Anti-inflammatory effect of amalgam on periapical lesion cells in culture

Anti-inflamacijski efekat amalgama na ćelije iz periapeksne lezije u kulturi

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Abstract

Background/Aim. Amalgam has been used for years in dentistry, but the controversy on its adverse effects, both on local oral/dental tissues and systemic health, still exists. When used for retrograde filling in apical surgery, amalgam comes in close contact with the periapical tissue, and it is sometimes responsible for the induction of periapical lesion (PL) or its exacerbation. Therefore, the aim of the study was to examine the effect of amalgam on cytotoxicity and production of pro-inflammatory cytokine by cells isolated from PL. **Methods.** Conditioned medium from freshly prepared amalgam (ACM) was performed according to the ISO 10993-12 by incubating the alloy in RPMI medium (0.2 g/mL) for 3 days at 37°C. Cells were isolated from 20 human PLs after apicoectomy by collagenase/DNA-ase digestion and cultured with different dilutions of ACM. Cytotoxicity was determined by MTT assay (n = 7 cultures) and apoptosis/necrosis assays (n = 8 cultures), whereas cytokine production was measured by a Flow Cytomix Microbeads Assay (n = 8 cultures). **Results**. Undiluted (100%) and 75% ACM was cytotoxic due to induction of apoptosis of PL cells. Non-cytotoxic concentrations of ACM (50% and 25%) inhibited the production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8), concentration-dependently. **Conclusion.** For the first time, our results showed an unexpected anti-inflammatory property of amalgam on PL cells, which could be beneficial for PL healing after apicoectomy.

Key words:
dental amalgam; periapical tissue; cytokines; cytotoxicity, immunologic; inflammation; apicoectomy.

Apstrakt

Uvod/Cilj. Amalgam se godinama koristi u stomatologiji, ali i dalje postoje kontroverze o njegovim neželjenim efektima na lokalno oralno/dentalno tkivo i sistemsko zdravlje. Kada se koristi za retrogradno punjenje u apikalnoj hirurgiji, amalgam dolazi u blizak kontakt sa periapeksnim tkivom, što je ponekad povezano sa indukcijom periapeksne lezije (PL) ili njenom egzacerbacijom. Zato je cilj ovog rada bio da se ispita efekat amalgama na citotoksičnost i produkciju pro-inflamacijskih citokina od strane ćelija izolovanih iz PL. **Metode.** Od sveže napravljenog amalgama pripremljen je kondicionirani medijum (ACM) inkubiranjem legure na 37°C u RPMI medijumu u toku 3 dana (0.2 g/mL) kako je predloženo standardom ISO 10993-12. Ćelije su izolovane iz 20 humanih PL nakon apikoektomije, digestijom tkiva pomoću kolagenaze/DNA-aze, a zatim su korišćene za kulturu u prisustvu različitih razblaženja ACM. Citotoksičnost je ispitivana pomoću MTT testa (n = 7 kultura) i detekcijom apoptoze/nekroze (n = 8), dok je nivo produkovanih citokina meren simultano pomoću eseja sa mikrokuglicama uz pomoć protočne citometrije (n = 8). **Rezultati.** Nerazblažen ACM (100%) i onaj od 75% pokazali su citotoksični efekat, indukujući apoptozu PL ćelija. Necitotoksične koncentracije ACM (50% i 25%) inhibirale su produkciju pro-inflamacijskih citokina (TNF-α, IL-1β, IL-6 i IL-8) na dozno-zavisan način. **Zaključak.** Naši rezultati po prvi put pokazuju neočekivano antiinflamacijsko svojstvo amalgama na PL ćelije, što može biti korisno za zarastanje lezije nakon apikoektomije.

Ključne reči:
amalgam, stomatološki; periapeksno tkivo; citokini; citotoksičnost, imunološka; zapaljenje; apikoektomija.

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Introduction

Dental amalgam is one of the most versatile restorative materials that has been used in dentistry for about 170 years, particularly as the first choice for restoring posterior teeth. However, it has myriads of uses, including root-end filling in periapical surgery [1–3]. This procedure prevents the invasion of irritants from infected root canals into the periapical tissues. The advantage of using amalgam for retrograde filling for such a long period of time is its self-sealing capacity, easy manipulation, radio-opacity, and insolubility in tissue fluids [2]. The preferred amalgam is a high copper-zinc-free amalgam, composed of silver 40%–70%, tin 12%–30%, and copper 12%–24%. However, it has many disadvantages, such as the production of corrosive by-products [4, 5], cytotoxicity of mercury and other dissolved metal ions, moisture sensitivity, and staining of hard and soft tissues [1, 6, 7]. There is a possibility of releasing non-resorbable scattered particles during amalgam manipulation, which may be difficult to retrieve [2]. Moreover, amalgam does not properly seal the root end three-dimensionally, has poor marginal adaptation, and does not prevent the leakage of microorganisms and their products in the peri-radicular tissue [2]. However, despite these disadvantages and evidence of a decrease in its use, amalgam's cost, durability, and ease of manipulation have persuaded many dentists to continue to use it, and amalgam remains a standard to which other materials are compared [2, 8].

The major concern for using amalgam in dentistry is its cytotoxic effect, which has been documented in many human and animal cells as well as in established cell lines *in vitro* [6, 9–11]. In the past few decades, however, potential systemic and local toxic effects have been described *in vivo* [2, 3, 12, 13]. Patients may suffer from hypersensitivity reactions to mercury or other amalgam components. Other reactions to amalgam with a variety of clinical symptoms, collectively termed "amalgam disease," have been reported, including adverse immunological effects and autoimmune phenomena [12, 14, 15].

Clinical and histopathological studies show that amalgam, implanted subcutaneously or in the bone, is well tolerated [16, 17]. This is in contrast with some studies showing the capability of amalgam particles to cause periapical lesions [18] and to cause a cytotoxic effect on periodontal ligament cells and periodontal fibroblasts [19–21]. However, there is no study investigating the effect of amalgam on human periapical lesion (PL) cells *in vitro*, which was the main goal of our study. This knowledge is important since the alloy communicates with the periapical tissue for a long period of time. Our results showed for the first time an unexpected anti-inflammatory effect of amalgam on PL cells which could be beneficial for PL healing.

Methods

Periapical lesion samples

Human PLs (n = 20) were extracted during apicoectomy at the Department of Oral Surgery, Clinic for Stomatology, Military Medical Academy (MMA), Belgrade, Serbia. The study was approved by the Ethics Committee of the MMA, followed by informed consent from patients. The exclusion criteria included the following: patients with malignant, autoimmune, and other chronic inflammatory diseases, as well as those on immunosuppressive/immunomodulatory therapy. The patients included had not been treated with antibiotics for one month prior to the PLs excision. PLs were diagnosed by clinical and radiographic criteria. No distinction was made between age, sex, tooth type, size, and clinical presentation of PLs. After extraction, PLs were immediately placed in a medium consisting of RPMI-1640 (Sigma, Munich, Germany) and antibiotics/antimycotics and transported to the laboratory.

Isolation of cells from PLs

The cells from PLs were isolated by a procedure that has been previously introduced by our research group [22]. Briefly, periapical tissue was placed in a Petri dish containing 1 mL RPMI-1640 medium and cut into 2–3 mm diameter pieces using a scalpel. The tissue was then digested for 20 min with 0.05% collagenase type IV (Sigma) and 0.02% DNA-ase (Sigma) dissolved in RPMI-1640 medium in a cell incubator at 37 °C. After that, the tissue was pressed through a stainless-steel mesh using a syringe plunger, filtered, and resuspended in RPMI-1640 medium containing 1 mM EDTA. The released cells were pooled, washed twice by centrifugation in the RPMI medium at room temperature (400 g for 10 min), and counted. The viability of cells, determined by Trypan Blue dye, was 93% ± 3%. The cells were used for *in vitro* experiments. Eight periapical lesions were used to study cytokine production and apoptosis/necrosis. Twelve PLs containing either a larger number of cells (higher than 2.0×10^6 cells; n = 4 PLs) or pooled PLs from the same donors (n = 8 PLs from 3 patients) were used for the MTT assay. The total number of individual cultures for this assay was 7.

Preparation of conditioned medium

Amalgam, consisting of the encapsulated alloy (Extracap) and mercury, was purchased from Galenika, Belgrade, Serbia. One-gram (g) powder of the alloy contained silver (500 mg), tin (299 mg), and cooper (201 mg). The alloy mass was 0.360 g, and the mercury mass was 0.400 g. Amalgam specimens were prepared by triturating amalgam alloy powder with pure mercury in an amalgamator, and after the mixture, disc-form specimens, diameter around 10 mm, thickness about 1–2 mm, were prepared. The freshly prepared amalgam discs were used for the preparation of amalgam conditioned medium (ACM) by placing the amalgam disc in a glass tube containing RPMI-1640 medium with an addition of antibiotics/antimycotics. The mass of amalgam to the volume of RPMI medium was 0.2 g/mL according to ISO 10993-5 and ISO 10993-12. The conditioning lasted for 3 days. Control CM was prepared by incubating control inert material, polystyrene, under the same conditions. ACM and control (C)-CM were supplemented with 10% FCS. There was no need for pH adjustment, which remained 7.4. Such prepared CM were further used for PL cell culture experiments.

Cell cultures

The cells isolated from PLs were cultivated in 96-wells, with round-bottomed plates (ICN, Costa Mesa, CA) (1×10^5 cells/well, 200 µL) in the complete culture medium consisted of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Sigma) and standard culture solutions of antibiotics [22]. The cultures were treated with different dilutions of ACM or C-CM. Undiluted CM was considered 100% CM. After 24 h, the cell supernatants were collected, centrifuged, and frozen at -70 °C until the levels of cytokines were determined. The cells were used for apoptosis/necrosis assay.

MTT assay

PL cells were cultivated in 96-well plates (1×10^5/well; triplicates) in either fresh complete RPMI medium, different dilutions of ACM or C-CM. After a 24-hour incubation period, the plates were centrifuged, and the medium was carefully removed. The solution of 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) (100 µL/well, final concentration 100 µg/mL), was added. Wells with an MTT solution without cells served as blank controls. The plates were incubated with MTT for 3 hours in an incubator at 37 °C. Dissolution of formazan was done by incubating the MTT-treated cultures with 0.1N HCl/10% SDS (sodium dodecyl sulphate) (100 µL/well) overnight. The next day, the optical density (OD) of the developed colour was read atw 570/650 nm (ELISA reader, Behring II). The results were expressed as the relative metabolic activity compared to the metabolic activity of control cultures.

The relative metabolic activity was calculated as follows: metabolic activity (%) = (OD of cultures with ACM/OD of cultures with control fresh medium) × 100.

Apoptosis/necrosis assay

Apoptosis/necrosis was detected by Annexin-V–fluorescein isothiocyanate (FITC) and Propidium iodide (PI) staining kit (R&D), following the manufacturer's protocol. Briefly, cultivated PL cells were collected, washed with binding buffer, followed by incubation with Annexin-V–FITC and PI. The labeled cells were analyzed on a flow cytometer (Partec, Cube 6). Annexin-V-FITC⁺ cells were recognized as primary apoptotic cells (early phase of apoptosis), PI⁺ cells were primary necrotic cells, whereas double-positive cells were apoptotic/secondary necrotic cells (late phase of apoptosis).

Cytokine assays

The concentrations of interleukin (IL)-1β, IL-6, IL-8, and TNF-α in culture supernatants were detected by a FlowCytomix Microbeads Assay. This is a bead-based ELISA-like assay optimized for flow cytometry, allowing the simultaneous detection of several cytokines in a volume of samples (50 µL). The inflammation kit, containing microbeads coupled with antibodies to pro-inflammatory cytokines, was purchased from Biolegend. The levels of cytokines were determined by constructing standard curves based on the known concentration of these cytokines.

Statistical analysis

The Student's *t*-test was used for comparison of parametric variables between two groups. The Friedman's test (paired one-way ANOVA) was used for comparison between groups for non-parametric variables with Dunn's multiple comparison post-test. The values of $p < 0.05$ were considered to be statistically significant. Software SPSS version 23.0 (IBM, Armonk, New York, USA) was used to analyze the data.

Results

The first aim of this study was to examine the cytotoxicity of ACM on PL cells in culture. By using the MTT test (Figure 1), we showed that only concentrated (100%) and 75% ACM significantly reduced the viability of PL cells ($p < 0.001$ and $p < 0.01$, respectively). The cytotoxicity was due to the induction of apoptosis (Figures 2A and 2B). Figure 2B shows that ACM increased the proportion of late apoptotic/secondary necrotic cells.
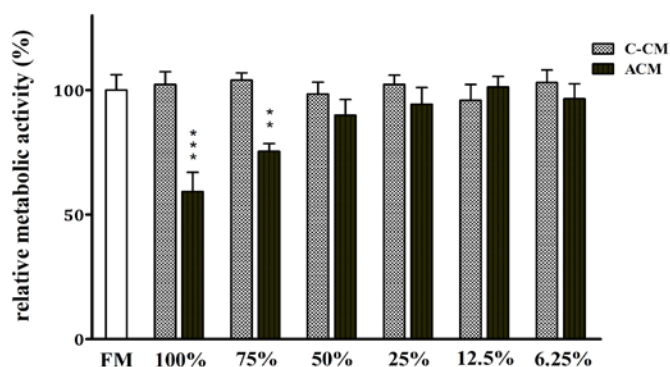
Fig. 1 – Cytotoxicity effect of amalgam on periapical lesion cells (PL) in culture. PL cells, prepared as described in Materials and methods, were cultured with different dilutions of amalgam conditioned medium (ACM) for 24 hours. The viability of PL cells was determined by the MTT test, as described. Values are given as mean ± SD (n = 7 cultures) of relative metabolic activity of cells. **$p < 0.01$**; *****$p < 0.001$** compared to control cultures. FM – fresh medium; C-CM – control-conditioning medium.**
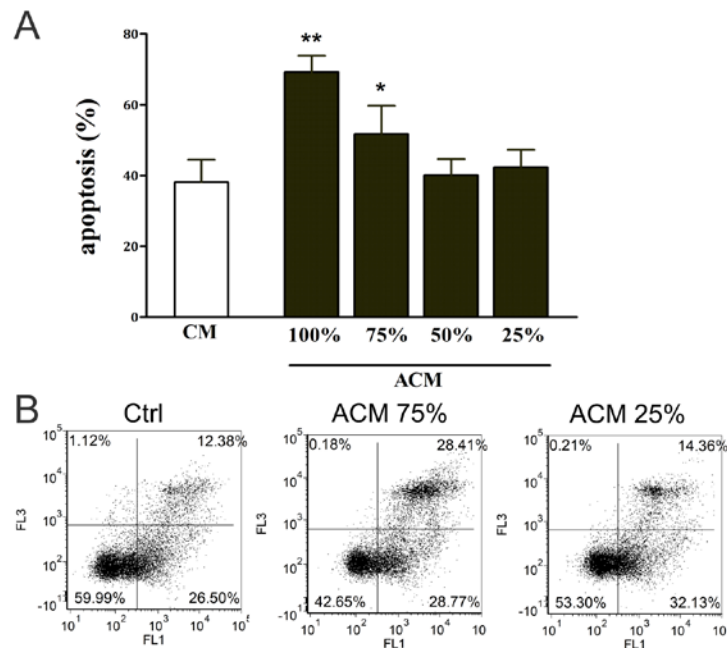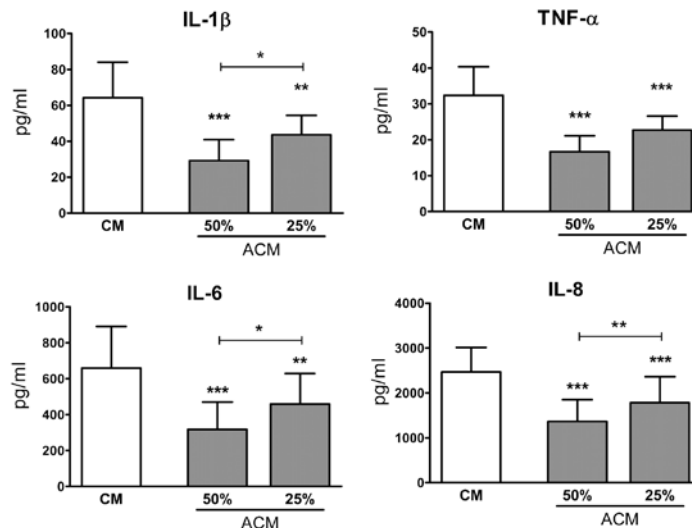
Fig. 2 – Effect of amalgam on apoptosis of periapical lesion (PL) cells in culture. PL cells, prepared as described in Materials and methods, were cultured with different dilutions of amalgam conditioned medium (ACM) for 24 hours. The apoptosis of PL cells was determined by the Annexin V-FITC/ PI assay, as described. A) Values are given as mean ± SD (n = 8 cultures) of apoptotic cells (*$p < 0.05$; **$p < 0.01$ compared to control cultures). B) Representative histograms showing that ACM accelerate apoptosis of PL cells, manifested by an increase of late apoptotic/secondary necrotic cells. CM – control medium.

The second aim was to investigate the effect of ACM on the production of pro-inflammatory cytokines (IL-1β, TNFα, IL-6, and IL-8) by PL cells. We used non-cytotoxic concentrations (50%, 25%, and 12.5%) of ACM. The 50% and 25% concentrations of ACM suppressed the production of all four cytokines dose-dependently (Figure 3), whereas the 12.5% concentration did not show any modulatory effect (data not shown).

Fig. 3 – Effect of amalgam on the levels of pro-inflammatory cytokines in the culture of periapical lesion (PL) cells. PL cells, prepared as described in Materials and methods, were cultured with different dilutions of amalgam conditioned medium (ACM) for 24 hours. The levels of pro-inflammatory cytokines in culture supernatants were determined by Flow Cytomix Microbeads Assay. Values are given as mean ± SD (n= 8 cultures) levels of cytokines (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to control cultures or compared to 50% ACM, indicated by corresponding bars). CM – control medium.

Discussion

The first aim of this study was to examine the cytotoxicity *in vitro* of a copper-zinc-free amalgam, which is the oldest root-end filling material in apical surgery. Apicotomy is a common procedure for removing periapical lesions (granuloma or cysts) when the conventional endodontic treatment is not efficacious. Amalgam is still used for this purpose because of its self-sealing capacity, radio-opacity, insolubility in tissue fluids, and low price. However, since amalgam does not properly seal the root-end three-dimensionally, has poor marginal adaptation, and does not prevent the leakage of microorganisms in the peri-radicular tissue successfully [1–3, 23], we hypothesized that amalgam, due to its cytotoxic effect, could aggravate periapical inflammation. Therefore, cells isolated from PLs, which are dominantly composed of infiltrating inflammatory cells [22, 24], were the most suitable target to test this hypothesis, and this was our original approach.

Before starting with crucial experiments, it was necessary to determine the cytotoxicity of amalgam by using this culture model. Up to now, many different tests have been used for assessing amalgam cytotoxicity, but MTT, based on the evaluation of cellular metabolic activity, is the most acceptable as a first screening assay [6]. It is known that amalgam causes cytotoxicity either in direct contact with examined cells or indirectly by metallic ions released from the alloy [2, 6, 9]. We decided to study the effect of amalgam indirectly by analyzing the effect of ACM in which its leachable products are present and which are considered dominant cytotoxic factors [9, 25]. The study was conducted exactly as recommended by the ISO 10993-5 standard. We showed that only high concentrations of ACM (concentrated and 75%) were cytotoxic for PL cells due to apoptosis induction, suggesting that amalgam is generally cytotoxic alloy as similarly shown on other target cells. A relatively high proportion of apoptotic cells were also observed in control PL cell cultures, and the most sensitive cells were granulocytes, followed by macrophages, whereas lymphoid cells were more resistant (data not shown). These observations are in line with the already known facts about the high apoptotic rate of extravasated neutrophils as terminally differentiated cells [26].

We did not examine the concentrations of released ions from amalgam because this has been extensively investigated and published [5, 6]. In fact, all metal ions can be released in CM from amalgam, such as mercury, silver, copper, and thin. Out of them, cooper is the most cytotoxic, but it can be hypothesized that other ions act synergistically in inducing cytotoxicity [1, 2, 5, 6, 9]. This hypothesis was based on previous publications which thoroughly investigated the release and cytotoxicity of metal ions from amalgams of different composition. In this context, Kaga et al. [9] have demonstrated that pure copper showed the highest cytotoxicity among the metals tested in zinc-free amalgams. Silver and mercury showed reduced cytotoxicity, while tin was non-cytotoxic. In contrast, zinc-containing amalgams are more cytotoxic due to the easy release of Zn ions. The toxic effects of mercury

are believed to exist due to the high reactivity of mercury species toward thiol-groups and other functional groups, notably in proteins [27]. It has been shown that both organic and inorganic mercury induce apoptosis of different cells, including human lymphocytes [27, 28].

The second part of this study was related to the effect of ACM on the production of pro-inflammatory cytokines by PL cells. We tested non-cytotoxic concentrations of ACM because toxic concentrations would not be relevant for a proper conclusion, partly due to the spontaneous release of cytokines from dead cells. We observed an unexpected result where ACM at non-cytotoxic concentrations significantly inhibited the secretion of pro-inflammatory cytokines (IL-1β, TNFα, IL-6, and IL-8). Therefore, our hypothesis was rejected.

The anti-inflammatory effect of ACM is contrary to the data published on the proinflammatory effect of amalgam particles which could induce the PL development if released into the periapical tissue during endodontic surgery [18]. Similarly, amalgam has been found to cause an inflammatory response in the dental pulp, which is transitory and significantly decreased in due time [29]. These differences (pro-inflammatory *versus* anti-inflammatory properties of amalgam) can be explained by the difference in setting experiments. Namely, cytotoxic effects of amalgam on periodontal tissue *in vivo* can provoke an inflammatory reaction due to direct contact, where, in the vicinity of the alloy, relatively high concentrations of cytotoxic metallic ions can be released. This effect dominates over anti-inflammatory effects seen at non-cytotoxic concentrations of leachable amalgam components.

No one has ever published a study related to amalgam nor examined the changes of multiple pro-inflammatory and other cytokines. The most relevant paper is the one published by Schedle et al. [30], who investigated the effects of dental amalgam on cytokine production by human peripheral blood mononuclear cells (PBMC) from healthy donors. To induce cytokine production, they stimulated PBMC in culture with lipopolysaccharide, phytohemagglutinin, or staphylococcal enterotoxin A in the presence of fresh amalgam, aged amalgam, or ACM prepared from fresh amalgam. They showed that freshly prepared amalgam, as well as ACM, reduced the production of interferon-γ (IFN-γ) and IL-10 but increased the levels of TNF-α. Both fresh amalgam and ACM had no effects on the levels of IL-2, IL-6, or granulocyte-macrophage colony-stimulating factor. Amal-gam aged for 6 weeks did not modulate the concentrations of any of the above cytokines. To investigate which heavy metal cations released from amalgam caused the observed immunomodulatory effects, Cu^{2+}, Hg^{2+}, and Sn^{2+}, which were detected in ACM, were added as salts to PBMC cultures. Cu^{2+} and Hg^{2+} decreased the IFN-γ and IL-10 levels. However, Hg^{2+} increased TNF-α concentrations, whereas Sn^{2+} had no modulatory effect.

It is evident that our results, showing a decrease in TNF-α production, are opposite. The difference could be due to the following reasons, respectively: different concen-trations of ACM (concentrated vs. diluted ACM); different

cells (stimulated PBMC vs. non-stimulated PL cells); different mass/volume ratio for ACM preparation (1.92 g/mL vs. 0.2 g/mL); different incubation time for cell cultures (48 h vs. 24 h). Some other studies investigated the effect of mercury. In this context, Soleo et al. [31] showed an increase in the number of CD4$^+$ cells in peripheral blood of subjects exposed to mercury from dental amalgam together with a decrease of serum IL-8 levels. Podzimek et al. [32] examined cytokine production (IL-1β, IL-4, IL-6, TNF-α, and IFN-γ) by human lymphocytes in cultures treated with mercury and found increased production of TNF-α and IFN-γ. Ilday et al. [33] observed reduced clinical periodontal findings in patients after overhang amalgam restoration removal, but these findings did not correlate with the changes in the levels of IL-6, IL-8, and TNF-α in the gingival crevicular fluid. This is in contrast with another study, published previously, which showed that removal of dental amalgam restorations was associated with decreased concentrations of Th1-type pro-inflammatory cytokines in serum, supporting the hypothesis that amalgam could be responsible for stimulating the Th1-type response *in vivo* [34].

It is known that cytokines play a key role in the pathogenesis of PLs [35]. Pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α, orchestrate the recruitment and activation of innate immune cells, presumably neutrophil granulocytes and monocytes in the early inflammatory phase and T and B cells in the later inflammatory phase, respectively. In this context, cytokines of T cells are the main controllers of the immune/inflammatory reactions. T-helper 1 (Th1) cells and Th-17 cells, by producing interferon-γ (IFN-γ) and IL-17, respectively, are involved in the progression of PLs and bone destruction, whereas T-helper 2 (Th2) cytokines, such as interleukin 4 (IL-4), IL-5, IL-10, and IL-33, are involved in the humoral immune response and attenuation of the tissue damage [22, 35, 36]. Therefore, further experiments investigating the effect of amalgam on this panel of cytokines could make a much better conclusion.

Conclusion

By using inflammatory cells isolated from human PL, we showed, for the first time, a potent anti-inflammatory effect of non-cytotoxic concentrations of ACM. This finding is in contrast with the previous findings, which state that particular amalgam particles released during retrograde filling can cause chronic apical periodontitis. Our results suggest that, in contrast to the high release of toxic ions from amalgam, slow release of leachable components from this amalgam, by down-modulating the production of pro-inflammatory cytokines, may control an excessive inflammation and promote PL healing.

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