Biocompatibility and acid resistance of preformed crowns in children

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Research Article

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Abstract

Objectives: To investigate the *in vitro* biocompatibility of human gingival fibroblasts (HGFs) with PPC, and resistance to acid exposure at levels that simulate the oral environment.

Materials and Methods: This laboratory study investigated primary HGFs viability, metabolic activity, cytotoxicity, and apoptotic events on preformed metal crown (PMC) discs, composite resin (CR)-coated wells, and monolithic ZR fragments at 24, 48, and 72 h using the ApoTox-Glo Triplex assay. The PPCs were also immersed in 0.1% lactic acid, 0.2% phosphoric acid, or 10% citric acid for 7 days at 37^{o}C to reproduce conditions associated with dietary intake or gastric reflux. Samples were then subject to inductively coupled plasma optical emission spectrometry (ICP-OES) to quantitate the release of ions.

Results: The viability of HGFs on stainless steel and CR significantly declined at 48 and 72 h, representing potential cytotoxicity ($p < 0.05$). Cytotoxicity of HGFs was also higher for stainless steel and ZR compared to control ($p < 0.05$). PMCs and ZR crowns gave minimal ion release. Meanwhile, significant quantities of metallic ions, including copper (Cu), iron (Fe), nickel (Ni), and zinc (Zn), were present in eluates from veneered-preformed metal crowns (V-PMCs).

Conclusions: As PPCs can be exposed to highly acidic environments for many years, thus the release of metallic ions from V-PMCs should form the further investigated in future studies

Clinical Relevance: The study showed that paediatric restorative materials are mildly cytotoxic to HGFs depending on the oral environment.

INTRODUCTION

Since the 1950s, preformed paediatric crowns (PPCs) have been widely used as one approach to treat paediatric dental caries [1]. PPCs allow retention of masticatory function and preserve the dental arch until permanent successors erupt. The survivability of PPCs is paramount, whether used in the oral cavity until natural exfoliation or as a definitive long-term treatment in adulthood for conditions such as hypomineralised first permanent molars. To that end, understanding the behaviour of the oral tissues to the foreign crown materials is vital to supporting and improving their design, compositional constituents, and clinical indications.

PPCs can be manufactured from a range of materials including stainless steel, zirconia (ZR) ceramics, and composite resins (CRs) used as veneers on a base of stainless steel. The elemental atomic composition of the preformed metal crowns (PMCs) currently in use is iron (Fe; 64–70%), chromium (Cr; 18–20%), Ni (8–11%), manganese (Mn; up to 2%), silicon (Si; up to 1%) with traces of other elements including aluminium and molybdenum (1–2%) [2]. The Ni content of earlier versions of PMC was as high as 72%, and this was associated with Ni allergies in children [3], which is greatly reduced in newer crowns. Some reports have suggested that the levels of Ni release from dental alloys is too low to be significant, and have argued that placing Ni containing dental alloys is unlikely to cause allergic reactions [4–10].

However, research into whether Ni ions released from PMCs are cytotoxic and measures of cell viability, metabolic activity and apoptosis in human cells, are scarce [11, 12]. This is surprising given that PMCs have been shown to release metal ions [13]. A study investigating dental hard tissue absorption of metal ions released from primary molars covered with PMCs found that levels of Fe, Ni, and Cr were 6 times higher in cementum, compared with controls [14]. Gingival fibroblast cytotoxicity from metal ion solutions released from PMCs has also been reported [15]. Elevated levels of Cr in hair samples of children with PMC have also been reported when compared with control groups with no PMCs, however, the levels of Cr were so low that they were deemed unlikely to cause harmful effects [4].

On the other hand, the aesthetic monolithic ZR crowns are composed of zirconium oxide (88–96%), yttrium oxide (4–6%), hafnium oxide (5%), an organic binder (2–5%), and a proprietary pigment (1–4%). This choice of material affects clinical outcomes of aesthetic crowns on primary maxillary teeth, with improved gingival health and low plaque levels for ZR crowns [16]. In contrast, veneered-preformed metal crowns (V-PMCs) and CR strip crowns have been associated with the growth of dental plaque. However, the local effects of ion release on adjacent gingival tissues by these PPC's have not been reported. A clinical and radiographic assessment of effects of PMCs on the health of the periodontium was conducted by Sharaf and Farsi [17] and evaluated oral hygiene, gingival health, proximal contacts, crown adaptation, and crown extension. Gingivitis and interproximal bone loss were associated with poorly adapted margins or non-satisfactory placement when judged radiographically, indicating an iatrogenic effect from operator error as the primary cause, rather than effects from the material itself. As many as 42% of PMCs on primary molars may been reported to have deficient margins [18]. In addition, subgingival placement of crown margins places the crown material in contact with periodontal tissues. As the margin moves more sub-gingivally, the health of the surrounding tissues has been shown to decline [19–21].

The oral environment is challenging for dental materials because of the range of microbial, chemical, and enzymatic actions that operate [8, 22]. The acidic milieu from diet, poor oral hygiene, or systemic conditions such as diabetes or gastric reflux is a key concern. Crowns have similarities to stainless steel orthodontic appliances where metal corrosion issues been explored, and which share similar metal composition to stainless steel [9, 23]. Release of nickel (Ni) ions from Ni-containing stainless steel orthodontic wires has been reported [8, 24]. This has helped fuel the development and manufacturing of improved materials for orthodontic appliances, with higher corrosion resistance and lower cytotoxicity for human cells [25]. As Ni is a common cause of allergic reactions, and allergies to Ni occur more frequently than those to other metals, (Ramazani et al. 2014) affecting approximately 10% of the general population (Danaei et al. 2011). Clinical symptoms associated with the Ni allergy include allergic dermatitis, asthma, and mucosal ulcers (Burrows 1986; Hildebrand et al. 1989). Moreover, Ni ions can cause damage to the periodontium, which may act as a modifying factor for periodontal disease (Gursoy et al. 2007; Naranjo et al. 2006; Pazzini et al. 2009). While most patients may experience minimal or no side-effects, individuals with predisposing allergies or medical conditions could be adversely affected. Given the above considerations, the present study had two main objectives: firstly, to assess the biological

responses of HGF cells grown on paediatric crown materials, and secondly, to measure the release of ions from PPCs immersed in acidic solutions.

MATERIALS AND METHODS

The Study Sample and Preparation of Crown Materials: The study sample consisted of three types of commercially available PCs: NuSmile ZR Zirconia™ (ZR; NuSmile, Houston, Texas, United States), NuSmile Signature™ (NS; NuSmile, Houston, Texas, United States), and 3M ESPE Stainless steel crowns (PMC; 3M ESPE, St Paul, Minnesota, United States), with n = 3 samples per test condition.

Stainless steel crowns discs were created from type 316 L stainless steel sheets (3M) using a metal punch (PPS-7 Power Punch Set, Metalmaster; TW) to give 3.18 mm diameter samples. Meanwhile, ZR fragments (~ 30–40 mg) from ZR crowns (NuSmile®) were simultaneously tested. Finally, TPH Spectra CR low viscosity resin (Dentsply Sirona Inc.; Charlotte, USA) was used to coat 96-well plates using a ball burnisher. Materials were disinfected using 70% ethanol for 20 min then rinsed 3 times in 0.15 M phosphate buffered saline (PBS) immediately prior to use.

Establishment of Primary HGF Cultures

Gingival tissue was collected from two healthy patients aged 15–17 years following gingivectomy procedures undertaken as part of orthodontic treatment. Human Research Ethics Committee (HREC) approval was obtained from the Ethics Committee of Royal Bruisbane Hospital and The University of Queensland (Ethics Approval No. HREC/2019/QRBW/57321). Two HGF cultures were established using Zafar et al. 2014 methodology from cell suspensions were maintained in a growth medium of Dulbecco's minimal essential medium with GlutaMAX (DMEM with GlutaMAX; Gibco® Invitrogen, MA, USA) containing antibiotic-antimycotic reagent (0.5 µg/mL; Invitrogen), gentamycin (0.25 µg/mL; Invitrogen) and supplemented with 10% foetal bovine serum (FBS, Gibco® ThermoFisher Scientific; MA, USA) [26]. HGFs were used between passages 3 and 8.

Cell Viability, Cytotoxicity, and Apoptosis (ApoTox-Glo Triplex) Assay

The biocompatibility of HGFs cultured on crown materials (PMC discs, Zr fragments or control) was measured using the ApoTox-Glo Triplex assay (Promega; Madison, USA). This assay assesses cellular viability, cytotoxicity, and apoptosis events. HGF cells were seeded at 1.5 x 10^4 cells/cm^2 into 96-well plates in 100 µL growth media, then incubated for 24, 48, and 72 h. For each time point, the plates were processed according to the manufacturer's instructions. Viability ($400_{Ex}/505_{Em}$)/cytotoxicity $485_{Ex}/520_{Em}$ absorbance was measured using an Infinite 200 Pro Tecan spectrophotometer (Tecan Group Ltd; Männedorf, CH) whereas apoptosis luminescence was read on a Tecan Spark multimode plate reader (Tecan Group Ltd; Männedorf, CH). This assay was performed in triplicate (n = 3) for each HGFs culture.

Acid Exposure Assays of Preformed Crowns

Whole PPCs were immersed in 2 mL of 0.1% lactic acid, 10% citric acid, or 0.2% phosphoric acid solutions in 24-well plates for 7 days at 37^{o}C. The materials used were PMCs, V-PMCs, and ZR crowns (n = 3 crowns samples per condition). Liquid samples were then analysed for elements of interest using a PerkinElmer Optima 8300 (PerkinElmer; Waltham, USA) dual view inductively coupled plasma optical emission spectrometer (ICP-OES) located at the XXX University at Central Analytical Research Facility (CARF).

Briefly, the sampling system consists of a peristaltic pump set at 0.44 L/min for analysis, an ESI SC Fast Prep (version 2.9) auto dilutor and sampler with an S400V syringe unit, cross flow nebuliser with an argon gas flow rate of 0.60 L/min. Plasma was generated by a solid state 40 MHz radio frequency generator power set at 1500 Watts. The plasma argon flow rate was set at 12 L/min, and the argon gas for the auxiliary was set at 0.8 L/min. The ICPOES dual monochromators for ultraviolet and visible range emission had a wavelength range of λ = 165nm to λ = 800 nm. Emitted wave lengths were measured using a sealed charged coupled device (CCD) detector, which was cooled to a temperature between − 7 to -8^{o}C with an integrated Peltier cooler. Multi-elements calibration standards and a 5 µg/mL Lu and 2.5 µg/mL Y internal standard solutions were prepared gravimetrically that cover the concentration range of all samples. All elemental readings of ≤ 2 ppm were excluded from further analysis.

Statistical Analysis

The data were tabulated on a Microsoft Excel spreadsheet (Version 16.30,

Microsoft Corp.) and then imported into GraphPad PRISM 9.0 software (GraphPad Software, San Diego, CA, USA) for statistical analysis and creation of appropriate graphs. Specific data analysis tests that were performed for biocompatibility and acid immersion experimental data using a two-way ANOVA. The levels of statistical significance of $P \leq 0.05$ was considered statistically significant.

RESULTS

Effects Of Crown Materials on HGF Viability

The ApoTox-Glo assay was used to measure the viability of HGFs grown on PMC and ZR. In both HGF-1 and HGFs-2 cultures, viability on PMC and ZR materials was higher for all time points compared to control (Fig. 1).

Effects Of Crown Materials on HGF Cytotoxicity

HGFs from both donors showed consistently higher cytotoxicity to PMC and ZR materials, compared to control (Fig. 2A, B). For cells from HGF-1, cytotoxicity values were significantly ($p \leq 0.05$) elevated for PMC at 48 h and ZR at 24 and 72 h. Meanwhile, for cells from HGF-2, cytotoxicity values for both materials at each time points were above those for control. Neither material was associated with significantly higher cytotoxicity than the other.

Effects Of Crown Materials on HGF Apoptosis

The presence of caspase 3/7 apoptotic markers in HGFs seeded onto crown materials was recorded using the ApoTox-Glo assay. For HGF-1, apoptotic signalling increased with extended exposure to PMC discs being significantly higher at 72 h (Fig. 3A). Meanwhile, cells from HGF-2 were significantly less responsive, with marginal increases in apoptotic events over time (Fig. 3B). The apoptotic profile for cells from both donors seeded onto ZR showed increasing rates of apoptosis after 2 or 3 days. After this time, apoptosis declined steadily for cells from HGF-1, while the rate of apoptosis for cells from HGF-2 increased at 72 h.

Release of Ions from Acid-Exposed Paediatric Crowns

PPCs were immersed in acidic solutions for 7 days at 37^{o}C to determine their solubility by measuring ion release using ICP-OES. The minimum threshold for inclusion was ≥ 2 ppm. V-PMC was the most reactive, with high levels of Cu, Fe, Ni, and Zn ions (Fig. 4) in the acidic elutes. The most destructive solution was 0.2% phosphoric acid followed by 10% citric acid, and lastly 0.1% lactic acid. PMCs released marginal quantities of Fe and Si ions. ZR crowns were the least reactive material, with minimal acid solubility.

DISCUSSION

Paediatric preformed crowns are essential for preserving the paediatric dentition, and can be manufactured from various materials [27, 28]. The severity of early childhood caries means a range of materials may be needed to satisfy a child's functional and aesthetic needs. PPCs remain in contact with periodontal tissues until natural exfoliation and as such, these materials need to perform without local or systemic impacts on the host [29]. This includes the capacity of crown materials to withstand environmental stresses including oral acidity. In rare instances, some dental materials can participate in immunological reactions [30, 31]. For example, previous PMC iterations which contained significantly more Ni than contemporary counterparts [3]. The *in vitro* biocompatibility of periodontal cells grown on PPC materials has not previously been documented. Specifically, the ability of HGFs to withstand short term exposure to PPCs has, until now, not been explored, even though the response of gingival fibroblasts to CRs or ceramic materials has been reported [32–34]. Cellular responses to materials such as monolithic ZR are less well understood [28, 35]. As a result, this study chose to assess the biocompatibility of ZR ceramics using a series of assays.

The results of the viability assay showed increase in cell viability for both PMC and ZR crowns compared to control. Whether similar metabolic trends occur for PMC or ZR crowns *in vivo* remains unknown. Similar trends were observed in apoptosis assay. This observation could be a result of an increase in cell density, rather than the number of cells per se. The results of the study showed no real indication of a large induction of apoptosis. As described by others, the early release of ions may explain also explain the findings (Elshahawy 2011; Milleding et al. 2003; Rizo-Gorrita et al. 2019).

HGFs from both donors showed consistently higher cytotoxicity to PMC and ZR materials, compared to control. The exposure of periodontal cells to cytotoxic resin-based materials has been extensively reported, with unreacted monomers having potentially detrimental effects on periodontal tissues [36–38]. Both host and environmental factors may contribute to monomer release. For instance, daily exposure to dietary acids can adversely affect the CR matrix-filler interface, increasing the potential for loss of material [39]. Similarly, the corrosion of stainless steel crowns can release ions which then produce cytotoxic cellular effects [15]. Stainless steel orthodontic bracket eluates after immersion in acidic solutions have been shown to induce genotoxic effects on HGFs, due to the release of ions [40]. Also, free radicals may damage cellular DNA and lead to mutagenic or carcinogenic outcomes [41, 42]. In the present study, no significant difference in cytotoxicity was found between cells cultured on PMC discs or on ZR fragments (Fig. 3A, B; $p > 0.05$). However, HGFs experienced noticeably higher cytotoxicity when grown on crown materials compared with the control ($p < 0.05$). A study investigating the biocompatibility of HGFs cultured on ceramic-based materials found cytotoxicity levels significantly decreased over time [43]. Those authors postulated that the release of ions from ceramics as responsible for the initial cytotoxic response. However, in the present study, cells were more viable when grown on stainless steel discs after 48 h, when compared with ZR. This is despite the fact that ZR ceramics are considered highly biocompatible [33, 35].

Overall, ZR ceramics show a low corrosive potential with minimal cytotoxic constituents that would minimally affect cellular behaviour. Even so, the effects of increasing oral acidity on ion release is underreported. The present study found ZR crowns immersed in ultrapure H_2O were largely unaffected. However, the addition of acidic solutions initiated the release of Al and Si ions, albeit in small quantities. These elements are common ingredients in ceramic-based materials such as lithium disilicate or ZR-reinforced lithium disilicate, with reported low cytotoxicity [44, 45]. Others have described chemical interactions between gastric acid solutions and monolithic ZR that result in smoother surfaces, which may indicate that a corrosive process has occurred [46–48]. Some ion leaching due to corrosion of ZR could have occurred in the solutions used in the present study.

The leaching of ions from V-PMCs into acidic solutions raises potential health concerns [49, 50]. In particular, the release of the metallic ions Cu, Fe, Ni, and Zn warrants further investigation. The concentration of Zn in V-PMC eluates was 5 ppm (H_2O), 225 ppm (0.1% LA), 371 ppm (10% CA), and 432 ppm (0.2% PA) (Fig. 5). Others have shown that mouse fibroblasts exposed to Ni and Cu metal salt solutions displayed *in vitro* cytotoxicity at 10 ppm [51]. In contrast, the release of Cu and Ni ions from V-PMCs in 0.2% phosphoric acid was 592 ppm and 102 ppm, respectively. A similar article testing the acid resistance of glass ionomer cement restorative materials reported 0.1% lactic acid to be the least destructive solution [52]. The present study also observed that V-PMCs released significantly less material when immersed in 0.1% lactic acid, compared with the 10% citric acid and 0.2% phosphoric acid solutions.

Elsewhere, others have found that Zn ions released from dental materials could impair cellular viability.[187] Similarly, the leaching of Ni ions has the potential for eliciting delayed hypersensitivity

reactions, as well as carcinogenic and mutagenic cellular events [12]. Overall, metallic ions released from V-PMCs have been found to be highly cytotoxic [15]. Interestingly, this contrasts with traditional PMCs, which are largely unaffected.

CONCLUSIONS

The results of the present study show that paediatric restorative materials could be slightly cytotoxic to HGFs depending on the associated factors and the oral environment. Whether cytotoxicity is limited to the early phases of growth or continues over a longer duration remains to be determined. The release of metallic ions from V-PMCs should form the basis of future studies. It would be prudent to expose HGFs to similar ion concentrations (measured by molality) to better understand the relativity of these cytotoxic effects.

Declarations

a. **Ethics approval and consent to participate: This research is carried out in accordance statement with the Declaration of Helsinki standard. Human Research Ethics Committee (HREC) approval was obtained from the Ethics Committee of Royal Brisbane Hospital and The University of Queensland (Ethics Approval No. HREC/2019/QRBW/57321). The informed consent was obtained from all subjects and/ their legal guardian(s)**

b. **Consent for publication: Not Applicable**

c. **Availability of data and materials:** The data will be available on request from the corresponding author

d. **Competing interests:** The authors declared no conflict of interest

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f. **Authors' contributions: L.W and S.Z conceived the idea; T.H collected the data, analysed the data, and led the writing. D.C provided technical support. L.W, D.C and S.Z revised the manuscript and gave final approval of the version to be published and agreed to be accountable for all aspects of the study.**

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Figures

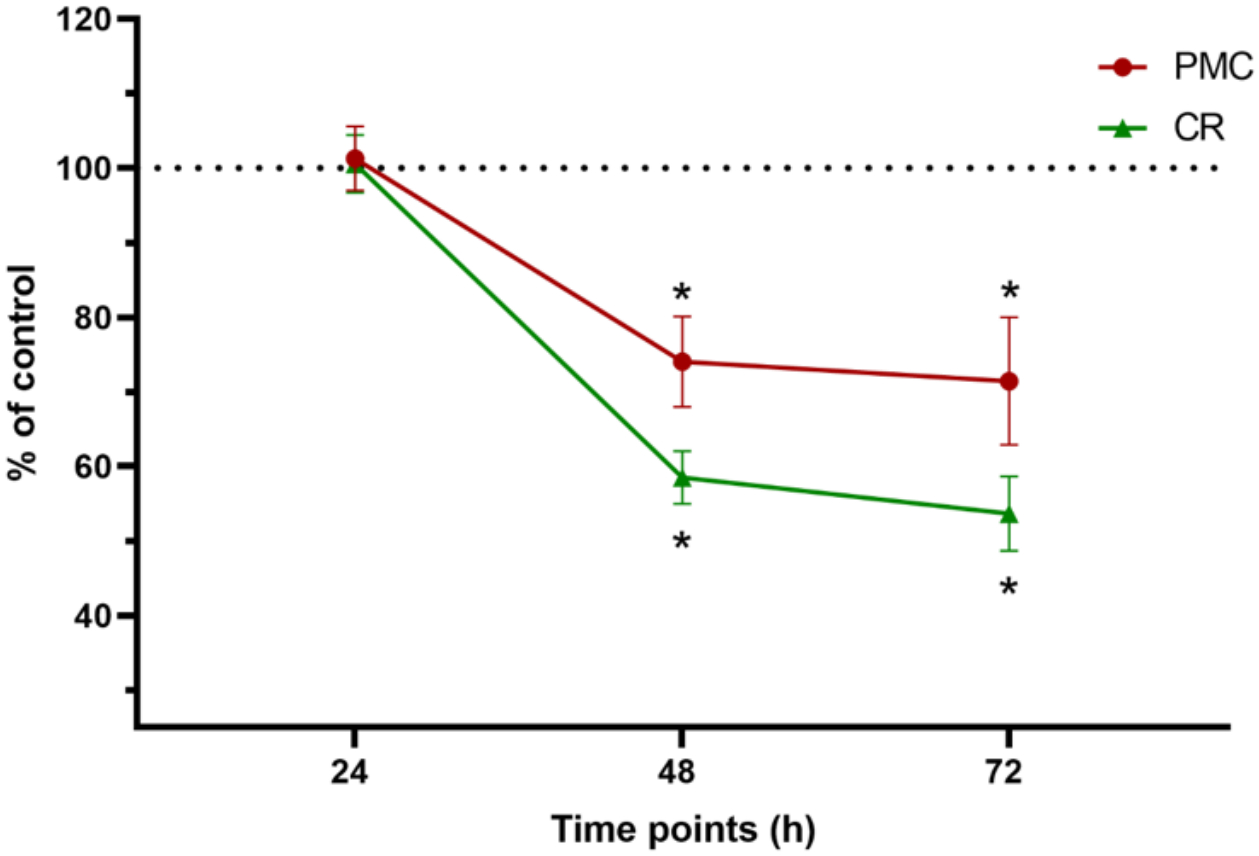

Figure 1

Mitochondrial dehydrogenase activity (MTT) of human gingival fibroblasts grown on preformed metal crown (PMC) discs or composite resin-coated wells (CR; 2) as a percentage of the tissue culture plastic (TCP; dash line represents percentage of control at 100%) control at 24, 48, and 72 h (n = 3; technical replicates, HGF-2). Asterisks indicate significant changes ($p < 0.05$) compared with TCP. Mean ± SEM.

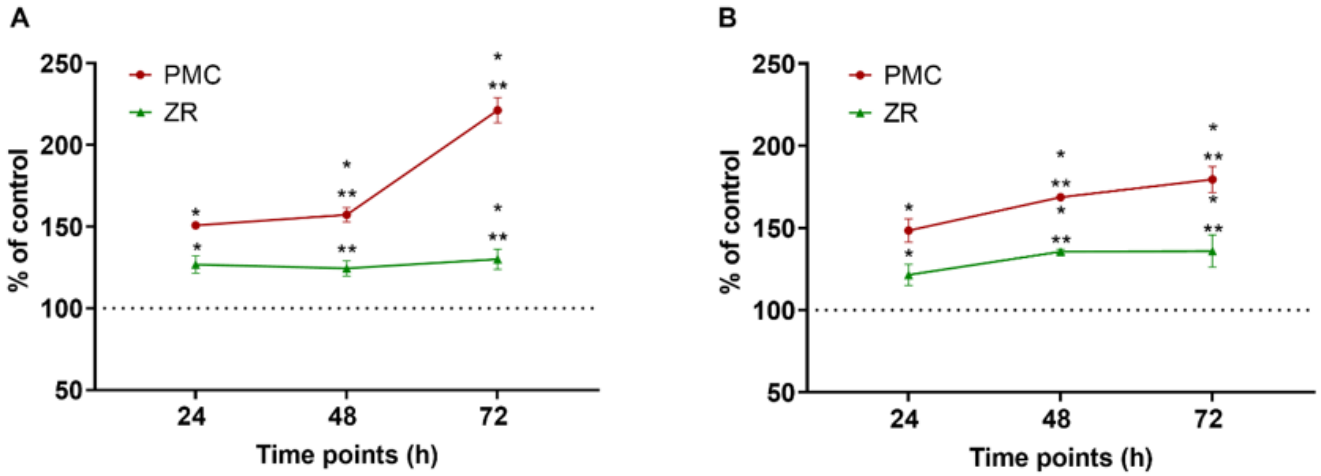

Figure 2

Cell viability of human gingival fibroblasts (HGFs) grown on PMC discs and ZR fragments compared with control at 24, 48, and 72 h (n = 3; technical replicates). **A.** Cytotoxicity assay with HGF-1 cells established from donor 1. **B**. Represents viability assay conducted with HGF-2 cells established from donor 2. Dotted line represents percentage of control at 100%. Asterisks indicate significant changes ($p < 0.05$) compared with the untreated control (*) or competing crown material (**). Mean ± SEM.

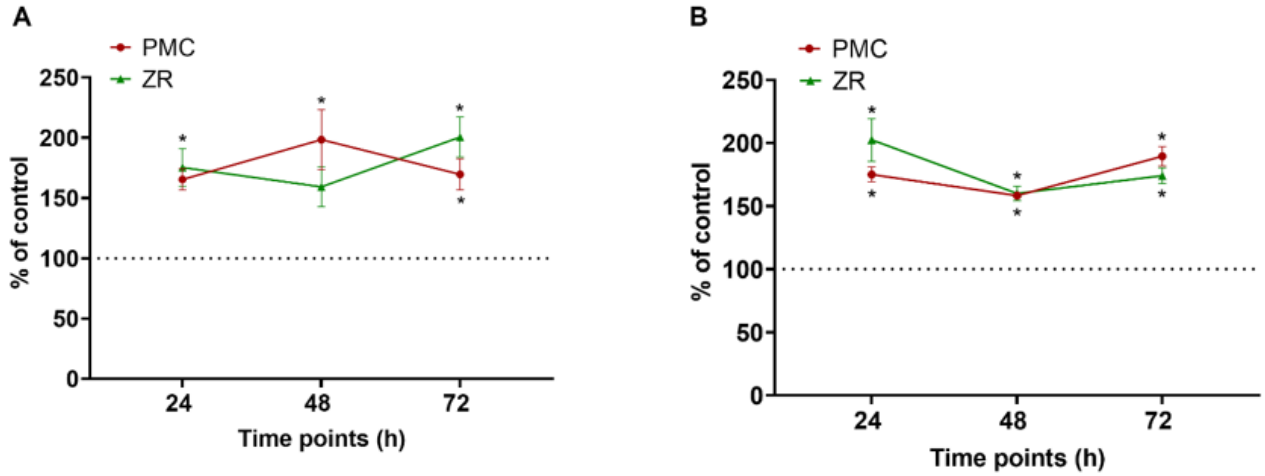

Figure 3

Cytotoxicity of PMC discs and ZR fragments compared with control on human gingival fibroblasts (HGFs) at 24, 48, and 72 h. **A.**Cytotoxicity assay with HGFs established from HGF-1 **B.** Cytotoxicity assay

conducted with HGFs-2 established from HGF-2. Dash line represents percentage of control at 100%. Asterisks indicate significant changes ($p < 0.05$) compared with the untreated control. Mean ± SEM.

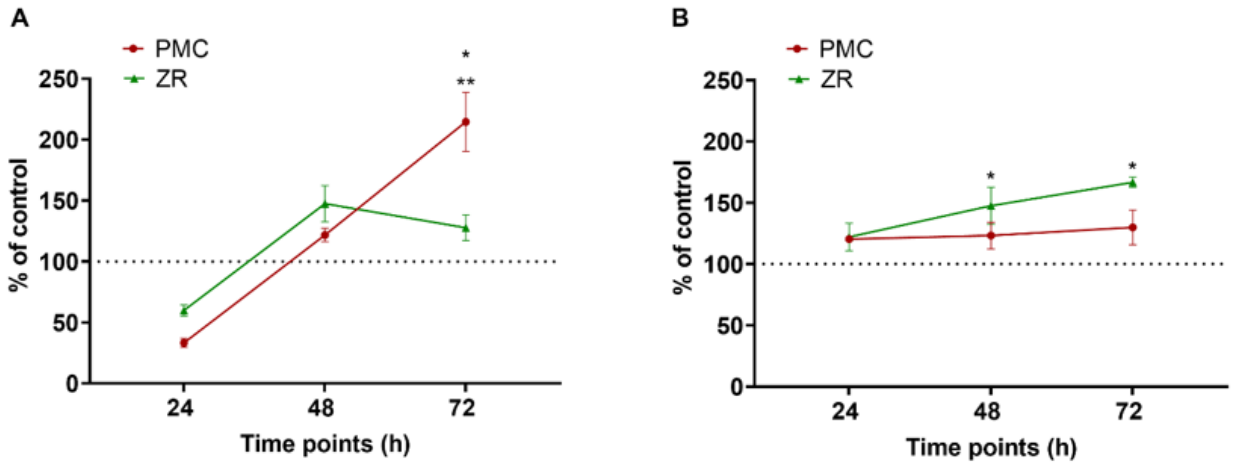

Figure 4

Caspase 3/7 levels in human gingival fibroblasts (HGFs) grown on PMC discs and ZR fragments compared with control at 24, 48, and 72 h. **A.** Apoptosis assay conducted with HGFs-1 established from HGF-1 **B.** Apoptosis assay conducted with HGFs-2 established from HGF-2. Dash line represents percentage of control at 100%. Asterisks indicate significant changes ($p < 0.05$) compared with the untreated control (*) or competing crown material (**). Mean ± SEM.

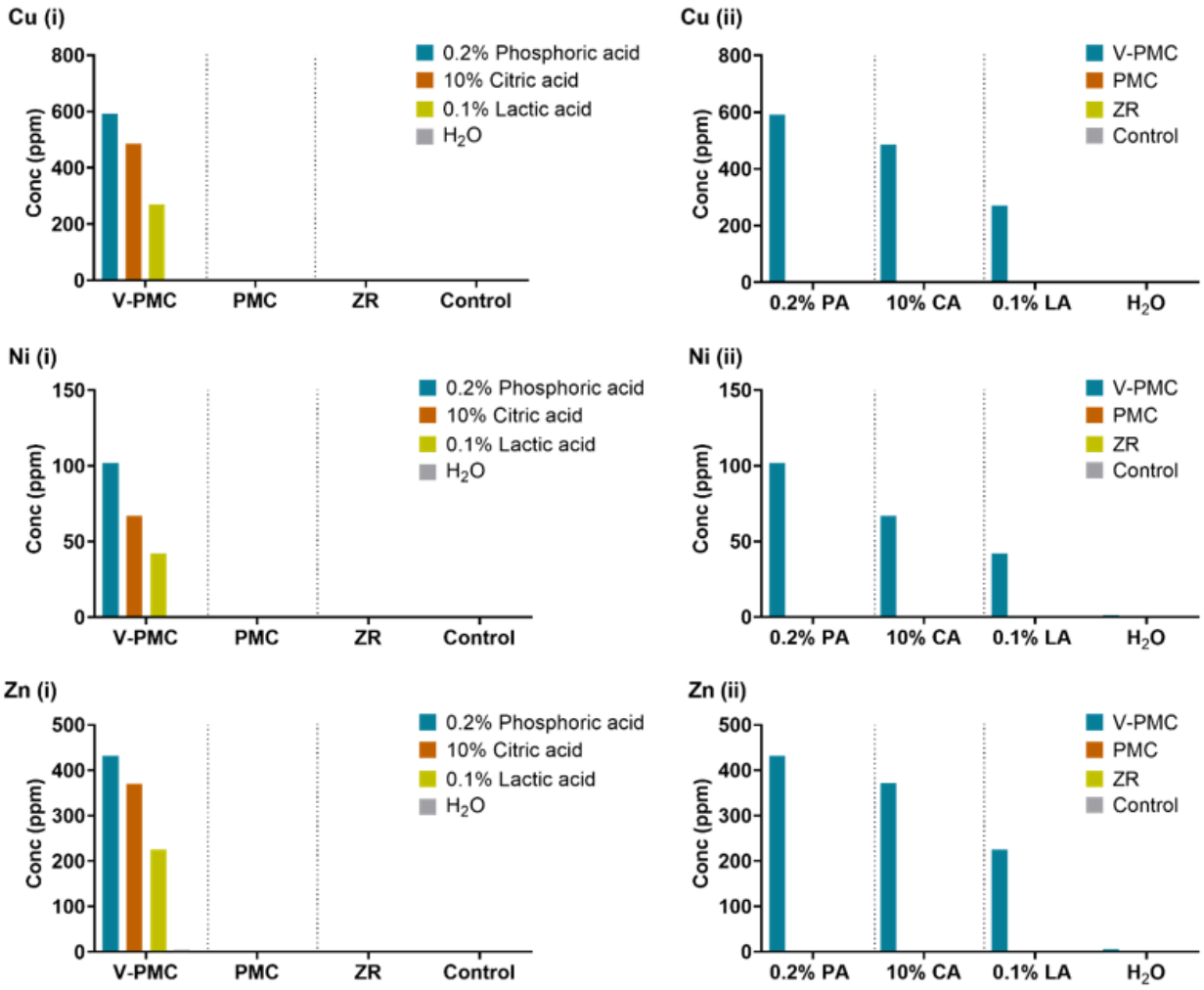

Figure 5

PPC solubility. Cu, Ni, and Zn ion release categorised by (i) materials; and (ii) acidic solutions.