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Cytotoxicity and microbiological behavior of universal resin composite cements

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ABSTRACT

Objectives: To investigate the cytotoxicity on human dental pulp cells (HDPCs) and *Streptococcus mutans* (*S. mutans*) biofilm formation on universal resin composite cements (UCs).
Methods: Three UCs (RelyX Universal, 3 M Oral Care - RXU; Panavia SA Cement Universal, Kuraray Noritake - PSAU; SoloCem, Coltene - SCM) and one 'gold-standard' multi-step cement (Panavia V5, Kuraray Noritake - PV5) were used following two polymerization protocols (light-cured - LC; self-cured - SC). Cytotoxicity (MTT) tests were performed after 1, 3 and 7 days of direct contact. Carboxy-2′,7′-dichlorodihydrofluorescein diacetate was used to detect the release of reactive oxygen species (ROS), and interleukin 6 (IL-6) expression was analyzed by IL-6 proquantum high sensitivity immunoassay. *S. mutans* biofilms were grown on UCs samples in a bioreactor for 24 h, then adherent viable biomass was assessed using MTT assay. For microbiological procedures, half of UCs samples underwent accelerated aging. Data were statistically analyzed ($\alpha = 0.05$).
Results: The highest cytotoxicity was observed for PSAU SC, RXU SC, and PV5 SC at day 1, then for SC RXU after 3 days, and SC PSAU, LC PV5 and SCM after 1-week ($p < 0.05$). There was no increase in IL-6 expression after 1 day, while it increased depending on the group at 3 and 7 days. The highest ROS expression after 12 h was recorded for PSAU SC, PV5 SC and PV5 LC. Biofilm formation was as follows: RXU > > PSAU = PV5 > SCM, while light-curing systematically decreased biofilm formation (≈−33 %). Aging leveled out differences between UCs and between polymerization protocols.
Significance: The choice of cement brand, rather than category, and polymerization protocol influence cell viability and microbiological behavior. Light-curing is beneficial for reducing the harmful pulpal effect that UCs may possess.

1. Introduction

Resin composite cements can be classified into 3 main categories: primer/adhesive-assisted or multi-step, self-adhesive/one-step, and universal resin composite cements (UCs) [1]. The latter have most recently been introduced following the tendency of dental industry towards simplification and versatility in everyday practice [2]. UCs can be essentially viewed as the latest generation of self-adhesive cements. However, unlike their predecessors, UCs can be successfully coupled with their own representative universal adhesive without issues related to chemical incompatibility and consequent adverse effects on polymerization reaction [3]. Other features displayed by UCs are the implementation of adhesion-promoting functional monomers and/or silane in their formulation, and dual-cure polymerization mechanisms (auto/photo) [1].

To date, only three UCs are available on the market [1], with more expected to come shortly. Each of them has peculiar characteristics regarding their chemical composition: the incorporation of a silane coupling agent alongside one functional monomer such as 10-methacryloyloxydecyl dihydrogen thiophosphate (10-MDP) in the case of Panavia SA Cement Universal ('PSAU', Kuraray Noritake, Tokyo, Japan); the integration of two functional monomers (10-MDP and

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4-methacryloxyethyl trimellitic anhydride (4-META)) in SoloCem ('SCM', Coltène, Alstätten, Switzerland), and the absence of bisphenol A (BPA) derivates as reported by the patent literature for RelyX Universal ('RXU', 3 M Oral Care, Seefeld, Germany). Furthermore, RXU is characterized by the presence of glycerol phosphate dimethacrylate (GPDM).

The physical properties and bonding performance of PSAU have been investigated in recent laboratory studies [4–7], along with one clinical case reporting its use during luting procedures to enamel [8]. Similarly, data from in vitro studies about biomechanical, optical, and adhesive properties of SCM [9,10] and RXU can also be found in literature [11–17]. Current scientific literature generally encourages using UCs since they showed comparable or improved bonding properties compared to their predecessors. Furthermore, they have proven to be a reliable luting material for indirect restorations, as they can achieve good bond strength values to various restorative materials such as composites and ceramics [1].

From a biological point of view, it is essential to mention that all resin composite cements can display cytotoxic properties towards mesenchymal cells and fibroblasts, independently from being primer/adhesive-assisted or self-adhesive [18]. Indeed, the most frequent biological complications seen in indirect restorations are the need for endodontic therapy and secondary caries [19]. The cytotoxic effect is especially noticeable when composite cements are not properly polymerized. The lower degree of conversion (DC) observed when dual-cure cements are not adequately light-cured facilitates leakage of potentially toxic monomers from the cement's matrix [18,20]. Considering that UCs were the last to be launched into the dental market, it is still not known if these materials may cause reduction in cell viability and lead to inflammatory reaction when in close contact with dental pulp cells. Furthermore, it is unknown to what extent biofilm masses of cariopathogens (such as *S. mutans*) can be formed to UCs surface. For that reason, this laboratory study aimed to investigate the potential cytotoxic effect of universal resin composite cements depending on their polymerization protocol (light-cure [LC] or self-cure [SC]) and in comparison to a 'gold-standard' [21] primer-assisted cement (Panavia V5, Kuraray Noritake: 'PV5'). The production of inflammatory cytokines (Interleukin 6 [IL6]) and generation of reactive oxygen species (ROS) from human dental pulp cells (HDPCs) in the presence of the investigated cements were additionally analyzed. The null hypotheses tested were that: (1) UCs do not exhibit cytotoxic potential and ability to trigger an inflammatory response from HDPCs, and (2) the polymerization protocol does not influence HDPCs viability and microbiological properties of UCs.

2. Materials and methods

2.1. Resin composite cements

Three commercially available UCs (Panavia SA Cement Universal, Kuraray Noritake - 'PSAU'; RelyX Universal, 3 M Oral Care - 'RXU'; SoloCem, Coltène - 'SCM') and one 'gold-standard' primer-assisted resin composite cement (Panavia V5, Kuraray Noritake - 'PV5'), which served as a control, were investigated in this study. Table 1 details the composition of the cements investigated with their batch numbers.

2.2. Resin composite cement specimen preparation for cytotoxicity testing

On the day of the experimental procedure, resin composite cement disks (5 ×1 mm) were prepared by applying the cement into a custom-made polyvinyl mold positioned between two microscopic glass slides to prevent the formation of an oxygen inhibition layer. For each cement, 2 polymerization protocols were applied: light-curing (LC: Elipar Deep cure, 3 M Oral Care, operating at 1470 mW/cm^2 for 40 s in close contact with the specimen surfaces) and self-curing (SC: 90 min in a dark chamber at 37 °C). Subsequently, the disks were removed from their molds, disinfected with 80 % ethanol, rinsed with sterile distilled water, air-dried, and sterilized under UV light for 30 min (15 min from each side) [22]. For each time point, 4 disks per cement were prepared, and the experiments were repeated 3 times.

Table 1
The composition of the materials used in the present study.

Resin cement, shade and batch number	Composition
RelyX Universal, 3 M ESPE A3O (LOT 7182977)	γMPTES, reaction products with vitreous silica, DUDMA, TEGDMA, Mixture of GPDMA, bisGPDMA and trisGPDMA, silane treated silica, t-Amyl Hydroperoxide, 2,6-Di-tert-butyl-p-cresol, HEMA, Methyl Methacrylate, Acetic acid, copper(2 +) salt, monohydrate, Ytterbium (III) fluoride Silane-treated glass powder, L-Ascorbic acid, 6-hexadecanoate, hydrate (1:2), silane treated silica, Titanium Dioxide Triphenyl Phosphite
Panavia SA Cement Universal, Kuraray Noritake A2 (LOT AT0139)	10-MDP, Bis-GMA, TEGDMA, HEMA, silanted barium glass filler, silanted colloidal silica, Catalysts, Pigments, Paste B Hydrophobic aromatic dimethacrylate, Silane coupling agent (LCSi proprietary monomer), Silanated barium glass filler, Aluminum oxide filler, Surface treated sodium fluoride (Less than 1 %), Camphorquinone Accelerators Pigments, Peroxide, Camphorquinone
SoloCem, Coltène/Whaledent Dentin (LOT L48519)	2,6-di-tert-butyl- 4-methylphenol, 10-MDP, dibenzoyl peroxide (BPO initiator), 4-META, TEGDMA, DUDMA, Bis-GMA, HEMA, zinc oxide, ytterbium(III) fluoride
Panavia V5, Kuraray Noritake, A2 (LOT 970213)	Bis-GMA, TEGDMA, aromatic multifunctional monomer, aliphatic multifunctional monomer, new chemical polymerization accelerator, dl-camphor quinone, photopolymerization accelerator, surface treated barium glass, fluoroaluminosilicate glass, fine particulate filler

Abbreviations: 10-MDP, 10-Methacryloyloxydecyl dihydrogen phosphate; 4-META, 4-methacryloxyethyl trimellitic anhydride; APTES, (3-aminopropyl) triethoxysilane; Bis-GMA, Bisphenol A diglycidylmethacrylate; bisGPDMA, bis (gliceryldimethacrylate) phosphate; DEGDMA, Diethylene glycol dimethacrylate; DUDMA, diurethane dimethacrylate; GPDMA, glycerol phosphate dimethacrylate; HEMA, 2-Hydroxymethacrylate; LCSi, long carbon-chain silane coupling agent; TEGDMA, Triethyleneglycol dimethacrylate; trisGPDMA, tris (glyceryldimethacrylate) phosphate; γMPTES, 2-Propenoic acid, 2-methyl-, 3-(trimethoxysilyl)propyl ester.

2.3. Resin composite cement specimen preparation for microbiological analysis

Microbrush fine tips (Microbrush International, Grafton, WI, USA) were used to evenly distribute a fixed amount of each cement on the flat bottoms of 96-well, flat-bottom, tissue culture-treated black plates (Corning CLS3991 Microtiter plates, Thermo Scientific Italy, Rodano, MI, Italy). A total of 32 wells were obtained for each cement. Two holes (diameter=3.0 mm) were produced on top of the lid of each 96-well plate. The holes provided openings for polyethylene tubing that was connected to cylinders containing nitrogen gas. The lids were then hermetically sealed to the plate using impression material (Express™ 2 Light Body Standard, 3 M ESPE, Seefeld, Germany). A constant flow of nitrogen gas (1000 ml/min) was maintained over the surface of the specimens for 10 min to obtain the desired oxygen-depleted atmosphere [23], then, half of the samples (n = 16) were LC using the same polymerization protocol previously applied, fitting the tip of the light source over the top of the well, precisely matching its diameter. The other unpolymerized wells (n = 16) were covered with black cardboard until LC procedures were finished, upon which they were left in a dark chamber at 37 °C for 90 min (SC). During storage in the dark, nitrogen flow was kept to a minimum positive air pressure to ensure maintaining the oxygen-depleted atmosphere. Subsequently, the disks were removed

from the wells that acted as molds and stored in a dark room under running water for 24 h. Then, they were divided into two additional subgroups (n = 8), depending on the aging procedures. Non-aged samples were dried and stored in a dark room for 30 days, while plates containing the samples undergoing accelerated aging were immersed in excess absolute ethanol at 37 °C for 24 h (500 ml), and then stored under running water for 30 days [24].

2.4. Cell cultures and exposure of cells to resin composite cements

HDPCs were purchased from Lonza (Euroclone, Milan, Italy). They were cultivated in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Munich, Germany), supplemented with 10 % Fetal Bovine Serum (FBS) (Gibco, Thermo Fisher Scientific, Monza, Italy). The cells were then incubated at 37 °C in a humidified atmosphere of 5 % CO_2 in air and the medium was changed every 3 days until the cells reached a sub-confluent state. HDPCs between 3rd and 9th passages were used during all experiments.

In order to perform a 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide MTT (Sigma-Aldrich) assay, sub-confluent cells were detached with 0.25 % trypsin and 0.05 % EDTA for 1 min and seeded into 24-plates at a concentration of 3×10^4 per well, into which 300 µl of fresh DMEM with 10 % (FCS) was added. The cells were then incubated at 37 °C for the next 24 h, after which the resin disks were gently placed into wells under aseptic conditions [25]. Wells with HDPCs and no resin material served as control, while blank controls [26] consisted of wells with DMEM and FCS in which composite disks were placed. The plates were further incubated, and cytotoxicity tests were performed after 1, 3, and 7 days of HDPCs contact with the tested cements. Briefly, 30 µl of MTT dye at a concentration of 5 mg/ml was added to the wells; after 180-min incubation, the insoluble formazan produced by vital HDPCs was dissolved with 300 µl di-methyl sulfoxide. After complete solubilization of formazan crystals, optical density (OD=590 nm) was read using the GloMax Discover Microplate Reader (Promega Italia, Milan, Italy). The OD values of the experimental groups were divided by the control and expressed as a percentage of the control. Cement's cytotoxicity was rated based on cell viability (%) relative to the respective controls at each timepoint, according to the following equation [26–28]:

$$Cell\ viability = \frac{OD\ average\ of\ tested\ group - OD\ average\ of\ blank\ control}{OD\ average\ of\ negative\ control - OD\ average\ of\ blank\ control}\ x\ 100\%$$

For the purpose of visualizing the shape and viability of HDPCs, the cells were cultured on glass slides within wells of a 12-well plate, as described above. Cell-viability staining (LIVE/DEAD Viability/Cytotoxicity Assay kit, Invitrogen, Thermo Fisher Scientific) was performed after 1 day of cell contact with the cements, upon which the specimens were observed using a Nikon Eclipse E800 (Nikon, Melville, NY) microscope.

2.5. Analysis of IL-6 release

HDPCs seeded in 24-well plates were exposed to resin composite cements as described above; the supernatant was collected on days 1, 3, and 7 and stored until further measurements were performed. The level (pg/ml) of IL-6 within the supernatant was analyzed using human IL-6 pro quantum high sensitivity immunoassay (Invitrogen, Thermo Fisher Scientific, Monza, Italy) following the manufacturers' instructions.

2.6. Detection of ROS generation

The total reactive oxygen species generation was determined 12 h after HDPCs' contact with the cements by employing the fluorescent probe 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). Positive controls were introduced by adding 50-µM hydrogen peroxide on HDPCs 30 min before the reading procedure by the GloMax Discover Microplate Reader (Promega Italia, Milan, Italy) (excitation/emission = 485/535 nm, respectively).

2.7. Microbiological procedures

The culture media and reagents were obtained from Becton-Dickinson (BD Diagnostics-Difco, Franklin Lakes, NJ, USA). An artificial saliva medium (ASM) simulating the average electrolyte composition of human whole saliva was prepared from 0.1 L of 150 mM $KHCO_3$, 0.1 L of 100 mM NaCl, 0.1 L of 25 mM K_2HPO_4, 0.1 L of 24 mM Na_2HPO_4, 0.1 L of 15 mM $CaCl_2$, 0.1 L of 1.5 mM $MgCl_2$ and 0.006 L of 25 mM citric acid. The volume was made up to 1 L, and the pH was adjusted to 7.0 by pipetting 4 M NaOH or 4 M HCl solutions under vigorous stirring [29]. A pure suspension of *S. mutans* strain ATCC 35668 in brain-heart infusion broth (BHI) was obtained at 37 °C in a 5 % supplemented CO_2 environment after overnight incubation. Cells were harvested by centrifugation (1.500g, 19 °C, 5 min), washed twice with sterile ASM, and resuspended in the same medium. The cell suspensions were subsequently subjected to sonication (Sonifier model B-150; Branson, Danbury, CT, USA; 7 W energy output, 30 s) to disperse the bacterial chains; then each suspension was adjusted to 0.5 McFarland.

Biofilm formation was obtained using a modified drip-flow bioreactor [30]. The bioreactor allowed the placement of PTFE sample carriers on the bottom of the flow cells to completely submerge the surface of the disks (n = 8/group and subgroup) under the flowing medium. The samples were randomly allocated across the carriers and flow cells, and the bioreactor was sterilized using a chemical peroxide-ion plasma sterilizer (STERRAD, ASP, Irvine, CA, USA) at a maximum temperature of 45 °C for one hour before starting the experiments. The bioreactor was assembled inside a sterile hood. Then, each flow cell was inoculated with 10 ml of the previously obtained *S. mutans* suspension, and the bioreactor transferred into an incubator to operate at 37 °C. Time was allowed under static conditions to promote microbial adherence (t = 4 h); then a nutrient medium flow through the bioreactor cells was initiated using a multichannel, computer-controlled peristaltic pump (RP-1, Rainin, Emeryville, CA, USA). The sterile culture medium included 2.5 g/L mucin (type II, porcine gastric), 2.0 g/L bacteriological peptone, 2.0 g/L tryptone, 1.0 g/L yeast extract, 0.35 g/L NaCl, 0.2 g/L KCl, 0.2 g/L $CaCl_2$, 0.1 g/L cysteine hydrochloride,0.001 g/L hemin, and 0.0002 g/L vitamin K_1, and was supplemented with 1 wt% sucrose. The flow rate was set to 20 ml/h. After 24 h, the culture medium flow was stopped, and all samples were gently extracted from the flow cells. Assessment of the viable biomass adherent to the surface of the samples was then performed.

2.8. Viable microbial biomass assessment

The viable biomass adherent to the surface of each sample was assessed using a modification of the MTT test [30]. Briefly, two starter stock solutions were prepared by dissolving, respectively, 5 mg/ml of

3-(4,5)-dimethylthiazol-2-yl-2,5- diphenyltetrazolium bromide (MTT), or 0.3 mg/ml of N-methylphenazinium methyl sulfate (PMS) in sterile phosphate-buffered saline (PBS). The solutions were stored at 2 °C in light-proof vials until the day of the experiment, when a measurement solution (MS) was made by mixing 1 ml of MTT stock solution, 1 ml of PMS stock solution, and 8 ml of sterile PBS. A lysing solution (LS) was prepared by dissolving 10 % v/v of sodium dodecyl sulfate and 50 % v/v of dimethylformamide in distilled water.

Samples extracted from the bioreactor were washed 3 times with sterile PBS at 37 °C to remove loosely attached cells, then were placed into 48-well plates containing 300 µl of MS each. They were left to react at 37 °C in a dark room for 1 h, and then the supernatant was gently discarded before adding 300 µl of LS. After 30 min at 37 °C in a dark room followed by 30 min in an orbital shaker, 100 µl the solution was transferred to 96-well plates and read using a dual-wavelength spectrometer (550 nm and 630 nm, Genesys 10-S, Thermo Spectronic, Rochester, NY, USA), subtracting the second reading from the first one. In this way, turbidimetry increases by elution of leachates from the cements were prevented from influencing readings.

2.9. Statistical analysis

After checking the normality (Kolmogorov–Smirnov) and the homoscedasticity (modified Levene's test) of the data, parametric or non-parametric tests were performed to analyze the data. In the experiments where the data were normalized relative to the controls in each aparteining time point (cell viability %, IL-6 release, ROS generation), in case of normal distribution of the data (cell viability and ROS release), one-way analysis of variance (ANOVA) was performed for each time point (if applicable), followed by Tukey's multiple comparison tests to assess the differences between groups [31]. When the data were not normally distributed (IL-6 release, failed Kolmogorov–Smirnov test; $p < 0.05$), the non-parametric Kruskal-Wallis and Dunn's post-hoc tests were run. The microbiological biomass data were normally distributed, and hence three-way ANOVA was performed considering the cement type, the polymerization protocol, and the aging as fixed factors. All the analyses were performed by a statistician blinded to the groups using SigmaPlot 14.0 (Systat Software, Chicago, IL, USA). The significance level was set at $\alpha = 0.05$.

3. Results

3.1. Cell viability (MTT) test

Fig. 1 shows that, compared to the control, the viability was significantly reduced ($p < 0.05$) after 1 day when cells were exposed to RXU SC, PSAU LC, PSAU SC and PV5 SC. Microscopic analysis of HDPCs after 1 day of exposure to resin cements can be seen in Figs. 2 and 3. Light microscopy images (Fig. 2) show control cells characterized by the standard fibroblast like morphology. Cells exposed to LC composite cements save their spindle-like morphology altough the numer seems lightly reduced compared to control samples, in agreement with the MTT data previously described. Cells exposed to SC materials showed a reduced cellular density compared to control samples, suggesting cellular toxicity presumably connected with the SC polymerization protocol.

Live and dead assay confirm the morphologidal data previously described and allow us to distinguish live vs. dead HDPCs after 1 day exposition to resin cements. Fig. 3 demonstrates a high density of cells in control samples, characterized by the normal fibroblast-like morphology. On the contrary, the cellular density is noticeable reduced in cells exposed to all the SC materials where several cells appear with a round shape morphology, likely connected to early cell toxicitological events. In particular, in RXU SC and PSAU SC samples it is possible to distinguish red round cells corresponding to dead cells.

After 3 days (Fig. 1), RXU SC continued to impair viability compared to the control group ($p < 0.05$). Further, PSAU SC, SCM LC and PV5 LC led to a significant reduction in cell vitality ($p < 0.05$) after 1 week of incubation compared to the control.

3.2. IL-6 release

No increased level of IL-6 was detected after 24 h of cell exposure to resin cements ($p > 0.05$) (Fig. 4). However, relative to the control, HDPCs produced a significantly higher level of IL-6 when exposed to RXU SC, PSAU SC and RXU LC after 3 days of incubation, with RXU SC demonstrating a higher IL-6 release compared to the other groups ($p < 0.05$). After 7 days, the production of IL-6 decreased significantly for RXU SC to a level comparable with that of the control group, while it notably increased for PSAU SC ($p < 0.05$). Overall, IL-6 results demonstrate a light influence of some resin cements in inducing the release of IL-6 up to 3 days, while the effect is almost absent after long exposition time, confirming the lack of any time dependet toxicity relation between HDPCs and resin cements.

3.3. Generation of ROS after exposure to resin cements

Cell exposure to PV5 SC, PV5 LC and PSAU SC generated higher ROS (3.3-, 1.7- and 1.6-fold, respectively) compared to the control ($p < 0.05$) (Fig. 5).

3.4. Viable microbial biomass

Multi-way ANOVA showed a highly significant influence of cement type, polymerization protocol, and aging on microbiological behavior ($p < 0.0001$), and a highly significant interaction between the type of cement and aging, indicating that each cement had a different aging behavior. No significant interaction was highlighted between the

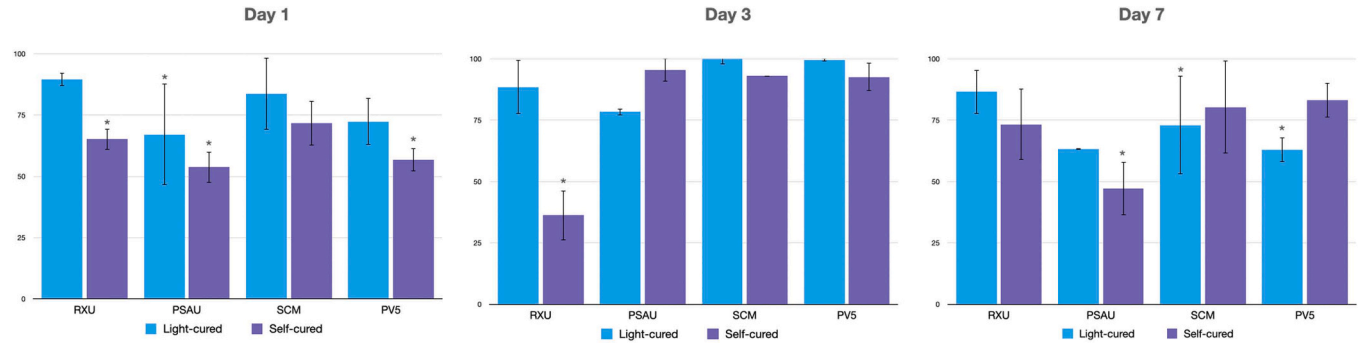

Fig. 1. Mean percentage of viable HDPCs after 1, 3 and 7 days of exposure to resin cements activated with different polymerization protocols (light-cured [LC] and self-cured [SC]). Data are expressed in percentages + standard deviation (y-axis), and are relative to the non-exposed HDPCs (control =100 % viability). * indicates statistically significant difference compared to the contro ($p < 0.05$).

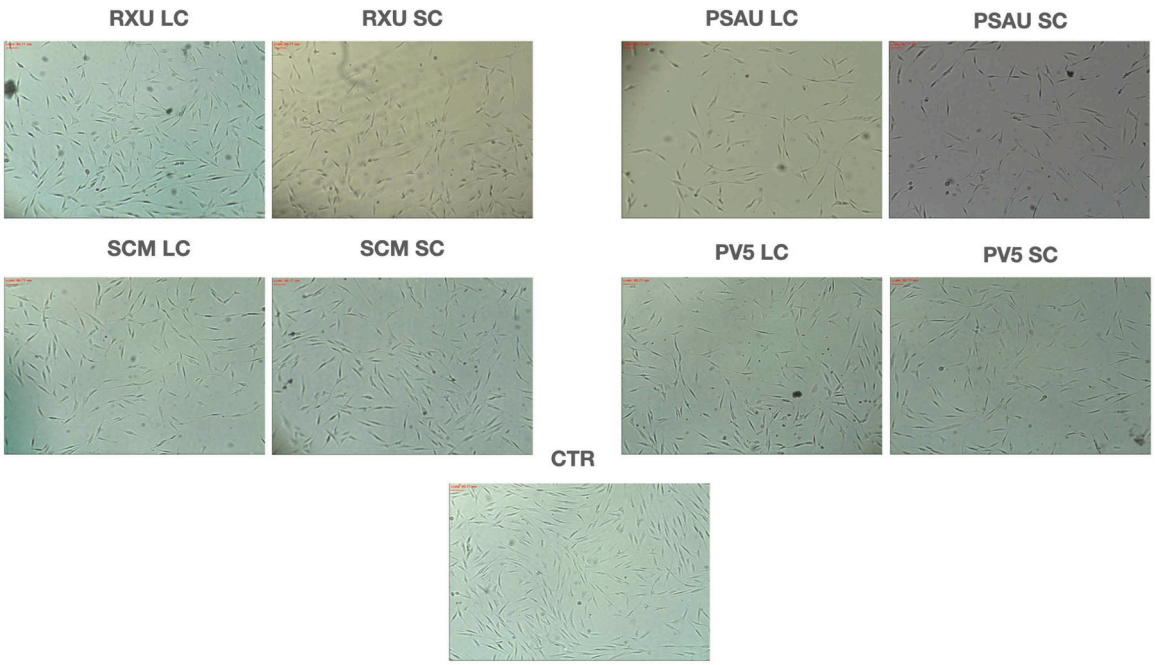

Fig. 2. Light microscopy images and morphological changes of HDPCs after 1 day of exposure to the investigated resin cements.

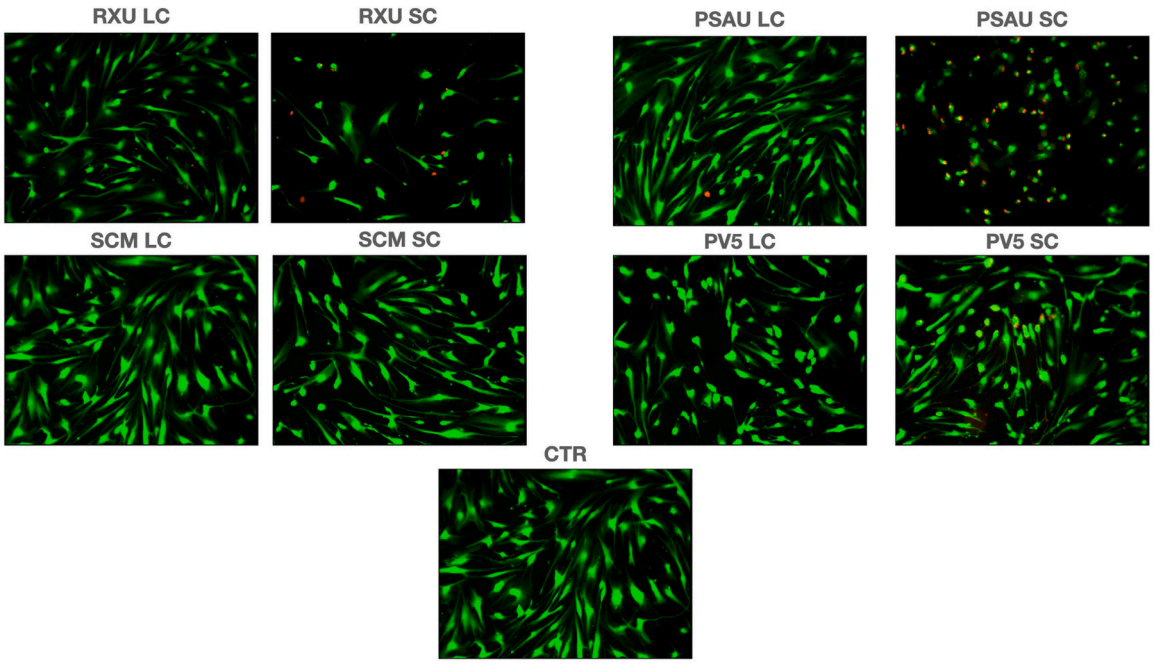

Fig. 3. Live/dead (green/red) staining with subsequent microscopic analysis of viability and morphology of HDPCs after 1 day of exposure to the tested resin cements (magnification 200X in all the images).

cement type and the polymerization protocol ($p = 0.23$), indicating that all tested cements showed a similar tendency when comparing SC with LC.

Considering the non-aged samples, the observed colonization was as follows: RXU > > PSAU = PV5 > SCM, with RXU showing about two times higher viable biomass values than PSAU and PV5, while SCM exhibited ca. 40 % lower colonization than PSAU and PV5 (Fig. 6). Light-curing significantly decreased the viable biomass by ca. 33 % for PSAU, PV5 and SCM ($p < 0.05$).

Accelerated aging caused a significant decrease in the viable biomass values of RXU and increased the values of all the other tested cements, ultimately leveling out the differences in the microbiological behavior of the tested cements (Fig. 6). PV5 was significantly less colonized than all other tested cements (ca. 15 % less, $p < 0.05$). No influence of polymerization protocols on microbiological behavior was recorded ($p = 0.12$).

4. Discussion

Various resin cements are routinely used for luting indirect restorations. The need for post-restorative endodontic therapy is listed among the most frequent biological complications in fixed prosthodontics [19]

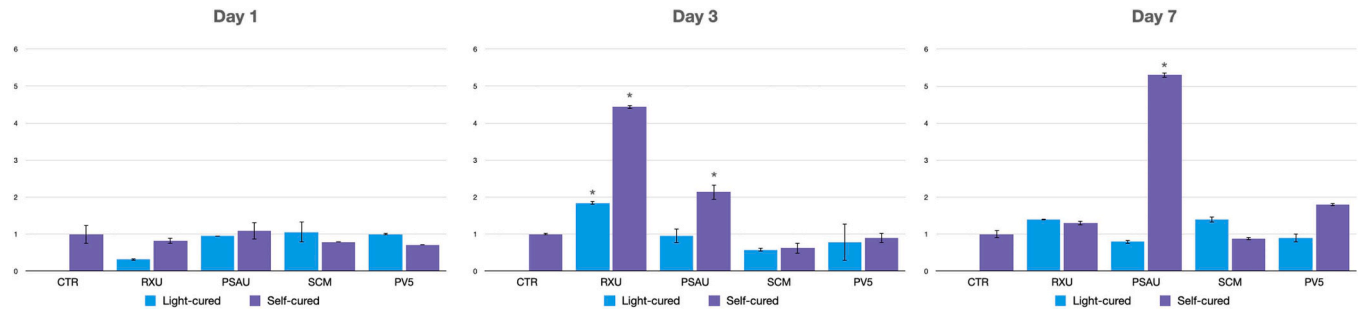

Fig. 4. Analysis of IL-6 expression levels (y-axis) within supernatants from the experimental groups after 1, 3 and 7 days of HDPCs' exposure to resin cements. The levels of detected IL-6 were normalized to non-exposed cells (=1.0). * indicates statistically significant difference compared to the control ($p < 0.05$).

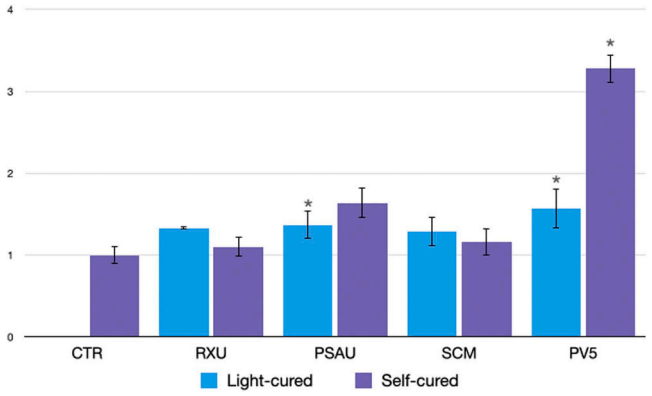

Fig. 5. Analysis of ROS generation from the experimental groups after 12 h of HDPCs' contact with resin cements. Mean fluorescence intensities (y-axis) were normalized to non-exposed cells (=1.0). * indicates statistically significant difference compared to the control ($p < 0.05$).

and might be due to an harmful effect of the resin cement on pulpal tissue [32]. Besides, secondary/recurrent caries mainly limit the longevity of prosthetic treatments, leading to the most frequent cause of crown replacement [33,34]. Considering modifications in the chemical composition of the latest generation of UCs, we aimed to assess potential harmful effects of these materials on HDPCs, as well as their microbiological properties. The results of the present study indicate that the newest group of resin cements can harm HDPCs and have different microbiological responses, by which both null hypotheses were rejected.

Most significant changes in cell vitality were observed after 1 day: 3 resin cements in the SC group (RXU, PAN, and PV5) and 1 cement in the LC group (PSAU) led to a significant reduction in HDPC viability, as compared to that observed for the controls. The percentage of viable cells in these groups was on average below 70 % (Fig. 1), which according to ISO 10993–5:1999(E) [35] can be considered as a characteristic of a cytotoxic material. Cement cytotoxicity is a direct consequence of its chemical composition and polymerization efficacy, since monomers can leach out from resin composite materials, damaging the integrity of HDPCs [36]. In particular, it was reported that the highest cytotoxic potential of resin-based dental materials is related to the release of Bis-GMA, followed by UDMA, TEGDMA, and HEMA [18]. As seen in Table 1, all investigated resin cements contain at least one of the mentioned molecules that could be responsible for causing cells' death.

One might have expected to see superior biocompatibility of RXU cement, since data retrieved from patent literature and safety data sheet indicated that RXU is a Bis-GMA-free material (Table 1) and that it also contains a novel amphiphilic polymerization-initiator system that enhances the polymerization process, thus decreasing the possibility that the cement is inadequately cured. Indeed, a laboratory study confirmed that RXU could achieve adequate DC in both self- and light-cure mode, [37] and that no bis-GMA traces were found in the eluates of this cement [16]. However, the same study reported that the total amount of eluted monomers, which mainly consisted of UDMA, was significantly higher for RXU SC compared to RXU LC, [16] therefore explaining greater cell death caused by RXU SC in our experiment. Similarly, the lower percentage of viable HDPCs observed for PV5 SC can be justified by hampered polymerization of this cement in the absence of light-curing, since it has recently been demonstrated that this cement requires light-curing to achieve optimal DC [38]. Additionally, it was reported that PV5 SC released more bis-GMA than PV5 applied in LC mode [16]; this undoubtedly contributed to the reduction of viable cells observed in our study.

An interesting feature of PSAU is the integration of a long carbon-chain silane coupling agent (LCSi), which should increase the

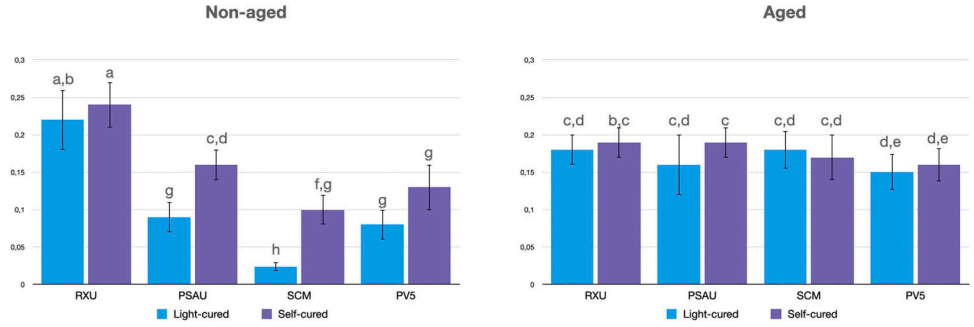

Fig. 6. *S. mutans* biofilm formation on the tested resin cements. The means (± 1 standard error) are shown on y-axis; different letters account for significant differences between groups (Tukey's test, $p < 0.05$). Universal cements were compared to the "golden standard" multistep PV5. Groups were divided by accelerated aging treatment. The cement type markedly influenced biofilm formation, and light-curing systematically decreased adherent bacterial biomass in non-aged samples (≈ −33 %). Aging leveled differences between groups, leaving only PV5 slightly less colonized (≈ −15 %) compared to the universal cements. No effect of polymerization protocol on biofilm formation was shown after aging procedures.

universality of this material regarding the substrates it can bond to, while reducing the number of clinical steps during cementation. Although the manufacturer does not reveal the exact chemical structure of LCSi, a previous paper hypothesized that LCSi is in fact trimethoxysilyl long-chain alkyl methacrylate [4]. This suggestion was supported by a recent study [7] that detected methoxy groups attached to siloxane oligomers and polymers, which may indicate incomplete silane hydrolysis and the presence of silanols on the cement surface. To the best of our knowledge, no data can be found regarding the cytotoxic or antimicrobial properties of commonly used silane coupling agents in dentistry. On the other hand, the negative effects induced by silanols have gained great interest in toxicology, and many efforts have been made to clarify the mechanism responsible for silica pathogenicity [39]. Today, it is known that some subfamilies, so-called "nearly free silanols", can lead to membrane lysis and induce the release of pro-inflammatory cytokines [40]. Consequently, we cautiously postulate that the reduction in cell viability observed for PSAU is most likely due to silanols on the cement surface [7]; future in-vitro studies need to verify this hypothesis.

At day 3, only RXU SC showed a significant reduction in cell viability compared to the controls (Fig. 1). Our study results align with previous research that found high cytotoxicity immediately after cell exposure to self-adhesive cements and composite materials, which gradually decreased in most groups with time [41–43]. In order to better understand the observed phenomenon, it is necessary to mention that the acute release of monomers within the first 24 h [44,45], alongside the high acidity of UCs in the first hours of the setting phase [1], is most likely responsible for the maximum reduction in cell viability at this early time point. Between days 1 and 3, the release of monomers from resin cements is expected to decrease due to medium saturation [46], while the cements' initially low pH increases [47]. This allowed cells to proliferate and regain viability rates above on average 70 % in most of the tested groups (Fig. 1).

It is well accepted that regular medium change keeps the cell cultures healthy by providing fresh nutrients and eliminating waste products generated by the cells [48,49]. After 7 days, the statistically significant drop in cell vitality in some LC groups (Fig. 1) compared to the control, that was considered to have 100 % cell viability for each time-point, can be explained by the cells prolonged exposure to the medium, which had not been refreshed. In this study, no medium change was done, as this would have removed the monomers released during the first days of cell exposure to the cements. The limited amount of nutrients between days 3 and 7, the accumulation of released monomers [36,50], as well as the accumulation of metabolic products and pro-inflammatory cytokines could have negatively influenced the biological processes and impaired the function of cellular enzymes [51], eventually having led to lower viability observed at day 7.

The generation of ROS induced by resin-based materials has been previously observed [52,53]. ROS synthesis is related to various monomers that leach out from the materials and can disrupt the redox equilibrium of cells [54]. Furthermore, the accumulation of ROS can lead to cell apoptosis and even DNA damage [55]. In our study, the amount of total intracellular ROS was increased 3.3-, 1.7- and 1.6-fold for PV5 SC, PSAU LC and PV5 LC, respectively; this was statistically higher compared to the non-exposed cells. The increased ROS levels induced by PV5 SC and PSAU LC may be considered as one of the mechanisms responsible for reducing cell viability in the respective groups after 24 h. As far as we are aware, only one study [52] investigated the potential of SCM and PV5 to induce ROS production. Contrary to our results, the authors reported increased ROS production, followed by reduced viability (69 % of reduction compared to control) when eluates of SC SCM were added to the cell culture of human gingival fibroblasts. Additionally, they found no significant increase of ROS for PV5, although the cement did produce higher cell death at day 1 and 3 as compared to the control. Taking into consideration our and the previously mentioned study [52], it becomes evident how complicated it can be to predict the cytotoxic potential of resin cements based solely on ROS production.

IL-6 is a pro-inflammatory cytokine that is upregulated when HDPCs are exposed to stress [56]. This cytokine can be regarded as one of the markers of the acute phase of inflammation, as elevated levels of IL-6 have also been reported in symptomatic pulpitis [57]. In deep cavities with only a thin layer of dentin, small hydrophilic molecules such as HEMA and TEGDMA (present in all tested materials, Table 1) can infiltrate dentin and diffuse towards the pulp, causing subsequent inflammation [58]. According to the results of our study, there was no significant increase in the production of IL-6 during the first 24 h of HDPC contact with resin cements. On the contrary, even though the threshold for statistically significant difference was not reached, the concentration of IL-6 within supernatant was in some groups even lower as compared to the control (Fig. 4). Although it may seem unexpected, our finding aligns with Cokic et al. [59], who observed lower levels of IL-6 when bronchial cells were exposed to the dust of commonly used composite materials and which have potential to relese monomers in the environement [60]. As suggested by the author, we also support the hypothesis that the reduction of cell viability in groups exposed to resin cements could have led to lower production of IL-6 compared to the controls [59]. The actual irritative effect of resin cements on HDPCs became evident at day 3, since the levels of the pro-inflammatory cytokine were significantly increased for RXU SC, PSAU SC, and RXU LC, as well as after one week for PSAU SC. The clinical relevance of this finding may be the occurrence of post-operative sensitivity within days of the cementation procedure, indicating the possible development of irreversible pulpitis [61,62] and the subsequent need for endodontic treatment.

Eluates of EGDMA and TEGDMA were demonstrated to promote proliferation of cariogenic bacteria, whereas BisGMA had the opposite effect [63]. Furthermore, composite resin degradation products from BisGMA upregulated the expression of genes associated with biofilm formation and other virulence factors in *S. mutans* [64]. In the present study, resin cements showed a significant reaction to light-curing that consistently decreased biofilm formation in all tested formulations, as compared to self-curing. The finding must most likely be attributed to the fact that light-curing produced better conversion, thus minimizing leakage of monomers that could promote biofilm formation. Besides, each resin cement had its own formulation, including different monomer blends, fillers, and antimicrobial agents, making it hard to separate the contribution of each component to stimulation/inhibition of the biofilm formation observed.

Data showed that microbial growth of aged specimens was similar in all groups, independently from the polymerization protocol applied. Aging procedures minimized monomer release, reducing the effect that such phenomenon may have had on biofilm formation. A deterioration of the chemical-physical surface properties following surface aging procedures, as previously observed [24], may have produced surfaces with similar characteristics, that ultimately leveled out biofilm formation between groups. It is unclear which composition difference could have provided the slightly lower colonization of the considered gold-standard resin cement (PV5), when compared to all universal cements. One may speculate on a slightly better degree of conversion, possibly due to the absence of functional monomers (10-MDP, 4-META, GPDM) or other agents (fluoride, butylated hydroxytoluene) that may have decreased the overall polymerization reaction. Another arguably better explanation could be provided by PV5's F-Al-Si glass filler content, which is widely known for releasing significant amounts of fluoride and is the main mechanism of glassionomers' most effective antimicrobial behavior. PSAU also contains fluoride in the form of surface-treated sodium-fluoride particles. The latter characteristic could be the reason of a similar microbiological behavior displayed by PV5 and PSAU [65]. On the other hand, fluoride contained in YbF_3 fillers (SCM, RXU) is considered relatively stable, not providing much release overall, especially after 14 days [66]. Actually, the main reason of its

presence in resin-based materials is to render dental products the desired radiopacity.

The recent role that butylated hydroxytoluene (SCM, RXU) was found to play as an antibiofilm agent in quorum sensing and regulation of biofilms is of note [67]. However, the results of the current study do not allow us to hypothesize any significant influence of such molecule on *S. mutans* biofilm formation, as other compounds can be responsible for the decreased colonization shown by SCM, while the relatively highest colonization was observed on RXU specimens.

Zinc oxide seems to be the most responsible component for the reduced biofilm formation observed on non-aged SCM surfaces, especially the LC ones. The broad-spectrum antimicrobial effect of zinc oxide, especially zinc-oxide nanoparticles, is indeed well-known in literature when incorporated into resin formulations [68,69]. The complete loss of such activity by aged SCM specimens can be explained by its elution from the resin cement's surface, given its relatively high solubility [70], especially at low pH.

Lastly, final considerations should be given to the methodology related to the preparation of the cements in our study. As stated in the introduction, all UCs are designed to be used either with their respective adhesive or without any pretreatment (self-adhesive mode). From a clinical point of view, it is interesting to mention that universal adhesives that accompany RXU and PSAU should not be light-cured before cement placement according to the manufacturer instructions; instead, when the cement and adhesive come in contact, a touch-cure activation occurs with consequent chemical polymerization [7]. In order to simulate a simplified clinical procedure, all UCs were utilized in self-adhesive mode and were either light-cured (LC) or allowed to self-cure (SC). It should be noted that pairing PSAU with its universal adhesive leads to better DC than when PSAU is applied in self-adhesive mode [7]; this may reduce monomer leaching from this cement. Analogously, the accelerators present in the universal adhesive (Scotchbond Universal Plus, 3 M Oral Care) recommended to be used with RXU should optimize the polymerization kinetics of this cement [1]. Unlike UCs, PV5 is a primer-assisted cement requiring a tooth primer that contains the functional monomer 10-MDP and an accelerator that not only optimizes the polymerization reaction but also enables polymerization to initiate at the interface with dentin [71]. Indeed, pairing PV5 with its primer enables very fast and high DC thanks to the 'touch-cure' mechanism, which happens as soon as the cement comes in contact with its primer [72]. Similar to our methodology, a previous laboratory study investigated the elution of monomers from PV5 without primer application [16]. By preparing resin cement disks without their respective adhesive/primer that could have improved polymerization process and DC, the cytotoxic effect may have been overestimated, especially for RXU SC, PAN SC and PV5 SC. Nevertheless, by standardizing the methodology and using all cements in the self-adhesive mode, it was possible to compare the cytocompatibility and microbiological behavior of recently launched UCs. In the future, it would be of interest to verify whether pairing a UC with its adhesive could improve the biocompatibility of these cements, especially in the absence of adequate light-curing.

5. Conclusions

Universal resin composite cements, as well as the considered 'gold-standard' primer-assisted cement, can display cytotoxic effects on HDPCs, depending on the observation time and polymerization protocol employed. Furthermore, the tested cements triggered immune responses from HDPCs, which eventually led to elevated concentrations of IL-6 and ROS, indicating potential adverse clinical effects. The tested resin cements also possessed a specific microbiological behavior depending on their resin blend, fillers, and the presence of active compounds. Nonetheless, light-curing generally improved biocompatibility and microbiological behavior independently from each composition. This specific behavior was lost after aging in terms of microbiological properties, suggesting that it might only be expressed in the first weeks after the application of the resin cement rather than being its permanent characteristic. Adequate light-curing and the choice of the resin cement brand (rather than cement class) are fundamental to avoid possible pulp injuries and reduce biofilm formation in the first period after material application.

Declaration of Competing Interest

The authors declare no conflict of interest. Uros Josic acknowledges Academy of Dental Materials Early Investigator Award.

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