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Cell physiological effects of glass ionomer cements on fibroblast cells

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ABSTRACT

The cytotoxicity of glass ionomer cements (GICs) was investigated using a novel, cost-effective, easy-to-perform and standardized test. GIC rings were made using in-house designed, custom-made moulds under sterile conditions; 10 with Fuji Equia and 10 with Fuji Triage capsules, placed in direct contact with primary human gingival fibroblasts (HGF) and immortalized human fibroblasts (HFF1). On day 1, 4, 14 and 21, an AlamarBlue® (resazurin) assay was completed towards determining the effects of the GICs on metabolic activities of the cells, whilst cell morphology was examined by light microscopy. The influence of the compounds released from the GIC rings on cell physiological effects (viability, proliferation and adhesion) during 24 h incubation was further investigated by impedimetry.

Result trends obtained from this battery of techniques were complementary. At 100 *v*/v% concentration, the released compounds from Equia were strongly cytotoxic, while at lower concentration (0, 4, 20 v/v%) they were not cytotoxic. In contrast, Triage elicited only slightly transient cytotoxicity.

The method proposed has been proved as being efficient, reliable and reproducible and may be useful in quick testing of the cytotoxicity of similar biomaterials by using an immortalized cell line.

1. Introduction

Since mercury was recognised as a global threat to human and environmental health, reduction of its use and disposal has been on the agenda of major health and environmental organisations (European Commission: Scientific Committee on Health and Environmental Risks (SCHER), n.d). However, the complete removal of existing dental amalgam has not been enacted since it is an effective filling material with infinitesimal adverse effects and it is unnecessary to remove clinically satisfactory restorations (European Commission: Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), n.d). Nonetheless, the design and optimisation of alternative dental materials remain a key area of growth, as dental amalgam lacks aesthetics and has poor tooth adhesion. Mercury-free restorative dental materials likewise present challenges, particularly with respect to their potential cytotoxicity, a problem compounded by the commercial secrecy withholding their full chemical specifications. Hence, testing cytotoxicity of these restoratives is requisite in the development of highly biocompatible restorative dental materials.

In the search and optimisation of alternatives to dental amalgam, glass ionomer cements (GICs) have shown great potential. GICs, in general, have sound biocompatibility, good aesthetics, natural adhesion to tooth structures and anticariogenic property due to their sustained fluoride release (Wilson and Kent, 1972; Forsten, 1998; Nicholson, 1998).

To achieve a perfect balance between biocompatibility and physical and mechanical properties appears to be the main theme throughout the development of GICs (Smith, 1998; Mohd Zainal Abidin et al., 2015; Goldberg, 2008; Silva et al., 2016; Najeeb et al., 2016; Moshaverinia et al., 2016; Kim et al., 2017; Senthil Kumar et al., 2017; Noorani et al., 2017). However, the changing atomic cohesion and fluctuating interfacial configurations during setting of GICs result in their falling short of the fracture toughness requisite for universal permanent restoration (Tian et al., 2012, 2015), though they are acceptable for niche

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applications (Frencken et al., 2012). Thus, the fast development of GICs is anticipated. Consequently, the challenges must be met with evolving biocompatibility testing methods. Although different tests suggested by ISO 10993-5 and qualitative and quantitative measurements in investigating dental materials are correlating well, they were not standardized and there are limitations (Bruinink and Luginbuehl, 2012). Amongst these, the major limitations are the following: short test period, the use of cell lines that do not behave identically with primary cells, very mild extraction of compounds from the material surface only, undefined surface to volume ratio, etc. (Bruinink and Luginbuehl, 2012).

Hence, the aim of the present study was to propose a novel, cost-effective, easy-to-perform and standardized method to investigate the cytotoxicity of GICs, suitable for testing existing, emerging and newly developed GICs of the future. For consistency of our results from an experiment to experiment in the future, we have used a human fibroblast cell line (HFF1) which represents a good in vitro model. Furthermore, in order to imitate in vivo conditions and verify our results from using HFF1 we have also prepared and cultured primary human gingival fibroblasts (HGF) for use in the experiment.

Fuji is a well-known manufacturer of dental materials so we chose two lines of GICs, Equia and Triage, to test the applicability of the new method. Equia is a unique long-term posterior restorative material with great aesthetic features and low moisture sensitivity. Triage, with good biocompatibility, has been used in root surface protection and hypersensitivity prevention. It was therefore chosen to serve as a positive biocompatibility control in our experiments.

2. Materials and methods

2.1. Cell culture

We used primary human gingival fibroblasts (HGF) and immortalized human fibroblasts (ATCC SCRC-1041 (HFF1)) (Varga, 2011) for the current study. The protocol of isolation and culturing of human gingival fibroblast cells was established at the Department of Oral Biology and Experimental Dental Research, Faculty of Dentistry, University of Szeged, Szeged, Hungary according to the ethical standards of the Helsinki Declaration (Ungvári et al., 2010), and the HGF cells for this experiment were prepared there (see Aknowledgements). Informed consent was signed by the two healthy adult donors.

The HGF cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Switzerland, Biocenter, Szeged, Hungary) with 1.0 g/L glucose, 10% fetal bovine serum (FBS) (Life Technologies, Budapest, Hungary), 1% penicillin-streptomycin (Life Technologies, Budapest, Hungary), and 1% L-glutamine (GlutaMax, Life Technologies, Budapest, Hungary). The HGF cells were used at their 2nd and 3rd passages. For the HFF1 cells, we used DMEM with high glucose concentration (4.5 g/L) (Life Technologies, Budapest, Hungary). Cells were maintained in a humidified chamber at 37 °C - 5% CO_2. After they had reached approximately 80% confluence, they were rinsed with phosphate buffer saline (PBS) (pH = 7.4, Life Technologies, Budapest, Hungary) and harvested by trypsinization using Trypsin-EDTA (0.05%) (Life Technologies, Budapest, Hungary).

2.2. Preparation of cement specimens

The tested commercial glass ionomer cements (GICs) were Triage and Equia (Fuji, GC Europe, Leuven, Belgium). In order to achieve the equal size and contact surface with the culture medium as well as a perfect fit with the bottom of the 24-well cell culture cluster dish, open stainless steel ring moulds with triangular cross-section were custom made (Fig. 1). The capsules were used according to the manufacturer's instructions. The GIC was slowly and evenly exuded into the ring mould with care taken to eliminate entrapped air. The sample was allowed to set in ambient environment for 1 min before taken out of the moulds

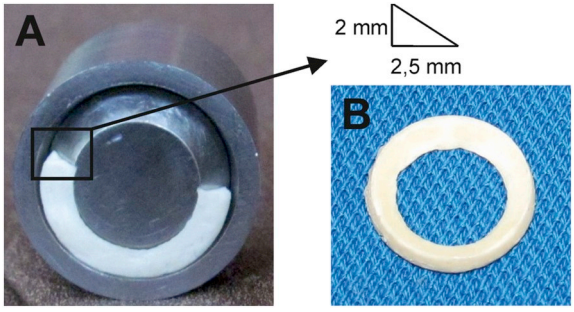

Fig. 1. Cement specimens with standardized mass/surface ratio moulded in a custom-made ring mould. (A) Photograph of the mould and cross-section geometry of the GIC sample; (B) Photograph of a GIC ring, top-view.

and weighed quickly with a weight balance (WLC 0,6/A1/C/2, Radwag Wagi Elektroniczne, Radom, Poland) at a resolution of 0.01 g. The weighting step was important in ensuring consistent sample mass. The calculated mean mass of the GIC ring samples ($n = 10$) was 0.263 g ± 0.007 SD. It was then transferred to the culture dish with the flat surface down and ridged surface up.

2.3. Placement of cell culture, initial pH measurement and cell morphology assessment

500 µL DMEM medium with 1.0 g/L glucose containing cell count of $\sim 7 \times 10^3$ was placed on top of each GIC ring sample and maintained at 37 °C. In the control wells there was only medium but no GIC sample. The medium was replaced every 24 h till day 4 and every 3 days afterwards. pH of the replaced medium was measured every 24 h for the first 4 days with an S2K712 pH meter (Ifsetcom Co., LTD, Saitama-ken, Japan), and cell morphology was examined by an inverted microscope (Nikon Eclipse TS100, Nikon Corp. Tokio, Japan) on day 1, 4, 14 and 21.

2.4. Viability and proliferation investigated by different assays

2.4.1. AlamarBlue® (resazurin) assay

Cell viability and proliferation were also evaluated with AlamarBlue® assay (Life Technologies, Budapest, Hungary). Determination of the effects on cell metabolic activities was done on day 1, 4, 14 and 21, using 5 h optimal incubation time, to following the manufacturer's protocol. Cell growth related to the chemical reduction of AlamarBlue® (resazurin) resulted in a change of the redox indicator from non-fluorescent (oxidized) to fluorescent (reduced) form. The amount of fluorescence produced is proportional to the number of living cells. Fluorescence emission spectra of AlamarBlue® were measured with a multilabel counter (PerkinElmer Wallac Victor3 1420, PerkinElmer, Inc., Waltham, MA, USA). The percent reduction of AlamarBlue® of every sample was then determined using the following equation:

$$\%\text{reduced} = C_{RED} \text{ test well}/c_{ox} \text{ negative control well} \quad (1)$$

where C_{OX} = oxidized form of AlamarBlue® and C_{RED} = concentration of the reduced form of AlamarBlue® and the negative control well contains medium + AlamarBlue® but no cells.

2.4.2. Impedimetry

Adhesion and proliferation of HGF and HFF1 were monitored in real-time mode in xCELLigence SP system (Roche Applied Science, Indianapolis, USA) by measuring the change of electrical impedance with a microelectrode array containing 96-well *E*-plate (ACEA Biosciences, Inc., San Diego, USA). Initially, the impedance of the control medium was recorded and used as the baseline and absolute

control. After 24 h pre-incubation of cement rings at 37 °C in 5% CO_2, 500 µL of DMEM medium with 1.0 g/L glucose and DMEM with 4.5 g/L glucose concentration was placed on top of each sample and cell physiological effects of the compounds released from cement samples during further 24 h of incubation were evaluated in 3–3 parallel wells. After 1 h, ~ 1×10^4 cells were loaded into each well with the released substances dissolved in culture medium in the following concentrations: (i) viability study: 0, 4, 20 and 100 v/v%; (ii) adhesion study: 0, 1, 4 and 20 v/v%. Since the 100 v/v% concentration proved to have almost total toxicity, its effect was not investigated on cell adhesion. Adhesion and proliferation of cells were monitored in real-time for 24 h at 37 °C in 5% CO_2 atmosphere. Based on the impedance change, Cell Index (CI) was calculated with the RTCA 2.0 software of the xCELLigence system using the following equation:

$$\text{Cell Index} = (Z_i - Z_0)/15 \quad (2)$$

where Z_0 is electrical impedance at time point 0 and Z_i is electrical impedance at time point i.

The mean value of three identical values of parallel samples was calculated and plotted against time. ΔCI, the difference between CI and the baseline at each time point i and the slope of the fitted curve were also calculated with RTCA 2.0 software.

2.5. Gel permeation chromatography (GPC)

GPC was performed following the protocol established by Yoshio et al. (1999). The possible presence of polyacrylic acid in the 500 µL of medium placed on cement rings after 24 h incubation at 37 °C in 5% CO_2 and humidified air was investigated by GPC. The relative molecular weight of polyacrylic acid in Fuji Equia and Triage was determined. 9 solution samples were prepared by dissolving 0.5 mL of the liquid component in 2 mL of water: 3 of the controls, 3 of Equia and 3 of Triage. Composition of the eluent was 0.7% Na_2SO_4, 0.1% preservative 1-chlorobutane in water. TSKgel G4000PWXL column (Tosoh Bioscience, Stuttgart, Germany), e2695 separation unit (Waters Corporation, Milford, Massachusetts, USA) and 2414 RI detector (Waters Corporation, Milford, Massachusetts, USA) were used. The temperature was set at 35 °C for the column and the detector and 25 °C for the sample compartment. A linear calibration curve was used and the calibration standards were PEG in the 400–8000 Da molecular weight range.

2.6. Gas chromatography-mass spectrometry (GC–MS)

The organic acid content in the 500 µL of DMEM with 1.0 g/L glucose pre-incubated on cement rings for 24 h in a humidified chamber at 37 °C - 5% CO_2 was investigated with GC–MS. The instrument used was 7890A, MS 5975C (Agilent Technologies, Wilmington, DE, USA), the capillary column was HP-35MS UI (J&W Pharmlab, LLC, Levittown, PA, USA), with the dimension of 30 m × 0.25 mm × 0.25 µm. 1 µL sample was injected by sample + air + solvent injection mode at 250 °C and He-pressure of 11.681 psi. Two differing sample preparation methods were applied. One sample was extracted with chloroform and after evaporation, it was injected in the GC–MS instrument. The other sample was first evaporated and the dry residual substance was dissolved in chloroform: ethyl-alcohol ratio of 1:1 and injected afterwards. The sampling split ratio used was 20:1. The program was set as follows: the temperature was maintained at 80 °C for 1 min, heating at a rate of 15 °C/min, then at 300 °C for 21 min. The temperature of the interface was 280 °C, and that of the MS source was 230 °C. Electron ionization was used for collecting mass spectra.

2.7. Statistical analysis

All experiments were performed twice and statistical evaluation was based on $n = 3$ in each group. Data were presented as mean ± SD and they were analyzed using one way ANOVA test with Post Hoc test (LSD) in order to determine the statistical significance of the differences between effects of the two types of cement (Equia and Triage) on the

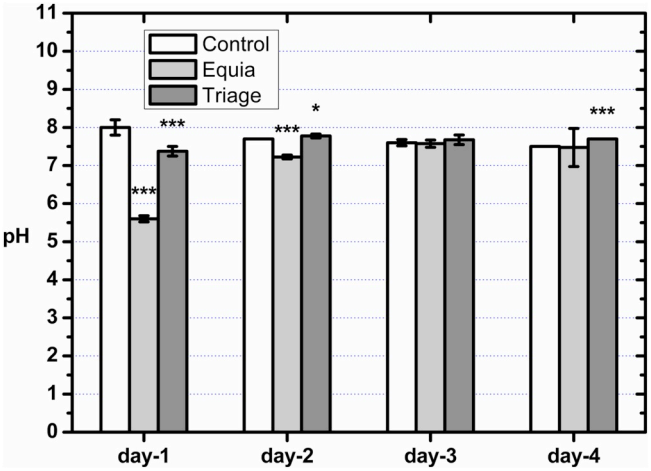

Fig. 2. pH changes of HGF cell medium placed on cement rings investigated for 4 days; $*p \leq .05$; $***p \leq .001$.

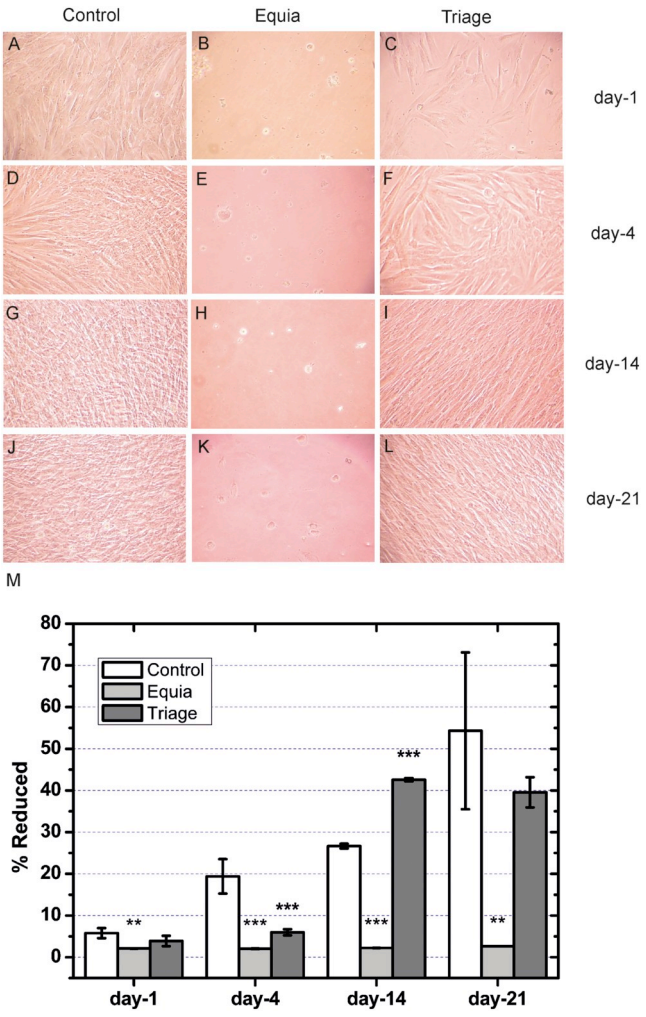

Fig. 3. Effect of Equia and Triage cements on morphology, viability and proliferation of HGF cells investigated for 21 days. Upper: Micrographs of the cell culture taken with light microscopy at a magnification of x200 (A-L); Lower: Results from AlamarBlue® assay in the form of % reduction plotted against time (M). $**p \leq .01$; $***p \leq .001$.

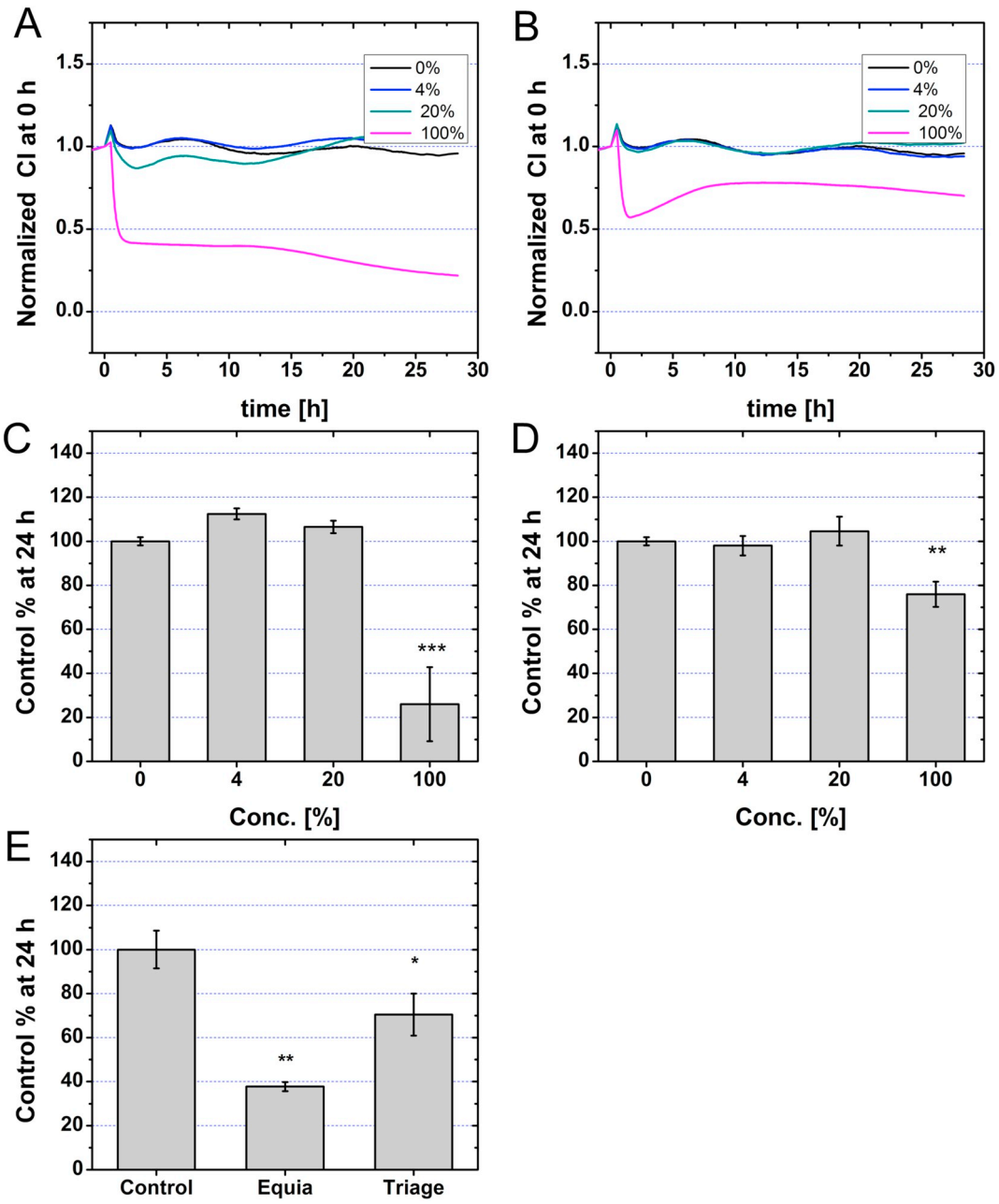

Fig. 4. Viability of HGF cells measured by impedimetry (A-D) and AlamarBlue® assay (E). Effects of 24 h direct contact with Equia (A and C) and Triage (B and D) on the viability of HGF cells; *$p \leq .05$; **$p \leq .01$; ***$p \leq .001$ (color!)

viability of human fibroblast cell cultures compared to identical controls. We used the IBM SPSS statistics 23 software and the differences were considered to be statistically significant at $p \leq .05$ (Confidence Interval: 95%).

Statistical analysis of impedimetric measurements (viability and adhesion assays) were performed by the statistical routine of ORIGIN Pro 8 (OriginLab, Northampton, MA, USA). The level of significance was calculated by one way ANOVA test. *$p \leq .05$; **$p \leq .01$; ***$p \leq .001$.

3. Results

3.1. Early pH changes of HGF cell medium

During day 1 a significant pH decrease from 8 to 5.6 ($p = .000$) was observed in the medium placed on Equia, while pH decrease from 8 to 7.3 in the medium placed on Triage was much less ($p = .000$) (Fig. 2). Although the pH recovered to be on the basic side by day 2, the pH of the Equia group (p = .000) still remained lowest amongst the three groups. On day 3 no pH differences were observed amongst the groups. The same pH changes were observed in the HFF1 cell medium, as well (data not shown).

3.2. Effect of cements on morphologic, cell viability and proliferation properties of HGF cells

After 24 h of direct contact with cements, well-spread healthy cells were observed in the Triage group (Fig. 3C). However, the confluency was lower than that in the control (Fig. 3A). Whereas there were no living cells in Equia group (Fig. 3B). By day 4 the cells in Triage group were proliferating well (Fig. 3F), although the cell density appeared lower than that in the control (Fig. 3D). By day 21 the difference

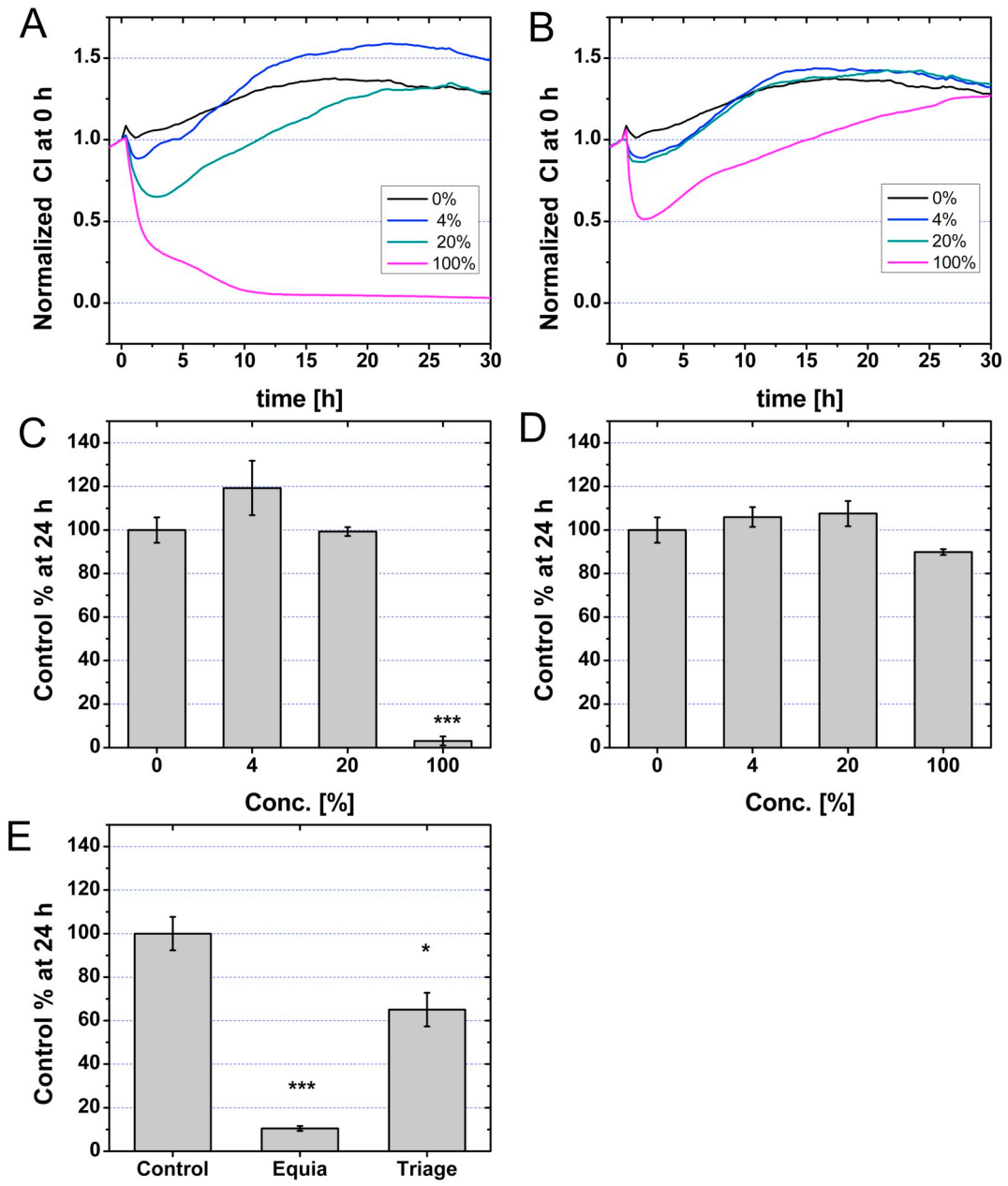

Fig. 5. Viability of HFF1 cells measured by impedimetry (A-D) and AlamarBlue® assay (E). Effects of 24 h direct contact with Equia (A and C) and Triage (B and D) on the viability of HFF1 cells; *$p \leq .05$; ***$p \leq .001$(color!)

between the Triage (Fig. 3L) and the control (Fig. 3J) practically diminished. No living cells were present in Equia group throughout the 21 day investigation period (Fig. 3B, E, H, K).

Cell viability of HGF cells was quantified as % reduction (Fig. 3M). The one way ANOVA test of the effect of the two types of cement on HGF cells showed a significant difference in each measured phase (day-1: $p = .012$, day-4: p = .000, day-14: p = .000, day-21: $p = .03$). Cells placed on Equia died on day-1 and no proliferation was observed afterwards. While cells placed on Triage survived throughout the investigation period of 21 days, showing similar viability to the identical controls. The statistical differences between Control-Equia, Control-Triage and Equia-Triage groups were shown by LSD post hoc analysis. At day-1, −4 and − 14 the differences were significant in each group. At day-21, the post hoc invention (LSD) showed a difference between Control-Equia and Equia-Triage groups (Fig. 3M). Effect of cements investigated on HFF1 cells gave similar results (Fig. A.1).

3.3. Impedimetric analysis of the viability

Cytotoxic effect of the released biologically active compounds from the two types of cement was also analyzed with real-time monitoring of electric insulator properties of living vs. dead cells. The tested 0 v/v%, 4 v/v % and 20 v/v% concentrations of Equia extracts had practically no effect on the viability of HGF fibroblasts, while 100 *v*/v% extract elicited a rapid and durable cytotoxic effect (Fig. 4A and C). The cytotoxic effects of Triage extracts were rather similar to the Equia: only the 100 v/v% concentration elicited a weaker but durable cytotoxic response, while the lower concentrations were neutral during the 24 h of the experiment (Fig. 4B and D).

For HFF1 fibroblasts low concentrations of 0 v/v%, 4 v/v% and 20 v/v% of the Equia extracts elicited only a transient negative response (3–7 h) which was followed by a gradual increase. Nonetheless, the 100 v/v% extract had a strong and irreversible cytotoxic characteristic

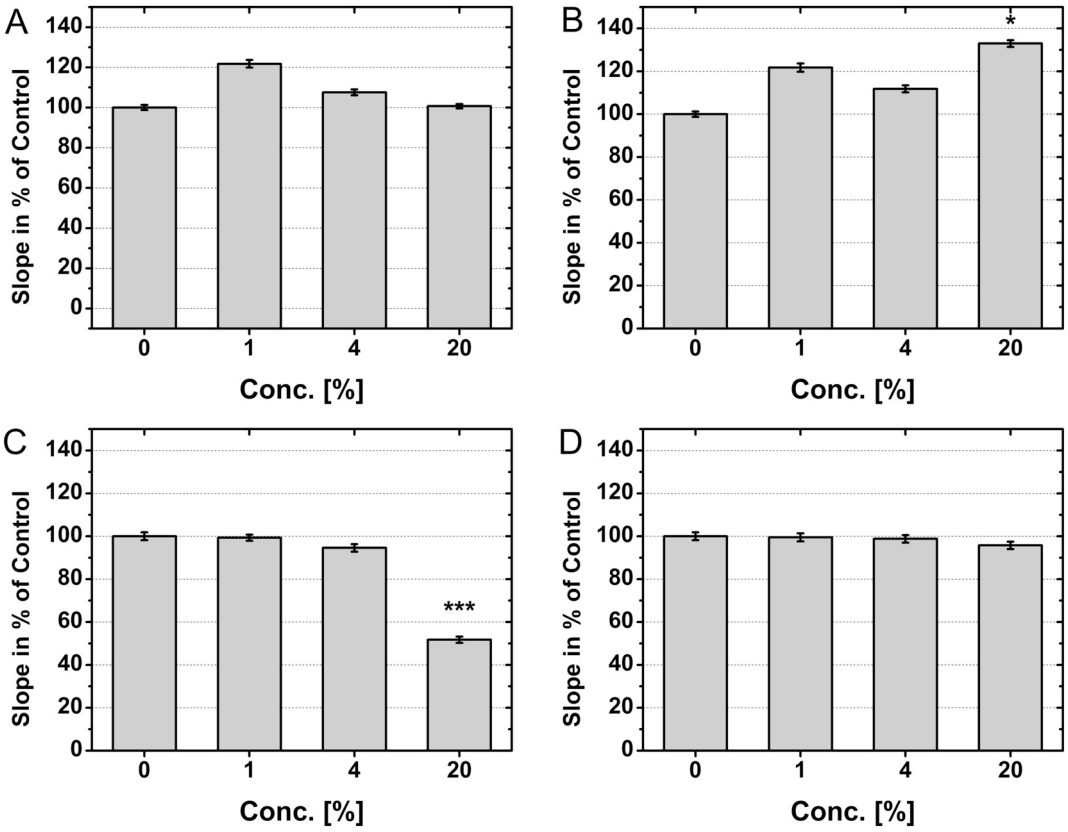

Fig. 6. Effects of Equia (A and C) and Triage (B and D) cement extracts on adhesion of HGF (A and B) and HFF1 (C and D) cells. Here 0% concentration represented the control; *$p \leq .05$; ***$p \leq .001$.

(Fig. 5A and C). The Triage extracts showed transient (3–8 h) cytotoxic effects, however, this negative response was followed by an induced cell proliferation in each concentration, even in the 100 v/v% Triage extract (Fig. 5B and D).

The comparative results gained by the AlamarBlue® test also presented significant differences between the toxic effects of the two types of cement (Fig. 4E and Fig. 5E).

3.4. Cell adhesion

Cell adhesion profile study showed that the responsiveness of HGF and HFF1 fibroblasts was significantly different in both extracts (Fig. A.2). The lowest tested concentration (1 v/v%) of the 24 h extracts of Equia seemed to have an increasing effect on fast adhesion of the HGF fibroblast cells, while the higher concentrations (4 v/v% and 20 v/v%) did not show a significant effect or no effect at all (Fig. 6A).

Triage extracts at all tested concentrations had cell adhesion inducing effect on the HGF, with the most effective response being elicited by the extract of the highest concentration (20 v/v%) (Fig. 6B).

On HFF1 a characteristically different response was elicited by both types of cement. After treatment with 20 v/v% Equia extract a strong reduction of adhesion was observed, while the lower concentrations were neutral (Fig. 6C). In contrast, Triage extracts had no cell adhesion modulating effect on HFF1 cells in any of the tested concentrations (Fig. 6D).

3.5. High molecular weight acid (polyacrylic acid) release from cement rings investigated by GPC

There was no high molecular weight acid (polyacrylic acid) released from Equia and Triage cement rings. The dissolved polymer concentrations of all samples were very low and gave similar results (average Mn = 13.963 g/mol). There was only a small difference in the polymeric region (above 500 Da).

3.6. Low molecular weight acid (organic acid) release from cement rings investigated by GC–MS

No low molecular weight acid was detected in any sample, although two different sample preparation methods were applied.

4. Discussion

A new method was developed to evaluate the undesirable cytotoxic effects of different dental cements. The biocompatibility of two GICs (Fuji Equia and Triage) towards human fibroblasts was investigated in a novel combination of varying in vitro reference assays used in dental materials research. The cement rings prepared made it possible to standardize the adhesion surface and the mass of the cements, and thus the release of cytotoxic compounds.

The cytotoxic effects of Triage cement were highly time-dependent. Significant differences in viability and proliferation of fibroblast cells compared to controls reduced constantly during the whole course of the experiment and by the 21st day, the differences disappeared. In contrast, Equia had a strong and irreversible cytotoxic effect on fibroblast cells, which could be explained by an acidic shock of the cells: the pH of the medium placed on Equia decreased dramatically (from 8 to 5.6), while the pH drop was very little from Triage (from 8 to 7.3). The pH drop was expected because the initial reaction of GICs is an acid-base one, where the hydrogen ions in the liquid are replaced by metallic ions released from the powder (Crisp and Wilson, 1974). The liquid component of GICs is mainly a polyacrylic acid aqueous solution, possibly containing organic acids. Gel permeation chromatography and gas chromatography–mass spectrometry (GC–MS) clearly showed that in

the first 24 h there was neither high molecular weight (polyacrylic) nor low molecular weight (organic) acid released in the medium, but an inorganic acid release could not be ruled out.

Equia liquid is a mixture of polyacrylic acid and tartaric acid, which is stronger than polyacrylic acid (Michaels and Morelos, 1955). 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) (Woolford, 1989). 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 zero proliferation.

The toxic effects of the two types of cement were similar on the HGF and HFF1 fibroblast cells, but the sensibility of the two fibroblast cells proved to be diverse in their responsiveness in adhesion assays. Equia did not have any effect on the adhesion of the HGF cells, which is the closest model to gingival sulcus being in direct contact with filling materials for class II and V cavities. The gingival HGF cells expressed a significantly wider range and more positive responsiveness to Triage than to Equia (1, 4, 20 v/v% vs. 1 v/v%). In contrast, for HFF1 cells the highest concentration (20 v/v%) of Equia extract worked as an adhesion blocker compound. Therefore sound conclusions cannot be drawn from cell lines alone, because immortalized cells do not replicate primary cells accurately (Kaur and Dufour, 2012), as has been proved again in our study.

5. Conclusion

The results gained by the different methods were complementary to each other and highlighted the advantage of the direct monitoring of cells by histology. Hence for quick testing of the newly developed GICs the immortalized HFF1 cell line is perfectly suitable, it can be used in larger studies ensuring the same genetic background and the morphological changes can be followed microscopically over time.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2019.104627.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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