. Nevertheless, the study of the curves indicates that these substances presumably result in selected subpopulations of HGEP cells, as suggested by the ascending trends of the

DOI:10.14753/SE.2022.2727
curves (e.g. Fig. 5a and 5d). All in all, the ClO_2 seems to be the most favorable considering its Safety Index value and morphometric data, too.

In the framework of the present PhD study, the cell physiological effects of additional components found in mouthwashes have not been studied, however, as shown in Table 6, these substances have a significant cell physiological effect on human cells. Of these, the cytotoxic effects are prominent, but may also affect proliferation, too. In mouthwashes, individual combinations of these substances can contribute significantly to the development of viability indices of gingival epithelial cells (and other human cells).

4.2 Glass Ionomer cements

As it was described in the chapter Results, a new method was developed to prepare GIC rings suitable for measuring cytotoxic and other cell physiological activities. With the help of the new method, GIC rings were made with a minimal error rate. Thus it was possible to prepare extracts under sterile conditions in the wells of plastic plates (and even to monitor the emigration of living cells to the surface of the rings under an inverted microscope).

Compared to other similar techniques mentioned in the literature (79, 80), this new method has proved to be suitable for both (i) in vitro cell physiological studies and (ii) preparation of extracts, too.

In the case of the two GICs examined, Fuji Equia was found to have a greater cytotoxicity in high concentrations on HGF and HFF1 cells (Fig. 13 and 15). The same result was obtained by colorimetric evaluations, which shows that Fuji Equia has a stronger cytotoxic character on fibroblasts than Fuji Triage. This can be considered for the future when making Fuji Equia posterior fillings.

In continuation of the Discussion of the issue raised in Objectives 7, we also evaluate the effects of the two GICs on viability and cell adhesion from a cellular perspective. The comparative study documented that both human fibroblast cell lines - regardless of their tissue affiliation (gingiva or foreskin localization) -, showed greater sensitivity to Equia extracts. This responsiveness reducer character of Equia GIC was demonstrated when measuring cell adhesion as well as in both methods evaluating cytotoxicity. Our understanding that Equia liquid is a mixture of polyacrylic acid and tartaric acid, which is stronger than polyacrylic acid (81). Thus the initial pH of Equia was expected to be lower than that of Triage. Also, the molecular weight of tartaric acid is much lower compared to

polyacrylic acid (150 vs 20,000) (82). Therefore faster diffusion of a stronger acid may be the cause of the early mortality of the cells in contact with Equia, and the continued lower pH may contribute to the observed depressed proliferation.

4.3 Polyethyleneimine complexes

The dental evaluation of the cell physiological effects of PEI / PEI-Ag complexes are complex. On the one hand, the antibacterial effects of the release of Ag from the PEI-Ag complex, on the other hand, the effects on the patient's cells must be taken into account. The present work aimed to analyze the latter. This requires a specific interpretation for both cell adhesion and cytotoxic effects, as the decreased cytotoxicity and increased adhesion values induced by PEI-Ag for the patient's cells (PDL and HGEP model cells) can be considered a beneficial effect. Because of the above, we give our answer to the question in the Objectives.

Our results show (Fig. 19), in terms of the physical character of the PEI and PEI-Ag extracts studied with Zeta-sizer Nano, that the size of the released nanoparticles fall in nearly the same nanometric range (10-13 nm) over the time periods of 1-5-10 days extractions. Further characterization of the silver-containing particles by impedimetric analysis (Fig. 20) also confirmed our above mentioned results, according to which our cell physiological studies were performed with PEI-Ag nanoparticles in the same size range.

Reviewing our results, we found differences in both the presence of silver in the complex (PEI-Ag vs PEI) and the target cell specificity. These indicate that the presence of silver in the extracts is advantageous for high dilutions (1/1000) resulting in increased adhesion and decreased cytotoxicity. This may have a beneficial effect on the prolonged (48-72h) effect of residual PEI-Ag in the oral cavity in clinical applications. Our observations are in good correlation with the test results of silver nanoparticles in the literature (83, 84). Our results indicate that of the two model cells examined the PDL cells proved to be more sensitive to the presence of silver (Figs. 25-26 vs Figs. 27-28).

The apoptotic effects found, suggested a different sensitivity of the two model cells, as a significantly higher degree of apoptosis was induced in PDL cells with either PEI or PEI-Ag (PDL: PEI-Ag vs PEI = 28.3 vs 23.6; HGEP: PEI-Ag vs PEI = 6.2 vs 9.6). As the data show, apoptosis can be induced more in PDL cells, as it is more sensitive to the tested PEI-Ag or PEI. It should be noted, however, that PEI was able to induce apoptosis in both

DOI:10.14753/SE.2022.2727
model cells at even higher dilutions (1/100), whereas PEI-Ag was only significantly apoptotic at the lowest dilutions (1/10).

Examination of the newly developed silver-containing dental material presented above indicated that the incorporation of silver into the PEI complex had a beneficial effect on their cell physiological nature. Thus, an (i) increase in adhesion, (ii) a decrease in cytotoxicity, and (iii) a shift in apoptosis-inducing concentration to the lowest dilution, all suggest that the new silver-containing complex has more favorable properties than PEI. Literature reports on migration and corresponding cell morphological changes induced by silver nanoparticles suggest an increase in migration/motility (85, 86), contrary to the results presented in our present work. There may be different explanations for the differences in the results. Contrary to the above-mentioned references, the differences in the model cells (retinal endothelium, fibroblast vs gingival epithelium, PDL stem cell) can also explain the discord in the results. However, the difference in the chemical structure of the nanoparticles seems to be a significant factor. This can be greatly influenced by the chemical stability, catalytic activity, localized surface plasma resonance and conductivity values (87, 88). In our work, the PEI-bound silver nanoparticles represent a new structure, therefore some of the cell physiological effects of PEI-Ag (see motility and morphometry) are different from those mentioned in the literature, but its overall cellular biological effects appear to be favorable.

DOI:10.14753/SE.2022.2727

5 Conclusion

The results proved that the new real-time methods, impedimetry and holographic microscopy can be used in studies for cell physiological effects elicited by materials used in dentistry.

Based on our results, the answers to subjects listed in Objectives, we can say that

1. The tested mouthwashes and their reference compounds had characteristic cytotoxic and proliferation-inducing effects on the human gingival epithelium cells.
2. Some of the negative effects mentioned above indicated direct cytotoxicity (H_2O_2, CHX, PerioAid 0.12%), while in other cases apoptosis induction was also found (ClO_2 and Gum Paroex).
3. Computer-based morphological analysis of ’Area’ and ’Perimeter’ show, that the most drastic change of the reference compounds was elicited by the 3% H_2O_2 and 0.05% CPC while the smallest morphological change was caused by 0.06 ppm ClO_2. For commercially available mouthwashes, the biggest change in cell morphology was caused by 0.001 %v/v PerioAid Maintenance, while Vitis Orthdontic proved to elicit the smallest change.
4. In contrast to the reference compounds used in oral disinfection (H_2O_2, CHX and CPC), the therapeutic concentration of ClO_2 is the least cytotoxic which is supported by the fact that ClO_2 has the optimal SI value (SI=34).
5. The aim of the present work was not to investigate the cell physiological effects of the addicional components of mouthwashes. However, numerous literature data suggest that these compounds (e.g. allantoin, ethyl alcohol (27%), NaF) may have a significant effect on human cells (e.g. epithel cells).
6. The new method, which gives cement rings a uniform morphology, can be used to prepare extracts from the cements which then can elicit cell physiological effects.
7. The two glass ionomer cements tested (Fuji Triage and Equia), Fuji Triage proved to be less toxic on both model cells, due to the presence of the faster diffusing tartaric acid component found in Fuji Equia extract.
8. Since no significant difference was found between the two model fibroblast cell lines (HGF and HFF1) in their cell physiological responses, the more

DOI:10.14753/SE.2022.2727

characterized, easier to maintain, immortalized cell lines can be used for GICs testing.

9. Particles in the nano-size range can be found in 1-5-10 day extracts prepared from PEI and PEI-Ag membranes.
10. Dilutions of supernatants containing PEI had concentration-dependent effects on cell adhesion and cytotoxicity in both PDL stem cells and HGEP cells.
11. Comparing the effects of PEI-Ag complexes with the reference PEI, the silver-containing PEI-Ag proved to have more favorable cell physiological parameters - although here the target cell specificity (HGEP vs PDL) showed some differences.

DOI:10.14753/SE.2022.2727

6 Summary

The main objective of our study was to analyze the cell physiological effects of **15 compounds** (4 reference compounds, 8 types of mouthwash, 2 glass ionomer cements, and 2 PEI complexes) already introduced in dentistry as advantageous on pathogens but **not characterized in the human gingival epithelium (3.1, 3.2, 3.3) and periodontal ligament stem cells (3.3)**. Our experiments were performed on a wide range of concentration- and time-dependence studies of the given substances and prepared extracts. The main parameters analyzed were: **(i) cytotoxicity / proliferation; (ii) cell adhesion; (iii) apoptosis; (iv) morphometry and (v) cell migration**.

Our results show that most of the mouthwashes and their reference compounds had both **cytotoxic and proliferative effects**, although cytotoxicity was mostly measurable at **higher concentrations**. In the case of commercially available mouthwashes, it can be assumed that some **known additives** may cause adverse effects. The **Safety Index (SI)** calculated from the maximal non-toxic doses and IC50 values indicate the safe use of **ClO_2 (SI=34) in HGEP cells**. Our results show that only ClO_2 and Gum Paroex can induce apoptosis, while 0.06 and 60 ppm **ClO_2 is the only substance** that does **not pathologically affect cell morphology**.

For the analyzed **glass ionomer cements,** the extracts prepared from **Fuji Triage proved to be less toxic** than Fuji Equia on both HGF and HFF1 cells. This cell physiological character was detected in impedimetric assays of **cytotoxicity and cell adhesion**, too. The observed increased toxicity of **Fuji Equia** cement can be explained by the higher content of tartaric acid of the extracts which results in **faster diffusion** and thus depressed proliferation.

In the case of **PEI-Ag nanoparticles**, the comparison with the reference PEI, **favourable properties (increased adhesion, decreased cytotoxicity)** were detected for the silver-containing complex.

Based on the results summarized above, in the case of all three tested groups of compounds, a **minimal, concentration-dependent and cell physiologically noxious effect** was demonstrated. However, it is worth mentioning that these negative effects are present in cells with a short cell cycle. The reported harmful effects are minimal in the dental stem cells.

The overall message of our results is that in the case of dental materials with a significant cytotoxic effect on the pathogenic flora, the harmful effects on the human cells is not

DOI:10.14753/SE.2022.2727
negligible. Although they may elicit negative character depending on the composition / chemical character they may be damaging even in the case of epithelial cells or stem cells in an uncontrolled environment and depending on the duration of exposure.

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DOI:10.14753/SE.2022.2727

Acknowledgement

First and foremost I would like to thank my Tutors Prof. Dr. Dobó-Nagy Csaba for his guidance and wisdom he showed me during my work.

I would also like to thank Prof. Dr. Buzás Edit for allowing me to work in her Department.

Very special thanks to the whole Chemotaxis Reasearch Group, especially Dr. Láng Orsolya my Scientific Supervisor for many years for her endless patience and guidance, and also to Dr. Lajkó Eszter, Dr. Takács Angéla and Dr. Zambaga Khorolsuren, to whom I could always turn to for advice. Thank you Orbán Andra for teaching me everything about cell cultures and how to keep my cells alive.

I would also like to thank Prof. Zelles Tivadar my former Scientific Supervisor and Dr. Géczi Zoltán for including me in their ongoing research.

Special thanks to all of my co-authors of my papers, especially to Dr. Gresz Veronika who introduced me to the world of glass ionomer cements.

I would like to thank my two homes – Semmelweis University's Department of Oral Diagnostics and the Department of Genetics, Cell- and Immunobiology where they believed in me, and made my work possible.

This PhD work was founded by EFOP-3.6.3-VEKOP-16-2017-00009 'Kiegészítő Kutatási Kiválósági Ösztöndíj'.

I am most grateful to my whole Family's support, especially my Mother and my Husband who made it possible for me to have some time to finish my PhD work.

Last but certainly not least, I would like to thank my Father, for believing in me.