INVESTIGATION OF THE CELL PHYSIOLOGICAL EFFECTS OF SUBSTANCES USED IN DENTISTRY

PhD thesis

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1. Introduction

A wide variety of materials are used in dentistry. During 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.
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.

2. Objectives

The main goal of my investigations was to contribute with novel data to better understand the biological and clinical effects performed on primary and secondary model cells, of three groups of substances (mouthwashes, glass ionomers and polyethyleneimine complex).
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 be assumed in the case of cell number decreases observed in viability changes?
3 Do the tested substances/mouthwashes cause cell morphological deviations detectable by computer-assisted morphometry in the tested eukaryotic oral cell line?
4 Which of the tested reference compounds have the least cytotoxic effect?
5 Are the additional components responsible for some of cytotoxic effects?

Glass ionomers

6 Development of a novel, cost-effective, easy to perform and standardized method to investigate the cytotoxicity of GICs.
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)? If so which characteristics of the cells or compounds are possibly responsible for the characteristics described?

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?

3. Methods

Cytotoxicity/Impedimetry

The cell physiological responses elicited by the dental substances tested were monitored by impedimetry using xCELLigence Real-Time Cell Analysis SP (ACEA Biosciences). In the assay system, gold electrodes are located at the bottom of each well of the 96-well array (E-plate). In the case of arrays connected to an AC system, the adhesive cells form an insulating layer on the gold electrode. Due to the electrical insulating property of the surface membrane of the living cell, an impedance signal (Z) is generated. The magnitude of this signal is increased in proportion to the number of adherent cells, making it suitable for tracking the number of viable and adherent cells. (The responses are explained using the impedimetric curve profile, depending on the phase the cells are in when the treatments were applied (Fig. 1). From the change in impedance obtained during the measurements, the Cell Index (CI) can be calculated using the following formula: $\text{Cell Index}_i = (Zt_n - Zt_0) / F_i$

In the formula, Zt_n is the impedance measured at the given time, while Zt_0 is the impedance measured at the starting time; F_i is the frequency constant, which is 15 in this case. Data normalized over the time (Delta Cell Index) were used to evaluate efects of treatments.

In the case of a cell adhesion study, the cells and the test compounds 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 compound and the registered signal indicates the cytotoxic or proliferative effects.

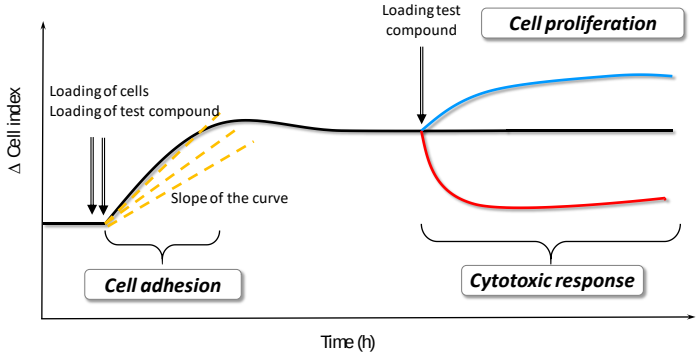

Figure 1 Understanding of impedimetric strategy to measure cell adhesion and cytotoxicity/proliferation

Apoptosis

Annexin V was used to measure apoptosis. Annexin V binds to an early apopstosis marker phosphatidylserine. In a living cell, phosphatidylserine is only present in the inner membrane (membrane asymmetry), while during/after apoptosis, it appears in the outer layer too, thus apoptosis can be detected with labeled Annexin V. Annexin V-PE Plus (MBL International) apoptosis detection kit was used. During the assay, the cells to be tested (5×10^4 cells/sample) were treated with mouthwashes that proved to be cytotoxic in our pilot experiments for 48 h. (By the end of 48 h, the efects elicited by the compounds did not change characteristically). The incubation with mouthwashes or active ingredients was followed by washing in PBS and centrifugation (1000 rpm/5 min). After removing the supernatant, the sample containing cells was resuspended in 300 µl assay bufer, after which 3 µ l of Annexin V-PE and 0.6 µ l of SYTOX Green dye (Invitrogen) were added to the cells. Incubation was performed at room temperature, protected from light, for an incubation time of 10 min. Annexin V-positive cells were evaluated with fow cytometry BD FACSCalibur (BD Biosciences) (Annexin V-PE: Ex=488 nm, Em=578 nm; SYTOX: Ex=488 nm, Em=525 nm) and under fuorescent cell movie analyzer JuLI FL (Nano Entek). Data acquired were then analyzed by CellQuest Pro software

Morphometry

Treatment with different dental materials also caused visible changes in the morphological parameters of model cells. The morphological changes elicited by treatments were recorded on native microscopic samples, and 50× magnifcation images of a Zeiss Axiovert A1-inverted microscope (Carl Zeiss) were used for these studies. Computer-assisted morphometric analysis was performed using video recordings (5 recordings/group). Model cells were incubated for 48 h with test compounds. 200 µl of the samples were placed onto predegreased surfaces. Each (video recorded) frame contained approximately 10–12 cells/microscopic felds for 120 s at a maximum frame rate of 3 frames/s. For this purpose, we used the Biomorph 1.1 program developed by Chemotaxis Research Group, GCI-SU. This morphometry analysis performed the cluster analysis of the data in addition to the basic morphometric parameters (Area, Perimeter) of the examined cells.

Holo Monitor

To measure cell morphological changes and swimming ability we used holo microspy (HoloMonitor M4). Holo microscopy uses a diode laser, this beam is split into two beams a sample beam and a reference beam. These beams come together and create a hologram. The hologram is recorded by an image sensor and computer-processed to produce the final phase image (quantitative phase images).

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.

Stainless steel mould

A novel stainless steel mould was created to produce high-level accuracy cement rings (Fig 2). These rings created are standardised in size and could be used in extraction experiments, under sterile conditions. 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/cell culture plates. With the help of the new method, the GIC rings were made with a minimum error rate.

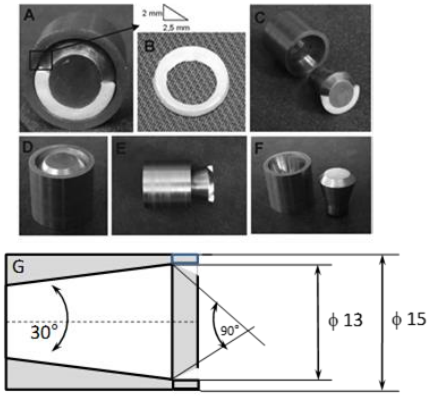

Figure 2 Open stainless steel ring mould designed. A: the outer and inner parts of the mold placed one on top of the other, indicating the surfaces forming the cement ring; B: a cast cement ring; C, D, E, F: the external and internal elements of the mold from different points of view; G: technical drawing of the mold

Statistics

In our studies, a minimum of four parallel measurements with three replicas (n=12) were performed in each case. For impedimetric studies, the slope analysis of the obtained curves was calculated by the xCELLigence SP equipment's statistical program (RTCA 2.0, Real Time Cell Analyzer; ACEA Biosciences). For the statistical evaluations of the obtained results, one-sample ANOVA and the statistical routines of Origin Pro 8.0 were used. The following symbols are used to denote the levels of signifcance: $z \leq 0.005$, $y \leq 0.01$, and $x \leq 0.05$. To determine the IC50 values, we used a ftting sigmoidal dose response curve with the nonlinear regression function of the Origin Pro8.0 program (OriginLab) based on the following equation: For the morphometric evaluation, the built-in statistical routines of Biomorph 1.1 were used.

4. Results

4.1 Mouthwashes

Cytotoxicity

4.1.1.1. Reference compounds

4.1.1.1.1 Hydrogen peroxide

The results of proliferation 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 in the case of concentrations, 6% and 3% the signals run low throughout the experiment. Lower concentrations had slight curves at some point in the experiment. In the case of 0.3%, this

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 control and the 0.3% line. 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.
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).

4.1.1.1.2 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. 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 the entirety of the 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).

4.1.1.1.3 ClO_2

The most concentrated solution in our experiment was 60 ppm ClO_2 which is outside the reported therapeutic range of ClO_2. 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, IC50 48h – 50.51) suggesting that the 24 hour incubation was enough to achieve the maximal decrease in cell viability (.

4.1.1.1.4 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 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 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.

4.1.1.2. Commercially available mouthwashes

4.1.1.2.1 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. 3a). 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; IC50 48h – 0.0015).

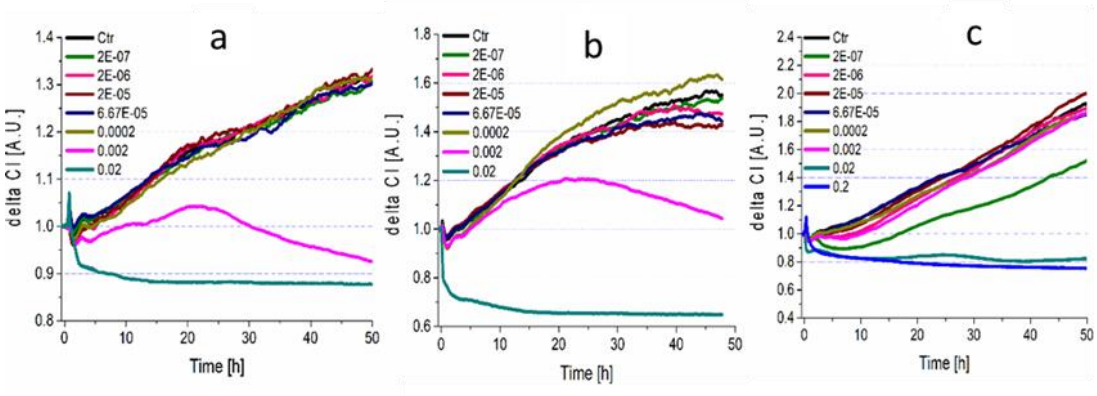

Figure 3 Impedimetric analysis (xCELLigence SP) of cytotoxicity modulated by commercially available mouthwashes on HGEP cells. (a - Gum Paroex; b – Vitis Gingival; c – Listerine Cool Mint)

4.1.1.2.2 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. 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.
Comparison of IC50 24h 0.04 and 48h 0.005 shows a 2-fold change which refers to a strong and time-dependent, early cytotoxic activity of the Perio Aid 0.12%. The IC50 values for Perio Aid 0.12% were also significantly higher than the ones for Gum Paroex were (IC50 24h 0.002 and IC50 48h 0.0015).

4.1.1.2.3 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.
The corresponding IC50 values show that the longer treatment meant a lower threshold for toxic effects. The IC50 value for 24h was 0.004 compared to the 48h 0.001 or the 72h 0.0009. The 72h IC50 value also means that this mouthwash still had some long-lasting toxic effect on the HGEP cells.

4.1.1.2.4 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. 3b). 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 clearly show that cytotoxicity is greater at 48h compared to 24h values (IC50 24h - 0.01, 48h - 0.001).

4.1.1.2.5 Vitis Orthodontic
The most concentrated solution of Vitis Orthodontic 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.

4.1.1.2.6 Dentaid Xeros

Dentaid Xeros 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) (see 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 hs did not show a significant change (IC50 24h – 0.069; IC50 48h – 0.063; IC50 72h – 0.065).

4.1.1.2.7 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. 3c). 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 7-20h. This mouthwash expressed also proliferative character, but this was only a weak and short-term (45-55h) effect.
IC50 values (IC50 24h – 0.01) of Listerine Cool Mint were consistent with the identical values for CHX, Vitis Gingival and Vitis Orthodontic. During the 0-72h course, the initial cytotoxicity became greater by 72h (IC50 24h - 0.01; 48h - 0.01; 72h - 0.009).

4.1.1.2.8 Listerine Fluoride Plus

In the case of the tested mouthwashes, uniquely, Listerine Fluoride Plus was found to have a very wide range of cytotoxicity. 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; 48h - 0.002; 72h - 0.002) show weak but still intensifying differences in cytotoxicity compared to the 24h value.

4.1.2 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.

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%).

4.1.3 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 effects of high and low concentrations of CHX, H_2O_2 and ClO_2, the 60 ppm (0.006%) and the 0.06 ppm (0.000006%) ClO_2 induced the smallest changes to the control. The 0.05 %v/v CPC elicited toxic effects resulting in more rounded cells.

These changes were also detectable with a computer-based morphometric evaluation of indices 'Area' and 'Perimeter'. 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).

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. 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. Unfortunately, separate subpopulations could not be identified by Biomorph 1.1.'s cluster analysis.

The mouthwashes caused a significant change in the model cells morphometric values. 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.

4.2 Glass Ionomer cements

4.2.2 Cytotoxicity

3.2.2.1 Human gingiva fibroblast cells (HGF)

On HGF cells (Fig. 4) 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.

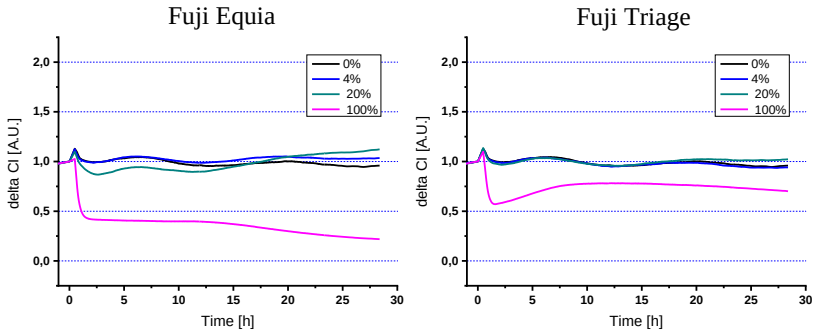

Figure 4 Impedimetric analysis (xCELLigence SP) of cytotoxicity modulated by Fuji Equia and Fuji Triage glass ionomer cements HGF cells

The cell viability and proliferation ability were further examined with colourimetry, AlamarBlue assay. Results show a significant difference between Fuji Equa's and Fuji Triage's effects on HGF cells. Fuji Triage was less cytotoxic than Fuji Equia was.

4.2.2.2 Human foreskin fibroblast cells (HFF1)

The obtained results of cytotoxicity induced by GICs on HFF1 cells (Fig. 5) 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.

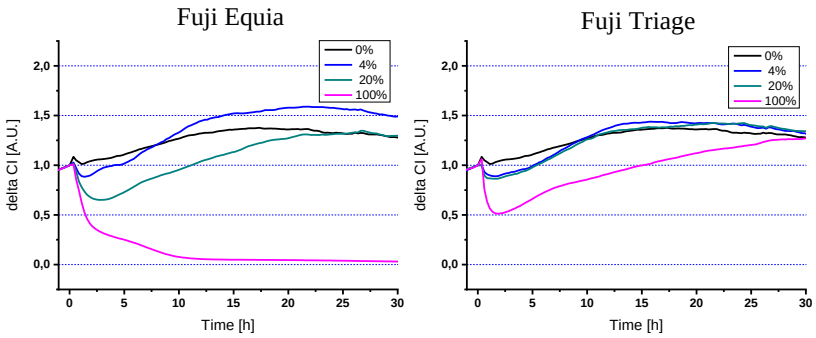

Figure 5 Impedimetric analysis (xCELLigence SP) of cytotoxicity modulated by Fuji Equia and Fuji Triage glass ionomer cements on HFF1 cells

Alamar Blue assay showed the same results presented at HGF cells. Fuji Triage was less toxic for the cells compared to Fuji Equia.

4.2.3 Cell adhesion

4.2.3.1 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.

4.2.3.2 Human foreskin fibroblast cells (HFF1)

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.

4.3 Polyethyleneimine (PEI)

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 dissolved in an FBS-free medium for 1, 5 and 10 days. There was a significant difference between the particles released from the silver-containing (PEI-Ag) and non-silver-containing (PEI) membranes by impedimetric analysis, the higher impedance signal was generated by the silver-containing complexes.

4.3.1 Cell adhesion (duration of impedimetry: 0-24h)

4.3.1.1 Cell adhesion – PDL cells

Particles released from the PEI-Ag membranes, only the 1 day 1/1000 dilution extract resulted in a similar adhesion signal to the control. The other dilutions of the 1 day extracts had a significant adhesion blocker effect. Every dilution in the other extracts (5 and 10 days) elicited a significant concentration-dependent inhibitory effect on adhesion.

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

4.3.1.2 Cell adhesion – HGEP cells

In the case of gingival epithelial cells, the undiluted (1/1), as well as the relatively low dilution level (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 lost their adhesion inhibitory effect but also became significant adhesion enhancers. This ability was sustained in the 1/1000 dilution but to a moderate extent.

Investigations of PEI effects on the cell adhesion of HGEP showed similar results to PEI-Ag, however, differences were also detected. The intense adhesion blocker character of 1/1 was detectable in 1/10 dilutions in both extracts. 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 the gradual transition.

4.3.2 Cytotoxicity (duration of impedimetry: 0-72h)

4.3.2.1. Cytotoxicity – PDL cells

The nanoparticles extracted from the PEI-Ag complex with 1, 5 and 10 day procedure 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.

In contrast to PEI-Ag, in the case of the silver-free extracts of PEI, 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.

4.3.2.2 Cytotoxicity – HGEP cells

PEI-Ag was also cytotoxic on HGEP cells 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 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.

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. 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.

4.3.3 Apoptosis

The results presented in this chapter are based on the rearrangement of phosphatidyl serine (Annexin V positivity) of the surface membrane at the 24 hour mark. This was since there was no significant change after the first 24h treatments with 1, 5 and 10 extracts of nanoparticles.

4.3.3.1 Apoptosis – PDL cells

Apoptotic mechanisms may also contribute to the cytotoxic character of nanoparticles elicited by PEI or PEI-Ag. Annexin V staining was used to evaluate this form of early apoptosis. In PDL stem cells the 1/10 dilution of PEI-Ag was the only apoptosis inducer of our samples. 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.

4.3.3.2 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).

In the case of PEI-Ag, only the lowest dilution levels (10x) induced apoptosis significantly. Apoptosis induced with PEI-Ag dilutions 100x and 1000x were weak. The treatments with compounds not containing silver (PEI) resulted in an apoptotic response that was more dependent on the degree of dilution (10x-100x1000x). The 10x dilutions had the most intense and significant apoptotic behaviour. In contrast to PEI-Ag, 100x dilution of PEI proved to be apoptotic also, nevertheless, only on a moderate level.

4.3.4 Morphometry

The morphometric changes caused by the treatment with the nanoparticles released from PEI or PEI-Ag membranes were detected with a holographic microscope. 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 resulted in decreased 'Area' compared to the control except for treatments with nanoparticles released from the 10 days extracts of PEI-Ag membranes. This 10 day extract had a moderate peak of 'Area' values at 160 sec. 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 sec, 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. From the two morphometry indices described above ('Area' and 'Optical thickness'), the HoloMicroscope also calculated the values of the volume-specific to each cell ('Optical volume'). 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 sec.

4.3.5 Cell migration and motility

The 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. 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 only the extracts obtained by shorter extraction times (1 and 5 days) were more effective while the 10 days extract (5 days > 1 day > 10 days extracts) proved to be neutral in respect to changes in morphology or cell movement.

5. Conclusions

The results proved that the new real-time methods of 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 most 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 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. and 11. Dilutions of supernatants containing PEI or PEI-Ag had concentration-dependent effects on cell adhesion and cytotoxicity in both PDL stem cells and HGEP cells. 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.

6. Bibliography of the candidate's publications

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