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Transdental cytotoxicity and macrophage phenotype of a novel quaternary ammonium silane cavity disinfectant

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ABSTRACT

Objective. To evaluate the transdental cytotoxicity and macrophage phenotype response to a novel quaternary ammonium silane (QAS) cavity disinfectant.

Methods. NIH 3T3 mouse fibroblasts were cultured in Dulbecco's Modified Eagle's Medium and incubated for 3 days. The cells (3×10^4) were seeded on the pulpal side of dentine discs and the occlusal side of the discs were treated with different cavity disinfectants: Group 1: de-ionized water (control); Group 2: 2% chlorhexidine (CHX); Group 3: 2% QAS; Group 4: 5% QAS, and Group 5: 10% QAS. Cell morphology of NIH 3T3 cells was examined using scanning electron microscopy (SEM) and cell viability was assessed using Trypan blue assay. The eluates were collected and applied on cells seeded in 24-well plates. The total protein production, alkaline phosphatase activity and deposition of mineralized nodules were evaluated after 7 and 14 days. Immunofluorescence staining was performed on the samples with primary antibodies of CD68+, CD80+, and CD163+ assessing the macrophage M1/M2 phenotypes. The macrophages were imaged using a confocal scanning light microscope with an excitation wavelength of 488 nm.

Results. No significant difference in cell viability ($p < 0.0001$), total protein production ($p < 0.01$) and mineralized nodule production ($p < 0.05$) was found between 2% QAS and the control, which was significantly higher than 2% CHX, 5% and 10% QAS after 14 days. Alkaline phosphatase production of 2% QAS was significantly lower than the control ($p < 0.001$), but higher than 2% CHX at 14 days. The M1/M2 macrophage ratio was also significantly lower in the 2% and 10% QAS groups ($p < 0.05$) compared to the control and 2% CHX groups.

Significance. The 2% QAS cavity disinfectant does not have cytotoxic effects on 3T3 NIH mouse fibroblast cells and the predominance of the anti-inflammatory phenotype after its application may stimulate healing and tissue repair.

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1. Introduction

Restoration of dental tissues aims to remove all infected dentine after cavity preparation. However, there is consider-

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able evidence that the complete removal of micro-organisms is impossible [1], with some bacteria persisting after soft dentine excavation [2]. Residual bacteria is one potential cause of recurrent caries and increased pulpal sensitivity if a restoration seal is breached [3]. Long-term degradation of the bonded interface results in the reduction of bond strengths, microleakage, interfacial gap formation and recurrent caries over time [4]. Previous studies have suggested the use of cavity disinfectants such as sodium hypochlorite, hydrogen peroxide, disodium ethylenediaminetetraacetic acid, and iodine for removal of residual bacteria [5]. With the use of cavity disinfectants, the sealing ability of dentine adhesives can be a concern for clinicians and researchers [6] making the interaction between adhesive bonding systems and cavity disinfectants a controversial topic in restorative dentistry [7].

Chlorhexidine (CHX) has been used as a pre-treatment for its antibacterial effects as well as inhibition of matrix metalloproteinases (MMPs) [8] and cathepsins K and B [9], thereby reducing degradation of the hybrid layer. However, the binding of CHX to demineralized dentine is electrostatic with no substantial interaction between the collagen fibrils and the antibacterial agent. Therefore, leaching of CHX from the bonded interface has been reported [10,11]. Furthermore, CHX has been shown to have dose- and time-dependent cytotoxicity on cultured MDPC-23 cells, causing direct damage and inhibition of cell metabolism [12]. In recent years, there has been improvement of antimicrobial activity and polymerisation capacity of quaternary ammonium-based compounds and monomers. These compounds with methacrylate monomers possessed methacrylate functionality undergoing linear polymerisation [13]. Quaternary ammonium salts have primarily been used as surface coatings in the food and textiles industries [14]. Due to the interaction between the positively charged quaternary amine (N^+) and negatively charged bacterial membranes, bacterial lysis occurs as a result of a disturbance in the electrical balance and osmotic pressure damaging the bacterial membrane. These cationic compounds penetrate bacterial membranes via needle-like structures [15]. Quaternary ammonium bis-phenol A glycerolate dimethacrylate had been proposed as antimicrobial dental monomer. The difunctional compound reduced degree of conversion slightly and led to an increase in water absorption of the cured polymer in presence of antimicrobial activity [16]. In addition to this, quaternary ammonium methacrylate was modified with silica nanoparticles for photocurable monomers functionally modified with bisphenol A glycerolate dimethacrylate and investigated for antimicrobial protection. Overall, the formulation had reduced cell viability with concentrations greater than 5 wt% [17].

The quaternary ammonium compound, 3-(trimethoxysilyl)-propyldimethyloctadecyl ammonium chloride (SiQAC; $C_{26}H_{58}ClNO_3Si$; CAS Registry Number 27668-52-6) is commonly used as an antimicrobial coating. Being a trialkoxysilane, SiQAC covalently attaches to other alkoxy-silanes or silanol-containing substrate surfaces via the formation of siloxane bridges [18]. The quaternary ammonium dimethyl-hexadecyl-methacryloxyethyl-ammonium iodide (DHMAI) has been reported to be effective against *S. mutans* biofilm when incorporated in experimental methacrylate-based composite resins used for dental restoration [19].

Recently, SiQAC has been coupled to other trialkoxysilanes with methacryloxy or epoxy functionalities via sol-gel platform chemistry that utilises tetraethoxysilane (TEOS) or dimethyldiethoxysilane as the anchoring unit [20–22]. Such sol-gel reactions between one tetra-alkoxy-silane and two tri-alkoxy-silanes generated a host of antimicrobial quaternary ammonium silane (QAS) molecules with methacryloxy or epoxy functionalities that can copolymerise with methacrylate or epoxy resins.

By using the same principles of platform chemistry, an ethanol-soluble, partially condensed and fully hydrolyzed version of QAS has been prepared. The hydrolysis by-product of the sol-gel reaction is ethanol instead of methanol with inclusion of tetraethoxysilane (TEOS) as the network-forming agent. The presence of TEOS aids the formation of three-dimensional organically-modified silicate by condensation of additional tetra- and tri-ethoxysilane molecules with remnant silanol groups within the quaternary ammonium compound. As a result of this, when QAS is applied to the dentine after smear layer removal, there is condensation of the modified network that may result in occlusion of incompletely-infiltrated hybrid layers and dentinal tubules that are not completely infiltrated by dentine bonding agents. The condensation has the potential of reducing nanoleakage and dentine hypersensitivity. As the QAS cavity disinfectant is applied as a pre-treatment on a demineralized dentine substrate prior to application of adhesive systems, it is therefore of great importance to demonstrate its safety and biocompatibility.

Macrophages are a heterogeneous subset of mononuclear cells that produce a host response to any implanted (foreign) material [23]. The cells are activated as a result of tissue infection, increasing the production of chemokines and cytokines after exposure [24]. The macrophage phenotype has been characterized based on functional properties, cytokine profile and cell surface markers of the microenvironment [25]. These polarized macrophages are referred as M1 or M2 phenotypes [26], where M1 is classically activated pro-inflammatory, cytotoxic macrophages induced by IFN- γ or in combination with lipopolysaccharide (LPS), predominantly characterized by classical signs of chronic inflammation. The anti-inflammatory M2 macrophages are induced by exposure to cytokines IL-4, IL-13 and IL-10, promoting constructive tissue remodelling. These macrophages can change their polarization in response to local stimuli, infection or response to any implanted biomaterial [27]. Hence, it is logical to speculate, that any reaction to any biomaterial is a “cross talk” between macrophages and the cells within the microenvironment.

The effect of QAS on macrophage phenotype response has not been investigated. Therefore, the objectives of this study were: (i) to compare the cytotoxicity of different concentrations of QAS cavity disinfectant on NIH 3T3 mouse fibroblasts (MFs) cells, and (ii) to determine M1/M2 profile of macrophages against different concentrations of QAS cavity disinfectants. The NIH 3T3 MFs cell-line are commonly used as target cells for cytotoxic assay of dental materials providing preliminary biosafety information on the application of the compound on mammalian cells [28]. These cells will be used in the present study to examine the cytotoxic effect of QAS. The

null hypotheses tested were that: (i) there is no difference in cytotoxicity among different concentrations of QAS and CHX, and (ii) there is no difference in M1/M2 profile of macrophages among different concentrations of QAS and CHX.

2. Materials and methods

2.1. Synthesis of QAS cavity disinfectants

The experimental versions of QAS cavity disinfectants used in the present study were synthesized by sol-gel reaction reported previously by Daood et al. [29]. The synthesized QAS solid was dissolved immediately in absolute ethanol to produce a 50% QAS solution. The 50% solution was further diluted with absolute ethanol to produce 2%, 5%, and 10% (w/v) QAS solutions. The three concentrations of QAS solution were stored in airtight vials at 4 °C until use.

2.2. Preparation of dentine discs

The study protocol was reviewed and approved by the Institutional Review Board of The University of Hong Kong (UW 14-406) including informed consent from patients for the use of teeth for experimental purposes. One-hundred and seventy extracted sound human third molars were collected and stored in 0.5% Chloramine T solution at 4 °C for up to 3 months. The roots were removed using a low-speed, water-cooled, diamond-impregnated disc (Isomet, Buehler, Lake Bluff, IL, USA) and the occlusal enamel of each crown segment was cut to expose deep dentine. A 0.5 mm thick dentine disc was prepared from the mid-coronal dentine of each tooth and the enamel was removed from the sides giving square-shaped dentine discs. A final disc thickness of approximately 0.4 mm was achieved by grinding the occlusal side with wet 320-grit silicon carbide papers. The thickness was evaluated using a pair of digital calipers (iGaging IP54 electronic digital caliper) providing a precision to ± 0.01 mm. For dentine permeability, thirty-five extracted teeth were used while twenty-five teeth were used for culture of NIH 3T3 fibroblasts cell line, viable cell counts, total protein production, alkaline phosphatase activity and alizarin red nodule analysis. For SEM observations, ten teeth were utilized.

2.3. Dentine permeability

Homogenous distribution of the dentine discs into 5 groups ($n = 7$) was performed by evaluation of the dentine permeability of each dentine disc. The smear layer was removed from both sides of the discs by applying 0.5 M EDTA (pH 7.4) for 60 s. After rinsing with deionized water, the discs were placed in the diffusion chamber connected to 180 cm column of water for 5 min [30]. The movement of a micro-bubble introduced through a metal cannula was recorded for 1 min. Calculation of the hydraulic conductance (L_p) of dentine was based on the mathematical equation as follows:

$$L_p = J_v / A t (P)$$

where L_p = hydraulic conductance in $\mu\text{L cm}^{-2}, \text{min}^{-1}$, H_2O^{-1} , J_v = fluid flow in $\mu\text{L min}$, A = surface area of the den-

tine in cm^2 , t = time, P = hydrostatic pressure applied in $\text{cm H}_2\text{O}$ [31]. After converting the values to hydraulic conductance, the dentine discs were allocated into 5 groups so that the mean hydraulic conductance was statistically similar among the groups (one-way ANOVA, $p > 0.05$). Then, an area of 0.28 cm^2 was standardized using a metallic ring on the dentine disc, and a fresh smear layer was created using 600-grit silicon carbide paper.

2.4. Culture of NIH 3T3 fibroblasts cell line

The NIH 3T3 MF cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Corp, St. Louis, MO, USA; DMEM) with 1% penicillin/streptomycin (10,000 U/100 $\mu\text{g/ml}$) and 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO_2 incubator at 37 °C. The cell culture was expanded to $4 \times$ dilution when 80% confluence was reached. Cells cultured to the sixth and seventh passages were utilized for the experiment. After 3 days of incubation, the cells (3×10^4) were seeded on the pulpal side of the dentine discs (0.28 cm^2) in 24-well plates ($n = 5$) in an incubator with 5% CO_2 and 95% air at 37 °C. The discs were transferred back to the same wells with the occlusal side up to receive the QAS and CHX treatments.

2.5. Application of QAS and CHX cavity disinfectants

The occlusal side of the dentine disc was etched using 35% phosphoric acid (Scotchbond etchant, 3 M ESPE) for 15 s, rinsed with de-ionized water for 10 s and blot-dried using Kimwipes (Kimtech Science, code 34120, USA). Five treatment groups ($n = 5$) were tested in the study: Group 1: de-ionized water (control), Group 2: 2% CHX (Millipore Sigma, St. Louis, MO, USA), Group 3: 2% QAS, Group 4: 5% QAS and Group 5: 10% QAS. The cavity disinfectants were applied onto the occlusal side of dentine discs using a sterile micro brush for 20 s, followed by blot-drying. All procedures were performed in a vertical laminar flow hood. After the treatment, the dentine discs were returned to the CO_2 incubator for an additional 24 h.

2.6. SEM evaluation of cell morphology

Two additional discs ($n = 2$) were used for examination of cell morphology grown on treated dentine discs. The NIH 3T3 MF cells on the pulpal side of dentine discs were grown for 24 h and later fixed using 2.5% glutaraldehyde in phosphate buffered solution (PBS) for 2 h at room temperature. Then, the glutaraldehyde was aspirated, the cells were rinsed with PBS, post fixed using 1% osmium tetroxide for 1 h and rinsed again using PBS. Subsequently, the samples were subjected to dehydration with an ascending series of ethanol solutions (75%, 85%, 95%, and 100%) twice for 60 min each. Specimens were mounted on aluminum stubs with conductive tape (double-sided carbon tape) in such a way that they could be observed in a cross-sectional view and stored in a desiccator for 24 h. They were sputter-coated with a 30 nm-thick layer of gold-palladium alloy for 120 s. After sputter-coating, the specimens were viewed using a scanning electron microscope (SEM) (Hitachi S-3400N, Hitachi High Technologies America, Inc., Schaumburg, IL, USA) operated at an accelerating voltage of

10 kV. All cell features and cell shapes were compared with cells from the control group.

2.7. Viable cell counts

The effect of cavity disinfectants on viable cells was evaluated using Trypan blue assay ($n=6$). The test provides a direct assessment of the total number of viable cells as the dye enters the porous cell membranes of the damaged cultured cells. After 24 h contact with cavity disinfectants, the DMEM medium was removed from the culture plates containing the discs ($n=5$) and 0.12% trypsin were introduced for 5 min to promote the detachment of cells from dentine. Around 50 μ L aliquots of the cells were mixed with 50 μ L of 0.04% Trypan blue dye (Sigma-Aldrich Corp., St. Louis, MO, USA) and left for 2 min. Ten μ L of the solution was then aspirated and placed on the hemocytometer and examined under an inverted light microscope (LW 0.52 Nikon Japan, Eclipse) for live and dead cells [32]. The final calculation was done by subtracting the total number of non-viable cells (trypan blue stained) from the total number of cells to obtain the number of viable cells [33] corresponding to $n \times 10^4$ cells per milliliter of suspension. Ten replicates were done for each group tested and analysis done in triplicates.

2.8. Total protein production

After dentine treatment ($n=5$), the eluate was collected and placed in contact with new-cultured NIH 3T3 mouse fibroblasts cells seeded in 24-well plates for 24 h. The eluate was carefully removed, replaced with DMEM medium and total protein production evaluated after 7 and 14 days. The cells were washed with 1 ml PBS three times and 0.1% sodium lauryl sulfate (Sigma-Aldrich) was added for 40 min at room temperature for cell destruction. After performing homogenization, a 1 ml of aliquot was transported to 24-well plates and the blank received 1 ml of distilled water. Lowry reagent solution (1 ml) (Sigma-Aldrich) was added to all samples, and later mixed with 500 ml of Folin-Ciocalteu's phenol reagent solution (Sigma-Aldrich). Three 100 ml aliquots were transferred to a 96-well plate for each group after 30 min and the absorbance read at 655-nm in a Thermo Plate reader. The average of three values was used for the statistical analysis.

2.9. Alkaline phosphatase activity

The alkaline phosphatase (ALP) activity was also assessed 7 to 14 days after 24 h contact with the eluates ($n=5$) [34]. With an ELISA microplate reader (Thermo Plate), absorbance was read at a wavelength of 590 nm. The values were converted into IU/L by means of a standard curve with known concentrations of ALP. The absorbance obtained was converted into mg/l by a standard protein curve. The final value of ALP was expressed in IU ALP/mg.

2.10. Alizarin red staining for mineralized nodule formation

The deposition of mineralized nodules on dentine surface ($n=5$) following application of cavity disinfectant was eval-

uated using Alizarin Red staining after 7 and 14 days. The cell culture and dentine discs were washed with PBS twice and fixed with 70% ethanol for 1 h. The specimens were later washed three times with de-ionized water and stained for 20 min with 40 mmol/l Alizarin Red solution (Sigma-Aldrich) adjusted to a pH of 4.0 with ammonium hydroxide. The excess dye was later removed and the cells washed twice with deionized water and representative photographs were taken using light microscopy (LW 0.52 Nikon Japan, Eclipse). The cells were counted (0.28 cm²) in 24-well plates. The cells were then incubated with 10% cetylpyridinium chloride (Sigma-Aldrich) for 15 min to solubilize the nodules. The absorbance of the resulting solution formed for each group was calculated based on the mean value of the control group at 7 days as 100% staining.

2.11. Macrophage isolation and culture

Human peripheral blood mononuclear cell line, SC (CRL-9855 #LOT: 61834527) was obtained from ATCC. A concentration of 2×10^5 cells for one flask was maintained. The cells were counted and using Iscove's Modified Dulbecco's Media (IMDM; ATCC, Lot:63331110-Manassas/VA), 1×10^6 cells/mL were cultured supplemented with 10% FBS, 0.05 mM 2-mercaptoethanol, 0.1 mM hypoxanthine and 0.016 mM thymidine.

Day 0: Coverslips (Fisher Scientific™ Richard-Allan Scientific) were placed onto 6-well plates (Sigma-Aldrich) for generation of resting macrophages (M0). The SC CRL cells were cultured in 60 ng/ml phorbol myristate acetate (PMA; Sigma P1585-Lot # SLBQ7454 V) for at least 6 h. After 6 h (without removing existing well contents), lipopolysaccharide (LPS-PG Ultrapure/Porphyromonas gingivalis-TLR4 ligand; 0.1 μ g/ml) was placed over the macrophage cells added with interferon gamma (IFN γ ; 20 ng/ml), respectively for M1 polarization. In addition, interleukin 4 (IL-4 Peprotech Human IL-4-Catalog # 200-04/Lot# 011614-1; 20 ng/ml) was added for M2 polarization. All wells were then incubated for 66 h. Day 3: The cells were pretreated with CHX and QAS as follows: Group I: no treatment (control); Group II: 2% CHX; Group III: 2% QAS; Group IV: 5% QAS and Group V: 10% QAS for 2 min inside the medium. The IMDM medium was completely removed and the cells were washed with PBS. The cells were later fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. The cells were again washed using PBS and 0.1% Triton X-100 (X100 Sigma-Aldrich in PBS) was later added for 20 min inside all wells for ~ 30 min at room temperature. The Triton X was removed and the wells blocked with 2% Bovine Serum Albumin - BSA (P1585-1 mg in PBS) for ~ 20 min at room temperature.

2.11.1. Immunohistochemical staining

Immunofluorescence staining was performed on the samples with primary antibodies of CD68+, CD80+ (M1 profile), and CD163+ (M2 profile) respectively. After removing the solution, primary antibodies [CD80+ > 1:100 (Gene Tex CD80 7E4-Catalog# GTX84704), CD68+ > 1:50 (Gene Tex CD68 514H12-Catalog# GTX75390), CD163+ > 1:50 (Gene Tex CD80 EDUHu-1-Catalog# GTX42364)] were added to the appropriate wells and incubated at room temperature for 1 h. CD 80+ is a

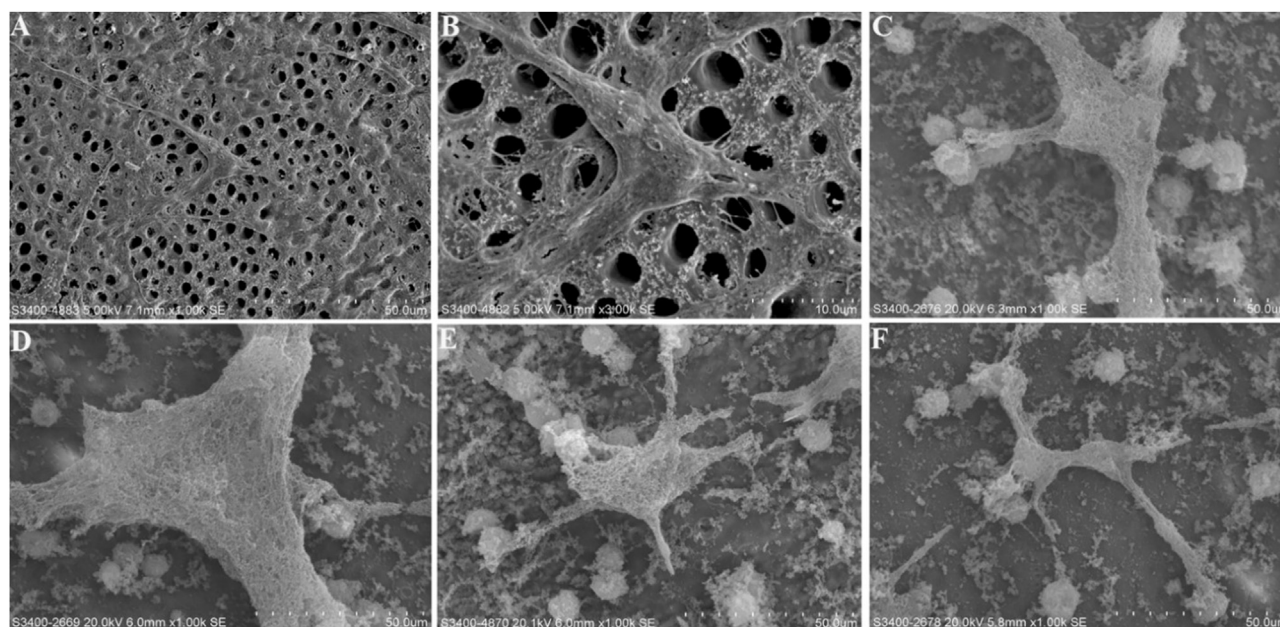


Fig. 1 – SEM images of NIH 3T3 cells seeded on the dentine substrate. (A) Control: Cells with large cytoplasm covering most of the dentine surface. (B) Control (higher magnification), cells appeared well attached with several cytoplasmic projections. (C) 2% CHX: Several small round-shaped cells with limited cytoplasmic projections remained adhered to dentine. (D) 2% QAS: Cells with large cytoplasm and projections similar to the control group were observed on dentine. (E) 5% QAS: Cells with different morphologies with limited projections are covering the dentine substrate. (F) 10% QAS: A small number of rounded-shaped cells remained attached to dentine.

surface marker indicative of an M1 phenotype and CD 163+ is a surface marker revealing the M2 phenotype. The wells were later washed with PBS and secondary antibodies (1:100) were added and incubated at room temperature for another 1 h. After washing with PBS, DAPI (Vectashield; Catalog #H-1200-Vector Laboratories/4', 6-diamidino-2-phenylindole) was added for analysis of blue fluorescence and coverslips were mounted onto the glass slides for CLSM (Fluoview FV 1000, Olympus, Tokyo, Japan) analysis, which was performed under dark, and macrophages were imaged on a confocal scanning light microscope using light emission between 500–550 nm with an excitation wavelength of 488 nm and 10, 20 and 40× objective lens for direct observation. The images were evaluated quantitatively by two independent examiners in a blinded fashion. The number of immune positive cells in six matched microscopic fields at 10× magnification was counted. The cell counts from negative controls (no primary antibody) were subtracted from the cell counts of the cells tested against QAS and CHX. The percentages of M1 and M2 were determined by dividing CD80+ (M1) and CD163+ (M2) by the number of CD68+ cells in each field tested [35]. An M1/M2 ratio was then calculated for each specimen as follows:

$$M1 : M2 = \text{percent M1 cells} / \text{percent M2 cells}$$

2.12. Statistical analysis

The Shapiro–Wilk test was employed to assess whether the data from each test (viable cell count, total protein production,

alkaline phosphatase activity, mineralized nodules deposition and percentage of M1 and M2) followed a normal distribution. Modified Levine test was performed to determine if the assumptions of equal variances was valid. After determining data normality and equality of variances, the data from the viable cell count was analyzed using one-way ANOVA, followed by Tukey's multiple comparison test. The total protein production, alkaline phosphatase activity, mineralized nodule deposition were analyzed using two-way ANOVA to examine the effect of disinfectants and time, and the interaction of the two factors on the respective parameters investigated. The percentage of M1 and M2 cells was determined by dividing CD80 (M1) and CD163 (M2) by the number of CD68 cells in each field tested. Values were analyzed using one-way ANOVA with Tukey's multiple comparison test. A significant level of $\alpha = 0.05$ was used.

3. Results

3.1. SEM evaluation of cell morphology

Representative SEM images of cells seeded on the dentine substrate after 24 h are shown in Fig. 1. No obvious differences in cell morphology was observed between the control (Fig. 1A) and the 2% QAS groups (Fig. 1D). The cells appeared mostly like fibroblasts, similar to the cells grown in the culture dish with dendritic cell extensions. The cells from the control and 2% QAS groups were flattened with large cytoplasm and were attached to the dentine by multiple small cytoplasmic projections (Fig. 1D). In contrast, the 2% CHX (Fig. 1C) and 5%

Table 1 – Mouse fibroblast cell line (NIH/3T3) viability in 24 h when in contact with 2% CHX and different concentrations of QAS

. Data are expressed as the mean $\pm$ SD. Lower case letters indicate significant differences between the experimental groups in the same period of time. A p value <0.05 was considered significant (Tukey's test).

Groups	Cell count	Viability reduction
Control	4.33 $\pm$ 0.47 a	–
2% CHX	1.92 $\pm$ 0.63 b	55.6%
2% QAS	3.00 $\pm$ 0.57 a	30.7%
5% QAS	2.00 $\pm$ 0.55 b	53.8%
10% QAS	1.13 $\pm$ 0.68 b	73.9%

Values are means $\pm$ standard deviation (n=5).

Groups with the same lower case letter are not significant (p < 0.0001).

QAS groups (Fig. 1E) showed irregular cells with few cytoplasmic processes. Some rounded-shaped cells with limited cytoplasmic projections were also observed. Conversely, the cells of 10% QAS group had lost the fibroblast-like morphology. A decreased number of round cells with poorly developed processes were seen, leaving large cell-free areas (Fig. 1F). The number of cells remained adhered to dentine surface decreased progressively with increasing concentration of QAS.

3.2. Cell viability

The highest reduction in cell viability was seen in the 10% QAS group (73.9%) (Table 1). The 2% QAS group (30.7%) showed significantly lower reduction in cell viability than 2% CHX (55.6%), 5% (53.8%) and 10% (73.9%) QAS groups (p < 0.0001), with no significant difference from the control group. No significant difference in cell viability was observed among the 2% CHX, 5% and 10% QAS groups. Cell viability decreased significantly with increasing concentration of QAS (p < 0.0001).

3.3. Total protein production

Total protein production by NIH 3T3 cells 7 and 14 days after application of cavity disinfectants are shown in Table 2. Results of two-way ANOVA showed that the factor “disinfectant” was significant (p = 0.001); while the factor “time” was not significant (p > 0.05). Interaction of the two factors was not

significant (p > 0.05). Total protein reduction was significant for both 2% CHX and all QAS groups after 7 days (p < 0.01); while it was significantly reduced in 2% CHX, 5% and 10% QAS groups after 14 days (p < 0.01). No significant difference in total protein reduction was found between 2% QAS and the control group after 14 days. Increasing the QAS concentration significantly reduced total protein concentration at 14 days (p < 0.01). No significant difference in total protein concentration was found in 2% CHX, 2% and 10% QAS groups between the two time points.

3.4. Alkaline phosphatase activity

Alkaline phosphatase activity (ALP) in NIH/3T3 cells 7 and 14 days after application of cavity disinfectants are shown in Table 2. Results of two-way ANOVA showed that both the factors “disinfectant” and “time” were significant (p < 0.001). Interaction of the two factors was also significant (p < 0.001). The ALP activity for all QAS groups was significantly lower than the control group at the 7th and 14th days (p < 0.05). However, the ALP activity for the QAS groups was higher than the 2% CHX group at 14 days. Increasing the QAS concentration had no effect on ALP activity at 14 days. No significant change in ALP activity was observed in all QAS groups between the two time points (p > 0.05).

3.5. Alizarin red staining for mineralized nodules

Deposition of mineralized nodules 7 and 14 days after application of cavity disinfectants are shown in Fig. 2 and 3. Results of two-way ANOVA showed that the factor “disinfectant” was significant (p < 0.001) and the factor “time” was not significant (p > 0.05). Interaction of the two factors was also not significant (p > 0.05). Similar to total protein production, mineralized nodule deposition was significantly reduced in both 2% CHX and all QAS groups after 7 days (p < 0.05); while it was significantly reduced in 2% CHX, 5% and 10% QAS groups after 14 days (p < 0.05). No significant difference in mineralized nodule deposition was found between 2% QAS and control group after 14 days. Increasing the QAS concentration significantly reduced total mineralized nodule deposition at 7 and 14 days (p < 0.05). No significant difference in total mineralized nodule deposition was found across all groups between the two time points (Fig. 3).

Table 2 – Total protein production (TLP) and alkaline phosphatase (ALP) by NIH 3T3 cells seven and fourteen days after application of cavity disinfectants on 0.4-mm-thick dentine discs. Data separated (black line) between TLP and ALP.

Specimens	Period after contact (TLP)		Period after contact (ALP)	
	7 days	14 days	7 days	14 days
Control	0.63 $\pm$ 0.100 A α	0.48 $\pm$ 0.025 a β	1.03 $\pm$ 0.163 A α	0.69 $\pm$ 0.027 a β
2% CHX	0.37 $\pm$ 0.069 C α	0.36 $\pm$ 0.052 c α	0.23 $\pm$ 0.042 C α	0.08 $\pm$ 0.012 c β
2% QAS	0.53 $\pm$ 0.128 B α	0.51 $\pm$ 0.024 a α	0.33 $\pm$ 0.050 C α	0.37 $\pm$ 0.040 b α
5% QAS	0.33 $\pm$ 0.090 C α	0.44 $\pm$ 0.024 b β	0.45 $\pm$ 0.029 B α	0.40 $\pm$ 0.012 b α
10% QAS	0.43 $\pm$ 0.108 B α	0.43 $\pm$ 0.017 b α	0.34 $\pm$ 0.055 C α	0.32 $\pm$ 0.029 b α

Values are mean $\pm$ standard deviation (n=5).

In the columns, groups identified by the same upper/lower case letter are not statistically different (p > 0.05).

In the rows, groups identified by the same symbol are not statistically different (p < 0.003). Groups identified by the same symbol are not statistically different (p < 0.001) in ALP.

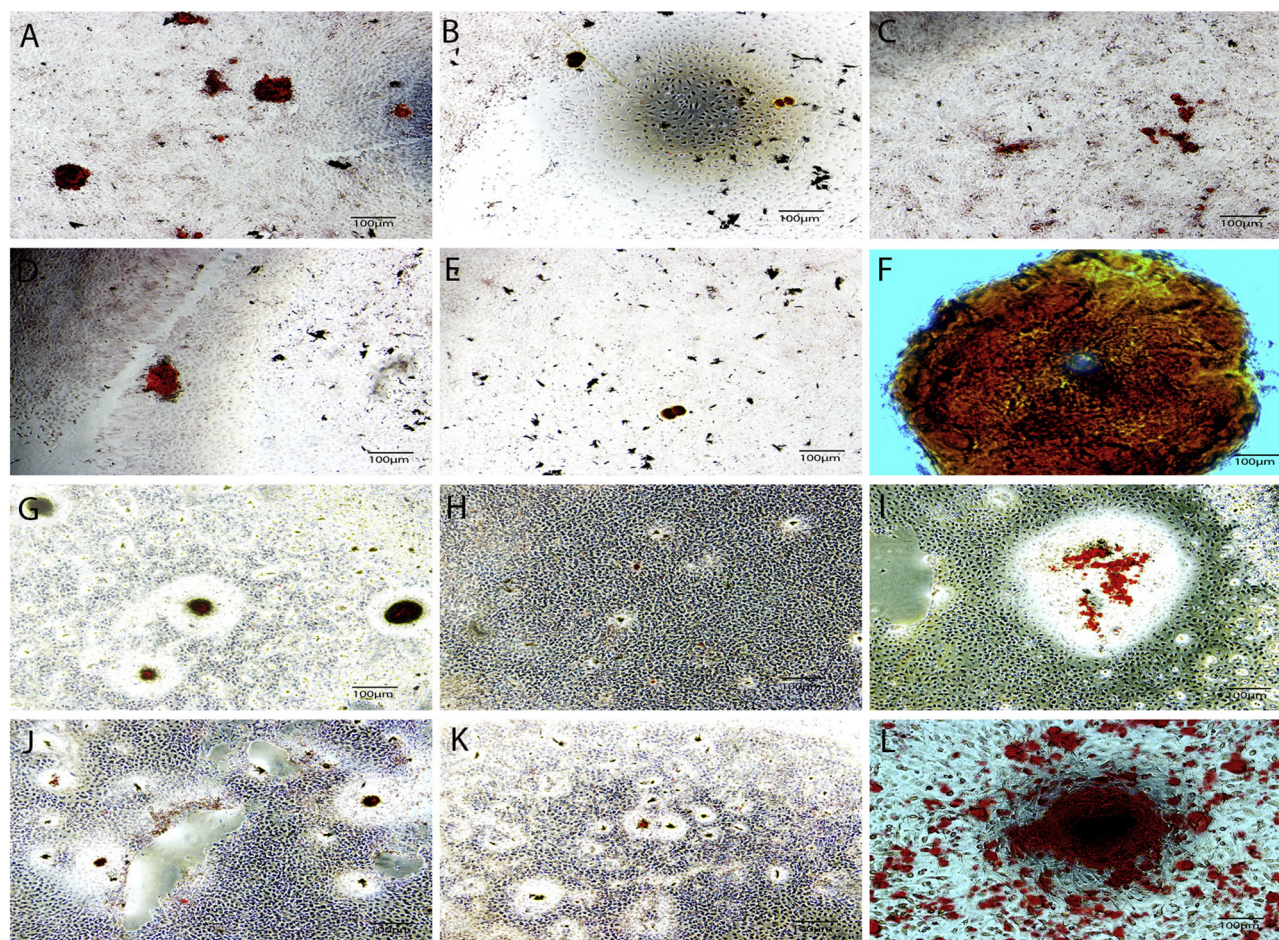


Fig. 2 – Light micrographs of mineralized nodule 7 (A–F) and 14 (G–L) days after application of different cavity disinfectants on 0.4-mm-thick dentine discs. Control: NIH 3T3 cells revealed calcium deposits and stained positively with Alizarin Red S after 7 (A) and 14 days (G); 2% CHX: Treated NIH 3T3 cells revealed lesser staining than control group after 7 (B) and 14 days (H); 2% QAS: Similar staining as control group was observed after 7 (C) and 14 days (I); 5% (D & J) and 10% (E & K) QAS groups also showed much less staining than the control group. Positive dark staining shows presence of calcium deposits i.e. mineralization. Positive dark red staining shows presence of calcium deposits i.e. mineralization in 2% QAS group after 7 (F) and 14 (L) days.

3.6. Immunohistochemical findings

The M1/M2 macrophage ratios after application of cavity disinfectants showed a distribution of macrophages to analyze for the different subtypes of macrophages are shown in Fig. 4. Results of one-way ANOVA showed that M1/M2 ratio was significantly reduced in the 2% and 5% QAS groups, when compared to control, 2% CHX and 10% QAS groups ($p < 0.05$). Values above 1.0 are indicative of an M1 type response (pro-inflammatory) while values less than 1.0 are considered an M2 type response (anti-inflammatory). Confocal laser scanning microscopy (CLSM) images of infiltrating CD163+ and CD80+ are shown in Fig. 4 B–F. The mononuclear macrophages CD68+ (without induction) were present in all tested cells throughout the study period according to confocal analysis. The qualitative analysis of the phenotype of the macrophages following application of the cavity disinfectants was prominently CD163+ in both the control (Fig. 4B) and 2% QAS groups, showing detection of M2 markers (Fig. 4D). Conversely, the

CD80+ markers were mainly found in the 2% CHX (Fig. 4C), 5% QAS (Fig. 4E) and 10% (Fig. 4F) QAS groups. The CD80+ and CD163+ markers distinguish between the two different types of macrophages. With double immunofluorescent staining and confocal analysis, these markers to a large extent separate the two different cellular populations.

4. Discussion

With current minimally invasive caries excavation strategies, residual bacteria may exist in prepared tooth cavity as a result of incomplete excavation. These bacteria may survive and proliferate under adhesives restorations, leading to pulpal inflammation, secondary caries and ultimate failure of the bonded restorations. The use of cavity disinfectant will help to eliminate or reduce bacteria under such restorations, increasing the long-term restorative success. Preservation of pulpal vitality is a major challenge in restorative dentistry. The

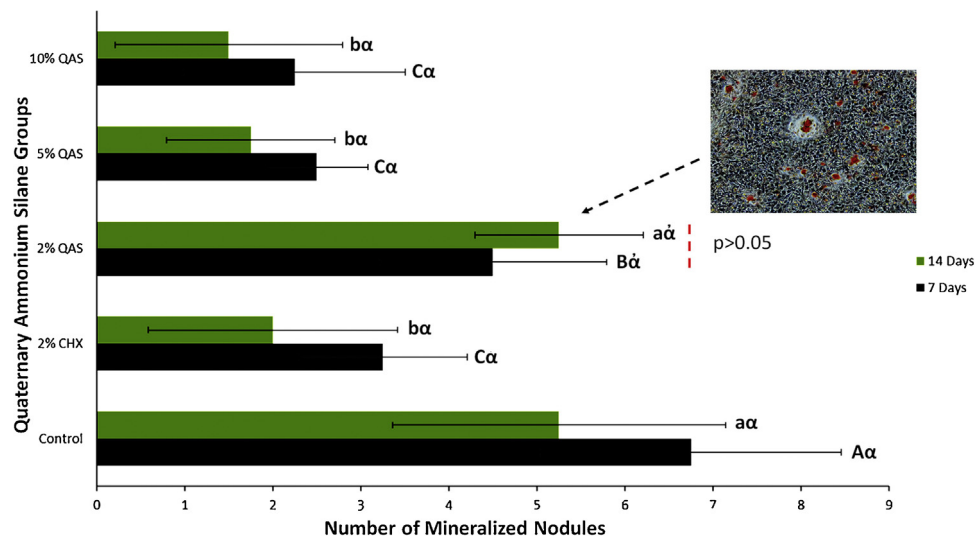


Fig. 3 – Mineralized nodule production by 3T3 NIH Mouse fibroblast cells seven and fourteen days after application of chemical disinfectants on 0.4-mm-thick dentine discs. Values are mean $\pm$ standard deviation ($n = 5$). Groups identified by the same upper/lower case letter are not statistically different ($p > 0.05$).

dentine-pulp complex needs to be protected to avoid additional pulpal challenge caused by operative procedures as a result of the toxicity of restorative materials [36]. Hence, an ideal cavity disinfectant should also be assessed for cytotoxicity and biocompatibility.

Although CHX have been studied extensively as a cavity disinfectant for its antimicrobial activity [37,38] and as a protease inhibitor for preservation of the resin-dentine

hybrid layer [10,39], there are concerns for its weak interaction with the collagen fibrils. In addition, chlorhexidine has been reported to have a high direct cytotoxic effect to cultured MDPC-23 cells after a 2-h exposure time, causing direct damage and inhibiting metabolism. The cell morphology was altered with more rounded and small-sized cells with increasing CHX concentration and exposure times. There were large cell-free areas correlating to the toxic effects of MPDC-23 cells

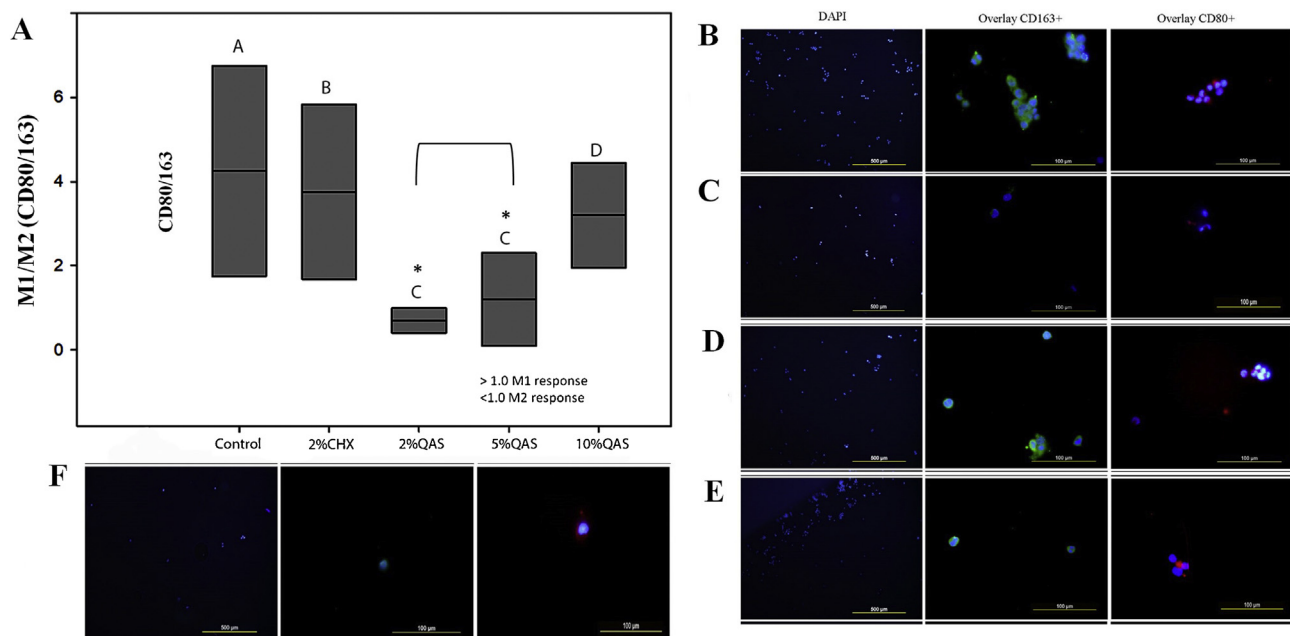


Fig. 4 – (A) Ratio of the percentage of M1/M2 macrophages ratio after application of different groups. Values above 1.0 are indicative of an M1 type response while values less than 1.0 are indicative of an M2 type response. CD80+ and CD 163+ distinguish between macrophage phenotypes. Confocal images of immuno fluorescence staining in (B) Control, (C) 2% CHX, (D) 2% QAS, (E) 5% QAS, and (F) 10% QAS specimens of Dapi (4',6-diamidino-2-phenylindoleblue), CD163+ (green), and CD80+ (red). Nuclei are clearly revealed by DAPI staining (blue), and overlay is fluorescence collected by all channels.

[14]. Therefore, it seems liable to assume that the use of CHX should not be recommended in cavities with pulp exposure because of its cytotoxic effects even after being rinsed off from the tooth surface.

Our previous work demonstrated that QAS is a good inhibitor of MMP and cathepsin K activities in demineralized dentine [40]. QAS also possesses antimicrobial activities and inhibits the growth of cariogenic biofilms. In addition to the protease inhibitory and antimicrobial effects, QAS when used as a cavity cleanser should also show low or preferably no toxic effects on pulp cells. *In vitro* cytotoxicity is widely used in the preliminary screening of biomaterials. In the current study, the cytotoxicity of different concentrations of QAS was evaluated. The transdental diffusion model, using very thin dentine discs (0.4-mm thickness) was adopted to simulate a clinical challenging situation to the dental pulp in extremely deep cavities. This model has been used widely to assess the cytotoxicity of different dental materials [32,41,42]. Cavity disinfectants were applied on the occlusal side of the dentine discs seeded with NIH 3T3 mouse fibroblast cells on the pulpal side. Fibroblasts are key in wound healing and are used as an established model for biocompatibility and potential cytotoxicity [43].

The present study demonstrated that cell morphology (Fig. 1) and viability of NIH 3T3 cells in the 2% QAS group were similar to the control group after application, showing that 2% QAS did not exert cytotoxic effects on NIH 3T3 cells. Though the alkaline phosphatase production was slightly reduced in the 2% QAS group compared to the control group, no detrimental effect on total protein and mineralized nodule productions was observed in the 2% QAS group after 14 days. The alkaline phosphatase is a membrane-bound ectoenzyme, highly expressed in mineralized tissue cells and is responsible for dentine matrix mineralization [44]. The normal deposition of mineralized nodules indicates that the dentine pulp reparative processes, which are essential tertiary dentine formation, were minimally affected following 2% QAS application, rendering it a safe cavity disinfectant for use in association with a dentine bonding agent.

By contrast, the 2% CHX, 5% QAS and 10% QAS groups showed alteration in cell morphology with more small rounded cells having limited cytoplasmic processes and a significant reduction in cell numbers, indicating that they are more cytotoxic to the NIH 3T3 cells. Also a direct correlation between QAS concentration and viability of NIH 3T3 cells was observed. The 2% QAS concentration has no cytotoxic effect on mouse fibroblasts as compared to 2% CHX. Similar results have been reported in studies that had used crosslinked quaternary ammonium polymers against murine 3T3 cells supporting normal cell adhesion and growth [45]. Furthermore, a reduction in total protein production, alkaline phosphatase activity and mineral nodule deposition was observed 14 days after these cavity disinfectant treatments. The change in cell morphology and metabolism observed in the present study could be attributed to the transdental diffusion of the cavity disinfectants. As 2% CHX, 5% and 10% QAS are more toxic to NIH 3T3 cells than 2% QAS, hence, the first null hypothesis that there is no difference in cytotoxicity among different concentrations of QAS and CHX has to be rejected.

Cytotoxic analysis to anaerobic bacterial cells and human cells should always be addressed when evaluating effectiveness of cavity disinfectants for future clinical applications. Many facultatively and obligately anaerobic bacteria dominate the microbial community of dental caries leading to lactic acid production via fermentation of carbohydrates [46,47]. Hence, the analysis of cytotoxicity against anaerobic cells is imperative. The cell viability of 2% QAS (3.0×10^4) is of particular interest as the *in vitro* cytotoxicity test results demonstrated reasonably high cell viability for this novel cavity disinfectant. The quaternary ammonium compounds can cause bacterial cell lysis by binding to cell membranes of bacteria and enabling cytoplasmic leakage [48]. When the positively charged (N^+) cationic sites of QAS contacts the negatively charged bacteria, there is a disturbance in the bacterial cell membrane and the bacteria undergoes explosion under its own osmotic pressure [49]. The fact that 2% QAS was lethal to bacteria but were not toxic to NIH 3T3 cells was surprising. The complete lack of effect of the 2% QAS on viable cell count revealed differences in bacterial and NIH 3T3 cell culture conditions. Bacterial culture studies were carried out in nutrient deficient conditions (ie PBS); while NIH 3T3 cells were grown in a complete cell culture medium. It was possible that proteins and other nutrients from the cell culture medium adsorbed onto the dentine surface almost immediately after contact and in this way masked the cytotoxic effects of 2% QAS on NIH 3T3 cells [50]. Further studies are needed to evaluate the cytotoxicity of 2% QAS on human dental pulpal cells.

The present study examined the effects of QAS on the macrophage phenotype, which can be identified and differentiated according to the cell surface markers, cytokine and gene expression markers [51–53]. M1 and M2 macrophages are involved in early or late inflammatory and tissue remodeling phases respectively [54]. CD 80+ identifies M1-activated cells and CD163+ show high expression in M2-activated macrophages. Any imbalances in M1/M2 ratios can lead to pathological changes [55], whereas changing the M1/M2 ratio through systemic infusion of polarized M1 and M2 macrophages can increase or decrease chronic inflammatory conditions [56]. The results of this study indicated the application of 2% QAS shifts the macrophage polarization profile to a predominantly M2 anti-inflammatory phenotype, facilitating tissue repair and regeneration. Hence, the second null hypothesis that there is no difference in M1/M2 profile of macrophages among different concentrations of QAS and CHX has to be rejected. These findings suggest that regulating the ratio of M1 to M2 may provide a novel therapeutic strategy.

The sol-gel synthesis provides a facile method for synthesizing organosilicates under mild conditions. The use of a tetrafunctional organosilicates as the anchoring unit for the antimicrobial trialkoxysilane molecules enables a three-dimensional network to be formed once condensation is brought to completion. Although deep dentine of vital teeth is a highly permeable membrane, condensation of QAS within the dentinal tubules may reduce dentine permeability and minimize the transudation of QAS into the dental pulp. The results of the current study demonstrated the cytotoxic effects of 2% CHX, 5% QAS and 10% QAS, therefore they are not recommended to be used as a cavity disinfectant in deep cavities

approximating pulpal tissue. However, the *in vitro* cytotoxicity results should be interpreted with caution and should not be directly extrapolated to the clinical situation. In vital teeth, the presence of cytoplasmic elongations of odontoblasts within the dentinal tubules and the exudation of dentinal fluid may reduce the inward transdentinal diffusion of toxic agents towards the dental pulp. However, this assumption should be tested *in vivo* before a final conclusion can be reached.

5. Conclusion

The 2% QAS cavity disinfectant does not have transdentinal cytotoxic effects on 3T3 NIH mouse fibroblast cells and the predominance of the anti-inflammatory phenotype after its application may stimulate healing and tissue repair.

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