Characterization of silver diamine fluoride cytotoxicity using microfluidic tooth-on-a-chip and gingival equivalents

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ABSTRACT

Objective: This study aims to characterize the cytotoxicity potential of silver diamine fluoride (SDF) on dental pulp stem cells (DPSC) and gingival equivalents.

Methods: DPSC cultured on 96-well plates was exposed directly to SDF (0.0001–0.01%) and cell viability (IC_{50}) quantified. Effect of SDF on DPSC viability under flow (with dentin barrier) conditions was evaluated using a custom-designed microfluidic "tooth-on-a-chip". Permeability of dentin discs (0.5–1.5 mm thickness) was evaluated using lucifer yellow permeation assay. Dentin discs were treated with 38% SDF (up to 3 h), and cell viability (live/dead assay) of the DPSC cultured in the inlet (unexposed) and outlet (exposed) regions of the pulp channel was evaluated. To assess the mucosal corrosion potential, gingival equivalents were treated with 38% SDF for 3 or 60 min (OECD test guideline 431) and characterized by MTT assay and histomorphometric analysis.

Results: DPSC exposed directly to SDF showed a dose-dependent reduction in cell viability (IC_{50}: 0.001%). Inlet channels (internal control) of the tooth-on-a-chip exposed to PBS and SDF-exposed dentin discs showed > 85% DPSC viability. In contrast, the outlet channels of SDF-exposed dentin discs showed a decreased viability of < 31% and 0% (1.5 and ≤1.0 mm thick dentin disc, respectively) ($p < 0.01$). The gingiva equivalents treated with SDF for 3 and 60 min demonstrated decreased epithelial integrity, loss of intercellular cohesion and corneal layer detachment with significant reduction in intact epithelial thickness ($p < 0.05$).

Significance: SDF penetrated the dentin (≤1 mm thick) inducing significant death of the pulp cells. SDF also disrupted gingival epithelial integrity resulting in mucosal corrosion.

Keywords: Tooth-on-a-chip, Microfluidics, 3D culture, Silver diamine fluoride, Biocompatibility, Dental pulp stem cells, Gingiva

Abbreviations: SDF, Silver Diamine Fluoride; DPSC, Dental Pulp Stem Cells; DMEM, Dulbecco's Modified Eagle Medium; EDTA, Ethylene Diamine Tetraacetic Acid; PMMA, Poly(methyl methacrylate); OECD, Organisation for Economic Co-operation and Development; PBS, Phosphate Buffered Solution; IC_{50}, Half-maximal Inhibitory Concentration

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1. INTRODUCTION

In recent years, silver diamine fluoride (SDF) has been increasingly promoted as a biological method of managing dental decay in children and patients with special needs for its ability to arrest over 80% of active lesions [1], [2]. A well-documented side effect of SDF is staining of the carious tooth structure after its application. However, this has not greatly impacted the acceptability of SDF especially in the non-esthetic zone [3]. Other lesser-known potential complications include the painful ulcerative lesions on the mucosa, gingival swelling and gingival bleaching if SDF is accidentally applied onto the oral mucosal tissue [4] and the induction of mild or chronic inflammatory response in the pulp tissues in teeth with deep carious lesions [5], [6]. While the mucosal injury from accidental SDF application is acute and self-resolving, the effect on the pulp is less predictable especially in an already inflamed pulp of a carious tooth. Moreover, SDF has also been shown to be able to penetrate deeply into dentin. *Ex vivo* studies have demonstrated the detection of silver precipitates deeper than 2 mm in dentinal tubules of artificially demineralized dentin [7] and deciduous teeth [8].

SDF is a alkaline (pH 9–10) solution with high fluoride concentration, silver and ammonium ions [9], [10]. It exerts its anti-microbial and remineralizing properties through the action of the fluoride and silver ions [10], [11]. However, silver ions can be toxic to human mesenchymal stem cells at concentrations as low as 0.5 ppm [12] and fluoride ions can be toxic to human pulp cells at 200 ppm [13]. Additionally, the high pH of SDF may result in damage to cells on direct contact and reduce the proliferation of pulp cells [14]. In in vitro studies, SDF was found to be 100% cytotoxic to human gingival fibroblasts at 0.01% concentration [15] and severely affected viability, mineralization ability and morphology of rat pulp cells at 0.0038% [16]. These concentrations are far lower than the 38% concentration that is used in the clinical setting. *In vivo* animal studies have similarly found that direct SDF application on pulp tissues resulted in necrosis in almost 100% of the time [17]. In a

recent systematic review, the authors concluded that direct application of SDF to pulpal tissue resulted in necrosis, while indirect (on dentin) application was generally biocompatible [18]. However, this conclusion was based on 30 teeth examined over 3 studies without controlling for dentin thickness. Despite the important contributions of these studies, more studies are needed to evaluate how the presence of a dentin barrier of different thicknesses would influence SDF's toxicity on pulpal cells as this more closely simulates the effect of SDF on the pulp tissues in the clinical setting.

Recently, microfluidic organ-on-a-chip devices have been developed to enable the culture of cells under dynamic flow conditions [19], simulating physiological blood flow and the pulp-dentin interface [20], [21], [22]. Advances in microfluidic devices also enable the assessment of compound and biomaterial permeation through a barrier tissue under dynamic flow conditions [19], [23]. The dentin barrier test has been used to examine the toxicity of dental materials with respect to dentin penetration and effects on dental pulp cells [22], [24], [25]. This method closely replicates the clinical situations where SDF is used and provides a more accurate reflection of what happens in clinical settings. Hence, organ-on-a-chip devices like tooth-on-a-chip can be designed to mimic the pulp-dentin-biomaterial interface and used to assess the permeation of SDF through the dentin to investigate the impact of the dentin barrier and the permeated SDF on the cells that are cultured beneath [26].

This study aimed to evaluate the effects of a commercially available 38% SDF on human dental pulp and gingival cells using a microfluidic tooth-on-a-chip device and full-thickness human gingival equivalents, respectively.

2. MATERIALS AND METHODS

2.1. Cell culture

Human dental pulp stem cells (DPSC, passage 4–6; Allcells, USA), primary gingival fibroblasts (passage 5–7), and immortalized human oral keratinocytes (OKF6/TERT1, passage 35–45) were cultured in Dulbecco's Modified Eagle Medium (DMEM) or keratinocyte serum-free medium (all consumables from Invitrogen, USA) as previously described [27], [28], [29].

2.2. Cytotoxicity and IC50 estimation

The DPSC were seeded in 96-well plates (5×10^4/well) and incubated at 37 °C for 24 h. Thereafter, the cells were treated with 150 µL of commercially available 38% SDF (Advantage Arrest, Elevate Oralcare, USA) diluted in basal growth media at concentrations ranging from 0.0001% to 0.01%. Subsequently, cells were subjected to MTS assay (CellTiter96 Aqueous One Solution, Promega, USA). Cell viability and half-maximal inhibitory concentration (IC_{50}) were calculated using the absorbance at 490 nm from SDF-treated and control (basal growth media) samples. All groups had four biological samples and the experiments were performed in independent triplicates.

2.3. Tooth slice (dentin disc) preparation

The use of teeth was approved by the NHG Domain Specific Review Board (DSRB ref: 2020/00147). Extracted permanent molar teeth (free from caries and visible dentin defects, subjects <40 years old) were used for the fabrication of the tooth-on-a-chip device. The teeth were disinfected in 1% chloramine-T solution (Sigma-Aldrich, USA) and stored in distilled water at 4 °C till use. Dentin slices (0.5, 1.0, and 1.5 mm thickness) adjacent to the roof of the pulp chamber were cut using precision sectioning saw (IsoMet 1000, Buehler, USA). The dentin discs were treated with 10% ethylene diamine tetraacetic acid (EDTA, Sigma-Aldrich, USA) for 1 min to remove smear layer, washed with phosphate-buffered saline (PBS, Sigma-

Aldrich, USA) under ultrasonic bath for 5 min and stored at 4 °C and 100% relative humidity till use.

2.4. Design and fabrication of microfluidic tooth-on-a-chip

The microfluidic tooth-on-a-chip device was designed using a computer-aided design software (Autodesk Inventor, Autodesk, USA, Fig. 1). The device was fabricated by thermally bonding four microstructured poly(methyl methacrylate) (PMMA, Whits Technologies, Singapore) sheets in vacuum oven (Eyela, USA). The chambers and microchannels within each PMMA sheet were microfabricated by computer numerically controlled (CNC) micromilling.

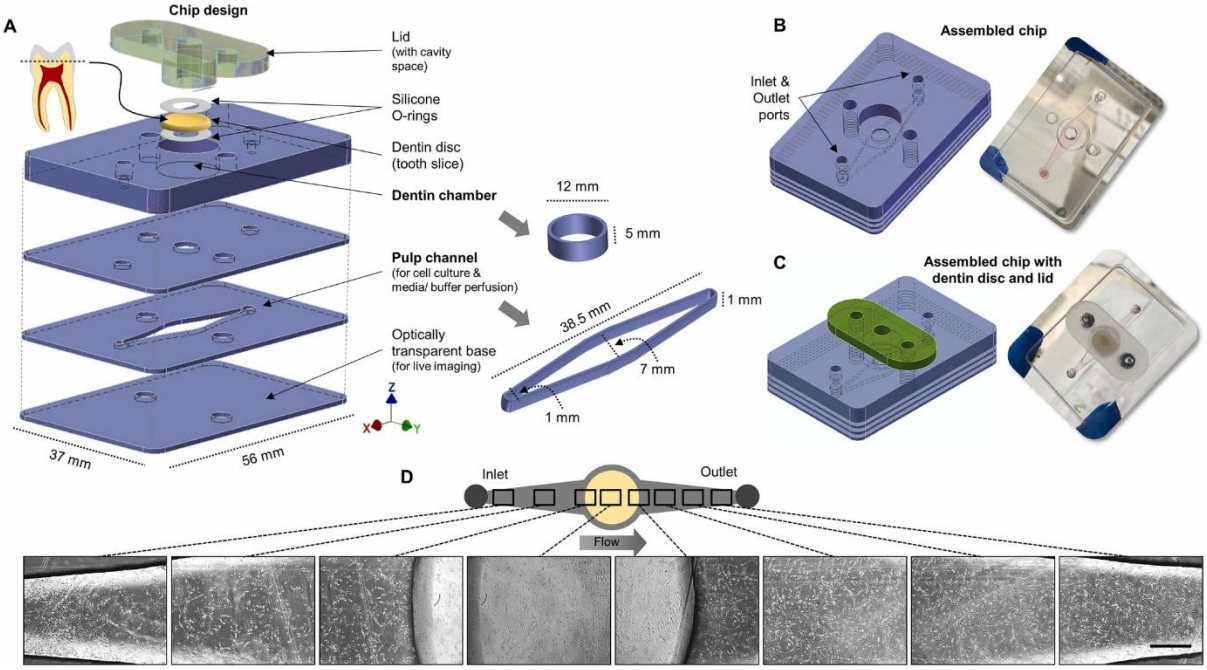

Fig. 1. Schematic illustration showing the features of the microfluidic tooth-on-a-chip device. The exploded view (**A**) and the assembled device (**B**, **C**) show the different features of the device that includes an optically transparent base, pulp channels, dentin chamber, removable lid and ports for inlet and outlet. (**D**) Sequence of phase-contrast images show the ability to visualize the cells and the even distribution of the DPSC cultured across the pulp channel and under peristaltic flow conditions. Scale bar: 1 mm.

Briefly, the device has a central cylindrical chamber (dentin chamber) to house the dentin disc. The dentin disc is clamped using silicone O-rings and a cylindrical hollow body removable lid. The hollow ("cavity space" 4 mm diameter) in the lid serves as the reservoir for

loading of test substances. Beneath the central chamber is a rhomboid-shaped perfusable microchannel ("pulp channel") with circular openings at either end (inlet and outlet ports) for cell seeding and perfusion of reagents using tubings connected to an Ismatec® peristaltic pump (Cole-Parmer GmbH, Germany). The microfluidic device was sterilized using low-energy X-ray radiation as described previously [19].

2.5. Assessment of permeability of dentin discs on-chip

The permeability of dentin discs of different thicknesses was quantified using the tooth-on-a-chip device. To this end, the dentin discs (n = 5 for each thickness) were clamped between two silicone O-rings placed within the central chamber of the tooth-on-a-chip device and fastened with the lid. Afterwards, 100 µL of lucifer yellow (1 mg/mL, Sigma-Aldrich, USA) was added on top of the dentin disc and allowed to permeate for 3 h. The pulp channel of the device was perfused with PBS (8 µL/min), and the perfusate was collected every 30 min in 96-well plates.

The amount of lucifer yellow in the perfusates was measured at 560 nm excitation and 590 nm emission using a fluorometer (Fluoroskan FL, Thermo Scientific, USA). The concentration of lucifer yellow was quantified using calibration plots from 11 serial dilutions over a range of 0.195 – 20 µg/mL (R^2 = 0.9998). The cumulative permeation per unit area of dentin disc was calculated using calibration plots from 11 serial dilutions of lucifer yellow over a range of 0.195–20 µg/mL (R^2 = 0.9998).

2.6. SDF treatment and on-chip assessment of DPSC viability

DPSC were seeded (1×10^5 cells/cm^2) on the pulp channel through the inlet and left undisturbed overnight (Fig. 1***D***). Dentin discs were sandwiched between two silicone O-rings, placed within the microchamber and clamped using the lid (n = 3 for each thickness). Culture medium was perfused through the pulp channel at a flow rate of 1.5 µL/min. Thereafter, 50 µL of SDF (38%) was added directly on top of the dentin disc through the cavity space in the lid, and allowed to

permeate for 3 h. Afterward, the flow of basal culture medium was replaced with medium containing 1 μg/mL calcein-AM (Thermo Fisher Scientific, USA) and 1 μg/mL propidium iodide (Thermo Fisher Scientific, USA) to stain the live and dead cells, respectively. After 15 min, the cells were gently washed with PBS and culture medium. The cells were imaged using confocal laser scanning microscope (Olympus FV1000, Olympus Corporation, Japan) at six regions of interest along the pulp channel, and percentage of live and dead cells were quantified (Image J, NIH, USA). The assays were performed at least three times for each dentin thickness, and discs were discarded after being used once.

2.7. Fabrication of full-thickness gingival equivalents

Full-thickness gingival equivalents were fabricated using gingival fibroblasts, and fibrin-based mucosal matrix [29]. Briefly, 3.5×10^5 oral keratinocytes were seeded on top of a 4-day-old oral fibroblast-populated mucosal matrix and cultured within culture insert (ThinCert™, Greiner Bio-one, Austria) under submerged conditions for 2 days, followed by the culture at air-liquid interface for an additional 14 days.

2.8. Assessment of in vitro mucosal corrosion

In vitro mucosal corrosion test was performed following Organisation for Economic Co-operation and Development (OECD) test guideline 431 [30]. Briefly, the top epithelial surface of the full-thickness gingival equivalents was exposed to 38% SDF for 3 min (room temperature) or 60 min (37 °C). Following the exposure period, the gingival equivalents were washed thoroughly with PBS. The negative controls were gingival equivalents exposed to PBS and eugenol (Pulp Canal Sealer™ liquid containing 60–90% eugenol in solution, Kerr Co, USA), and the positive control was 37% phosphoric acid solution (prepared from 85 wt% solution in water, Sigma-Aldrich, USA). Three independent gingival equivalents were used for both histological analysis and MTT cell viability assay.

2.9. MTT tissue viability assay

After exposure to the test substances from Section 2.8, the gingival equivalents were washed with PBS, transferred to a new plate with 300 µL of MTT solution (Promega, USA) and incubated for 3 h at 37 °C. The purple formazan product was extracted using 2 mL of isopropanol overnight in the dark at room temperature. Subsequently, 150 µL of the extract was transferred to a 96-well microplate, and absorbance values were measured at 570 nm using a microplate reader (Multiskan GO). Tissue viability was calculated as a percentage of the negative control. All data were expressed as mean ± standard deviation (SD) of three independent biological tissues. The mucosal corrosion potential of the applied substances was classified based on the prediction model recommended in OECD test guideline 431 (Table 1, [30]).

Table 1. Prediction model for in vitro mucosal corrosion test based on OECD test guideline 431.

Viability measured after 3 and 60 min	**Classification prediction**
Step-1	
• < 50% after 3 min of exposure	Corrosive
• ≥ 50% after 3 min of exposure AND < 15% after 60 min of exposure	Corrosive
• ≥ 50% after 3 min of exposure AND ≥ 15% after 60 min of exposure	Non-corrosive
Step-2 for substances/mixtures identified as Corrosive in step-1	
• < 18% after 3 min of exposure	Optional Sub-category 1A
• ≥ 18% after 3 min of exposure	Optional Sub-category 1B and 1C

2.10. Histology and analysis of epithelial thickness

Gingival equivalents exposed to the test substances were fixed in 10% neutral buffered formalin (Sigma-Aldrich, USA), processed for haematoxylin and eosin staining and images captured using brightfield microscope (Eclipse E600, Nikon, Japan). Thickness of the intact epithelium was quantified using a line tool (NIS-Elements, Nikon, Japan) at five random points in each image, and expressed as the mean± SD of three independent biological tissues.

2.11. Statistical analysis

All data were represented as mean ± SD of three or more independent biological replicates. Shapiro–Wilk and Lavene's tests were performed for checking normality and homogeneity. One-way ANOVA followed by Bonferroni-Holm post-hoc analysis was used to compare the results among three or more groups (GraphPad Prism, USA). A pre-set significance level of 5% was considered for all tests.

3. RESULTS

3.1. Effect of SDF on DPSC viability under static and direct exposure conditions

DPSC exposed directly (without a dentin barrier) to media containing SDF (0.0001–0.01%) under static culture conditions showed a dose-dependent reduction in cell viability (Fig. 2). The cellular viability was less than 50% for concentrations above 0.001% (IC_{50} = 0.001%; 95% confidence intervals: 0.0009 – 0.0014).

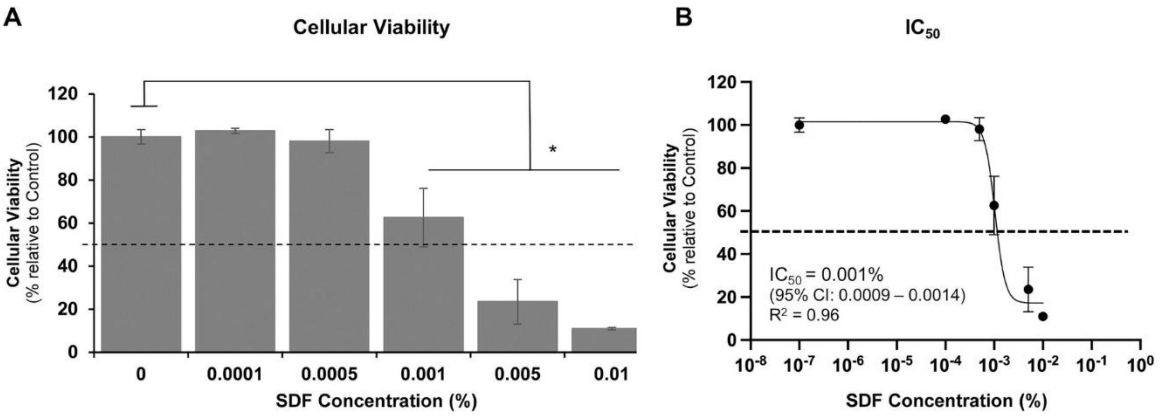

Fig. 2. Cellular viability of DPSC upon exposure to SDF. (**A**) Quantification of relative viability of DPSC exposed to SDF using MTS assay. (**B**) Computation of IC_{50} of SDF. Data presented as mean ± SD, n = 4, *p < 0.05.

3.2. Effect of SDF on DPSC cultured under flow and dentin barrier conditions

A microfluidic tooth-on-a-chip device fitted with dentin discs of varying thickness and peristaltic media flow conditions was fabricated to mimic the microphysiological conditions of

dentin barrier (Fig. 1). Permeation kinetics of lucifer yellow across the dentin barrier under peristaltic flow conditions is presented in Fig. 3. Dentin discs of 0.5 mm thick exhibited significantly higher cumulative permeation of lucifer yellow over 3 h compared to dentin discs of 1 mm and 1.5 mm thick ($p < 0.05$). Dentin discs of 1.5 mm thickness displayed the least permeation. Correlation analysis showed a linear and inverse relationship between dentin disc thickness and lucifer yellow permeability ($R^2 = 0.89$).

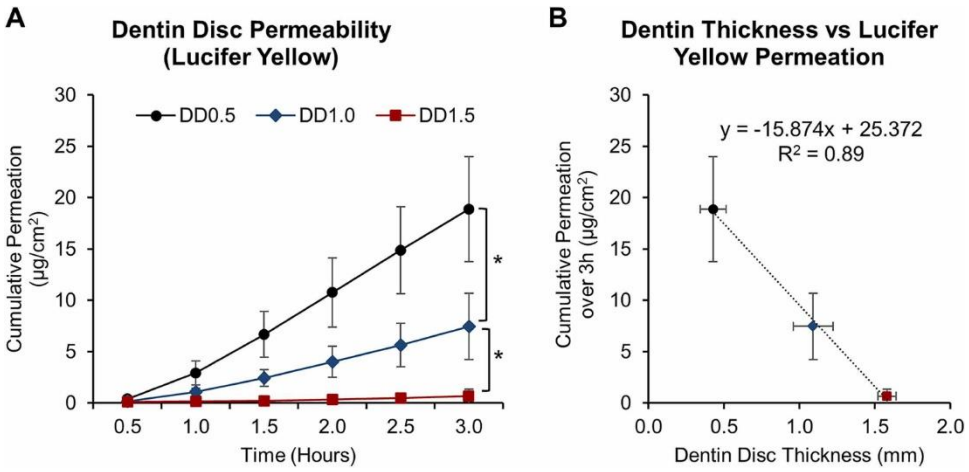

Fig. 3. Assessment of permeability of dentin discs using tooth-on-a-chip device. (**A**) Line graphs show the kinetics of permeation of lucifer yellow across dentin discs of different thickness (0.5, 1 and 1.5 mm) over 3 h period. (**B**) Graph representing the correlation between dentin disc thickness and cumulative permeation of lucifer yellow at the end of 3 h period. Data presented as mean ± SD, n = 5, *$p < 0.05$.

Next, we evaluated the impact of SDF permeability across the dentin barrier on DPSC cultured under peristaltic flow conditions. Phase-contrast images of the chip shows the presence of DPSC that were evenly seeded throughout the length of the pulp channel (Fig. 1***D***). Owing to the unilateral flow within the tooth-on-a-chip device, the viability status of cells in the inlet side of the pulp channel (unexposed to SDF) was used as an internal control for comparison against the viability of cells in the outlet side of the pulp channel (exposed to SDF) (Fig. 4***A,B***). Further, dentin discs exposed to PBS were used as the negative control. Fig. 4***C*** shows the sequence of on-chip fluorescent images (live/dead stained) captured on the inlet and outlet sides of the pulp channel. The inlet and outlet channels of chips with PBS-

exposed dentin discs (negative control) exhibited> 80% live cells. There was no significant difference in live and dead cells between the inlet and outlet channels (Fig. 4***E,F***). For SDF-exposed dentin discs of 0.5 mm and 1.0 mm thickness, there were no live cells (100% cell death) in the outlet channel compared to > 90% viability in the inlet channel ($p < 0.01$). In contrast, the 1.5 mm dentin discs exposed to SDF showed 30.6% (± 8.6) cell viability in the outlet channels ($p < 0.01$). However, the viable cells had a rounded morphology, compared to the elongated spindle-shaped morphology of the DPSC in the inlet channel or that of the control (PBS-exposed dentin discs). Further, on-chip phase-contrast microscopy showed the presence of cellular aggregation and rounded cells with pyknotic nuclei (Fig. 4***D***).

3.3. Effect of SDF on gingival epithelium

The histology of the gingival equivalents following 3 and 60 min exposure to negative controls (PBS and eugenol), positive control (37% phosphoric acid) and SDF is presented in Fig. 5***A***. Gingival equivalents treated with PBS and eugenol were intact, with minimal disruption of the corneal layers. In contrast, the tissues treated with phosphoric acid and SDF exhibited signs of epithelial barrier disruption. Qualitative analysis of epithelial integrity of the tissues exposed to SDF for 3 min, showed detachment of the corneal layer characterized by the wavy appearance and spaces (Fig. 5***A***, Table 2). Furthermore, the tissues exhibited a loss of intercellular cohesion in the basal layers of the epithelium. For the tissues that were exposed to SDF for 60 min, the effects were more substantial with desquamation of more layers and loss of intercellular adhesion in the basal and suprabasal compartments. Further, quantitative analysis of the thickness of intact epithelium showed a significant reduction in intact epithelial thickness ($p < 0.05$, Fig. 5***B***). These features of desquamation, loss of intercellular cohesion, and reduced thickness of intact epithelium were similar to those observed on tissues exposed to phosphoric acid. Overall, these histological findings are suggestive of pH-induced chemical burn or mucosal corrosion.

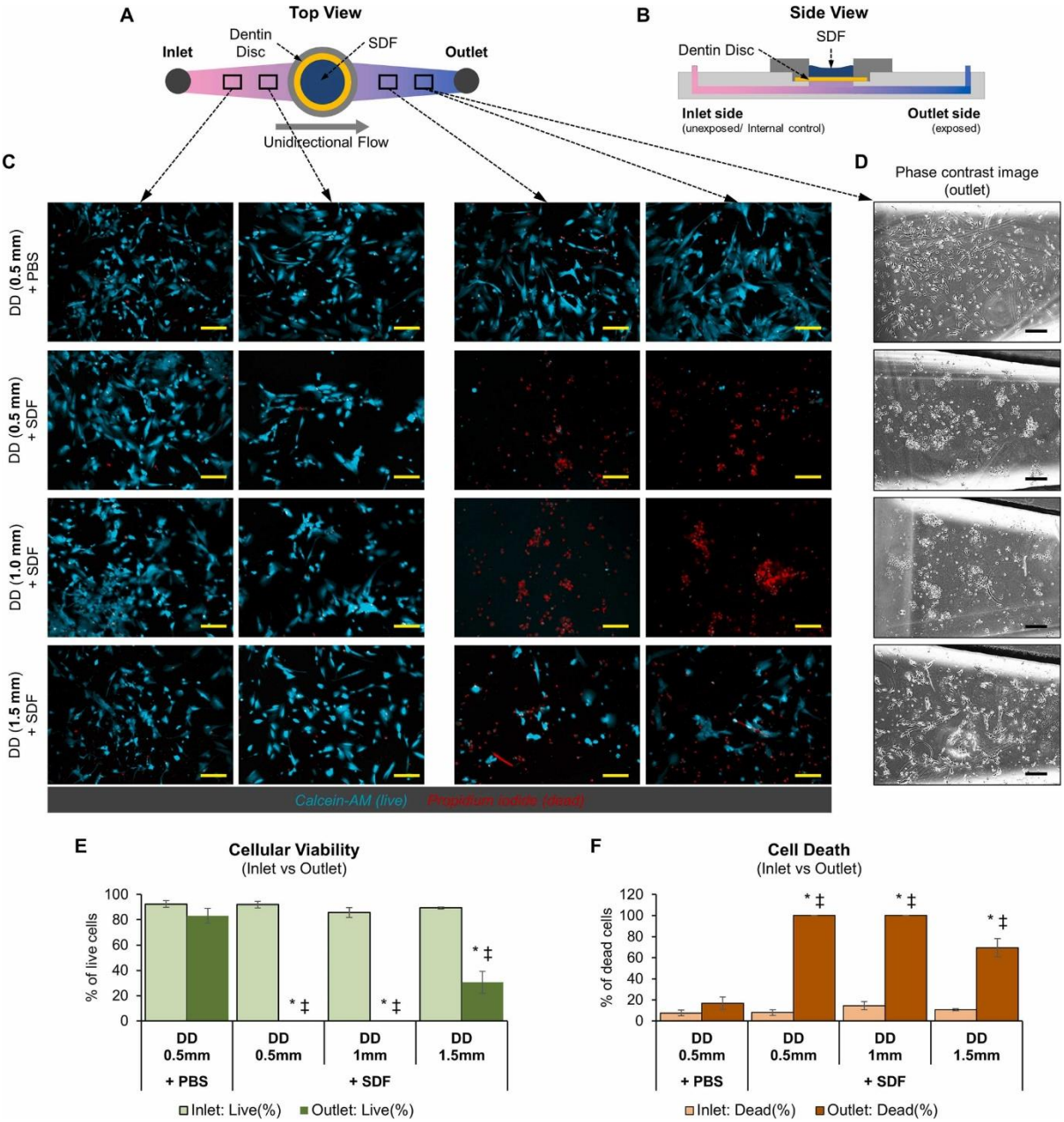

Fig. 4. Effect of SDF on DPSC cultured under flow and dentin barrier conditions. Schematic illustration demonstrating the working principle of the tooth-on-a-chip device as seen from the top (**A**) and side (**B**) views. Sequence of fluorescent (**C**) and phase-contrast (**D**) images from the inlet and outlet sides of the pulp channel show the DPSC stained with calcein-AM (live, cyan) and propidium iodide (dead, red). Scale bar in (D)− 200 µm, (D)− 500 µm. The bar graphs show the percentage of live (**E**) and dead (**F**) cells within the inlet and outlet sides of the pulp channel upon exposure to SDF and different dentin disc thickness. DD-dentin disc, Data presented as mean ± SD, n = 3, *$p < 0.05$ (inlet vs outlet), ‡$p < 0.05$ (PBS-exposed vs SDF exposed dentin discs).

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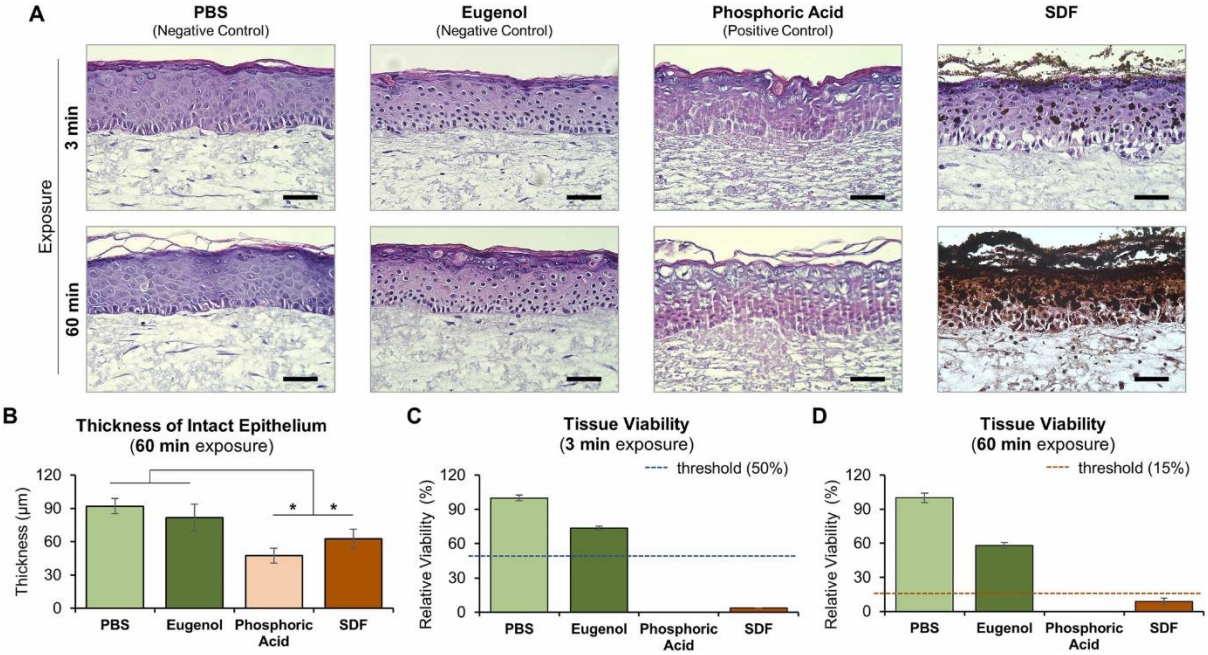

Fig. 5. Effect of SDF on gingival epithelium. (**A**) H-E-stained images show the integrity of the full-thickness gingival equivalents upon exposure to SDF and control substances. scale bar: 50 µm (**B**) Quantification of epithelial integrity expressed as thickness of intact epithelium upon 60 min exposure to SDF and control substances (mean ± SD, n = 5, *p < 0.05). (**C**, **D**) Quantification of tissue viability upon 3 min and 60 min exposure to SDF and control substances (mean ± SD). Dotted horizontal lines represent the viability threshold for 3 and 60 min exposure based on OECD TG431 guidelines.

Table 2. Qualitative analysis of the histological parameters of epithelial integrity of the gingival equivalents exposed to the test substances.

Histological Parameters	PBS (Negative control)	Eugenol (Negative control)	Phosphoric acid (Positive control)	SDF
3 min exposure				
Desquamation	–	–		
Cellular Vacuolization	–	++	+	+
Loss of intercellular Cohesion	–	–	++	++
60 min exposure				
Desquamation	+	–	++	++
Cellular Vacuolization	–	++	+	+
Loss of intercellular Cohesion	–	–	++	++

Note: -, no observable changes; +, mild (changes in few cells or few layers of the epithelium); ++, moderate-severe (changes observed in majority of the cells or all layers of the epithelium).

Next, we assessed the viability of the gingival equivalents following 3 and 60 min exposure to SDF and controls (Fig. 5***C, D***). The viability of the tissues exposed to SDF and positive control for 3 min was less than the 50% threshold, and significantly lower than the negative controls ($p < 0.05$). Similarly, the tissue viability following 60 min exposure was

lower than the 15% threshold. Based on the prediction model presented in Table 1, SDF exhibits the properties of a corrosive substance. Further, the viability of the tissues exposed to SDF for 3 min was 4% (± 0.2), which is below the 18% threshold for the subcategorization presented in Table 1. The intense corrosive action of SDF is further validated by the histological findings that demonstrated disruption of superficial corneal layers and cell-cell contacts in the deeper basal layers of the epithelium within the first 3 min of exposure (Fig. 5***A***). Overall, these findings suggest that SDF exhibits properties of a corrosive, sub-category 1 A.

4. DISCUSSION

The present study examined the effects of SDF on dental pulp and gingival cells. It was found that SDF was cytotoxic to DPSC at extremely low concentrations (IC_{50} = 0.001%). Additionally, 38% SDF penetrated dentin thickness of ≤ 1.0 mm resulting in complete death of DPSC cultured in a microfluidic device under peristaltic flow conditions (Fig. 4). When applied to a full-thickness human gingival equivalent, 38% SDF resulted in mucosal corrosion and epithelial desquamation even at exposure time as low as 3 min (Fig. 5).

The concentration (0.001%) whereby SDF was shown to be cytotoxic is similar to previous studies that applied diluted SDF directly on to human gingival fibroblasts [15] and rat dental pulp cells [16]. Furthermore, the study by Fancher et. al., [15] showed that this cytotoxic effect persisted even after 9 weeks of rinsing the dentin slices with artificial saliva. Collectively, these findings suggest that the application of highly concentrated SDF solution in deep cavity preparations close to pulp may be a source of cytotoxicity, nonetheless, this is yet to be confirmed by clinical studies.

Dentin is a semi-permeable barrier owing to the presence of dentinal tubules and hence, only partially limits the diffusion of biomaterial leachates into the pulp [31], [32]. Dentin permeability (a measure of barrier potential of dentin) varies significantly (3–10 folds) across

a few millimeters [33]. Hence, prior to experiments with SDF, we characterized the permeability of the dentin discs of different thickness. Using a lucifer yellow permeation assay, it was found that dentin permeability was inversely correlated with thickness up to a certain thickness (<1.5 mm), which corroborated with a previous study [34]. However, due to the propensity for SDF to precipitate in culture media, water, PBS and solutions with pH< 8, it was not feasible to determine the absolute permeability of SDF across dentin, which is a limitation of the present study.

To understand the cytotoxicity of SDF on dental pulp in vitro, it is essential to mimic dentinal barrier using models like the dentin barrier models, in vitro pulp chamber, and tooth-on-a-chip [20], [21], [22], [24], [25], [32], [35], [36], [37], [38]. Herein, a microfluidic tooth-on-a-chip device was used to examine the effect of 38% SDF on human dental pulp cells across a dentin barrier of various thicknesses. Furthermore, the device allowed direct visualization of the effects of SDF on pulp cells and enabled the flow of culture media. The pulp channel was designed with narrow inlet and outlet ports to mimic the narrow apical foramen, and a rhomboid central segment to mimic the wider pulp chamber (Fig. 1). The controlled flow of culture media using a peristaltic pump mimicked the physiological blood flow, providing constant supply of fresh nutrients and washing away any potentially toxic compounds that penetrated the dentin barrier. Due to the unidirectional flow of culture media from the inlet to outlet, the SDF permeating through the dentin disc can affect only the cells in the outlet channel. This permits the inlet channel to serve as an internal control for each technical and biological replicate, a unique feature offered by the microfluidic design and active control of fluid flow.

Using the tooth-on-a-chip device, this study found that 38% SDF was able to penetrate the dentin barrier ≤ 1.0 mm and resulted in cellular aggregation, pyknotic nuclei and 100% cell death. This occurred despite a constant supply of fresh media which may be attributed to higher

permeability of the dentin discs following removal of smear layer using EDTA [39], long exposure period (3 h) compared to a brief application clinically and/or the ability of SDF to maintain its toxic effects even after multiple rinses [15]. The cytotoxic effect cannot be attributed to potential remnants of lucifer yellow in the dentin discs (after permeation assay), as the PBS-exposed dentin discs (also exposed to lucifer yellow) showed> 85% viability in both inlet and outlet channels. Furthermore, cell viability studies on DPSC showed> 95% viability of DPSCs exposed to lucifer yellow (Fig. S1). The cytotoxic effect of SDF was found to be less pronounced at dentin thickness of 1.5 mm, suggesting reduced permeability at that thickness. Although previous studies concluded that indirect application (on dentin) of SDF only resulted in mild inflammation and tertiary dentin formation [5], [6], [40], a major limitation of these studies were the failure to consider the thickness of the dentin, which can have a major effect on SDF penetration as shown in this study. *Ex vivo* studies on artificially demineralized dentin [7] and deciduous teeth [8] have demonstrated the penetration of silver precipitates more than 2 mm into the dentinal tubules. Similarly, different tooth preparation approaches have been shown to impact the depth of penetration of silver into the dentinal tubules [41]. Though the permeation of substances through carious dentin is lower than that through sound dentin [39], [42], questions remain on the penetration through deep carious lesions with thin intact dentin. However, whether this would lead to pulpal inflammation and eventual pulpal death is uncertain, and further studies with the incorporation of immune cells and/or in vivo studies are required.

The mechanism of SDF cytotoxicity is still uncertain at this juncture. The cytotoxicity of SDF on human gingival equivalents was compared to the cytotoxic response against well-established positive and negative controls based on OECD guidelines [30]. Histologically, it was found that the process of cell death was similar to the effect of phosphoric acid, with the human gingival equivalent showing widespread desquamation, decreased thickness of intact

epithelium and loss of intercellular cohesion within 3 min of exposure. This rapid effect suggests that the toxicity may be due to the pH of the solution [43]. In a study on the effect of pH on human cells, fibroblasts and keratinocytes were found to have reduced viability at pH> 10, with keratinocytes being more vulnerable exhibiting almost complete cell death at pH of 11.5 [43]. The high pH of SDF solution can affect the integrity of corneodesmosomes [44], [45], resulting in the loss of adhesion between the corneocytes. Clinically, this may present as desquamation of the corneal layer, and separation or peeling of the whole epithelium from the underlying connective tissue when accidentally applied to the gingiva.

5. CONCLUSION

SDF was found to be cytotoxic to DPSC at extremely low concentrations (0.001%) and was able to penetrate dentin of low thickness (≤1.0 mm) resulting in death of pulp cells in vitro. It also disrupted the gingival epithelial integrity resulting in mucosal corrosion.

AUTHOR CONTRIBUTIONS

SH, CH, VR and GS conceived the idea and secured the funding; GM, AM, KH and JLL participated in data collection; GM, AM and GS conducted the data analysis and preparation of figures; GS and SH led the manuscript writing; CH and VR revised the manuscript for important intellectual content. All authors gave final approval to the manuscript submitted.

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APPENDIX A. SUPPORTING INFORMATION

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dental.2022.06.025.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author, GS, upon reasonable request.

REFERENCES

[1] Gao SS, Zhao IS, Hiraishi N, Duangthip D, Mei ML, Lo ECM, et al. Clinical trials of silver diamine fluoride in arresting caries among children: a systematic review. JDR Clin Trans Res 2016;1:201–10.

[2] Llodra JC, Rodriguez A, Ferrer B, Menardia V, Ramos T, Morato M. Efficacy of silver diamine fluoride for caries reduction in primary teeth and first permanent molars of schoolchildren: 36-month clinical trial. J Dent Res 2005;84:721–4.

[3] Hu S, Meyer B, Lai BWP, Chay PL, Tong HJ. Parental acceptance of silver diammine fluoride in children with autism spectrum disorder. Int J Paediatr Dent 2020;30:514–22.

[4] Duangthip D, Fung MHT, Wong MCM, Chu CH, Lo ECM. Adverse effects of silver diamine fluoride treatment among preschool children. J Dent Res 2018;97:395–401.

[5] Bimstein E, Damm D. Human primary tooth histology six months after treatment with silver diamine fluoride. J Clin Pediatr Dent 2018;42:442–4.

[6] Rossi G, Squassi A, Mandalunis P, Kaplan A. Effect of silver diamine fluoride (SDF) on the dentin-pulp complex: ex vivo histological analysis on human primary teeth and rat molars. Acta Odontol Latinoam 2017;30:5–12.

[7] Sayed M, Matsui N, Uo M, Nikaido T, Oikawa M, Burrow MF, et al. Morphological and elemental analysis of silver penetration into sound/demineralized dentin after SDF application. Dent Mater 2019;35:1718–27.

[8] Li Y, Liu Y, Psoter WJ, Nguyen OM, Bromage TG, Walters MA, et al. Assessment of the silver penetration and distribution in carious lesions of deciduous teeth treated with silver diamine fluoride. Caries Res 2019;53:431–40.

[9] Zhao IS, Gao SS, Hiraishi N, Burrow MF, Duangthip D, Mei ML, et al. Mechanisms of silver diamine fluoride on arresting caries: a literature review. Int Dent J 2018;68:67–76.

[10] Mei ML, Lo ECM, Chu CH. Arresting dentine caries with silver diamine fluoride: what's behind it? J Dent Res 2018;97:751–8.

[11] Hu S, Meyer B, Duggal M. A silver renaissance in dentistry. Eur Arch Paediatr Dent 2018;19:221–7.

[12] Greulich C, Braun D, Peetsch A, Diendorf J, Siebers B, Epple M, et al. The toxic effect of silver ions and silver nanoparticles towards bacteria and human cells occurs in the same concentration range. RSC Adv 2012;2:6981–7.

[13] Chang Y-C, Chou M-Y. Cytotoxicity of fluoride on human pulp cell cultures in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001;91:230–4.

[14] Hirose Y, Yamaguchi M, Kawabata S, Murakami M, Nakashima M, Gotoh M, et al. Effects of extracellular pH on dental pulp cells in vitro. J Endod 2016;42:735–41.

[15] Fancher ME, Fournier S, Townsend J, Lallier TE. Cytotoxic effects of silver diamine fluoride. Am J Dent 2019;32:152–6.

[16] Kim S, Nassar M, Tamura Y, Hiraishi N, Jamleh A, Nikaido T, et al. The effect of reduced glutathione on the toxicity of silver diamine fluoride in rat pulpal cells. J Appl Oral Sci 2021;29:e20200859.

[17] Hosoya Y, Aritomi K, Goto G. Pulpal response to diammine silver fluoride. (2). Application on exposed pulps. Shoni Shikagaku Zasshi 1990;28:327–37.

[18] Zaeneldin A, Yu OY, Chu CH. Effect of silver diamine fluoride on vital dental pulp: a systematic review. J Dent 2022:104066.

[19] Sriram G, Alberti M, Dancik Y, Wu B, Wu R, Feng Z, et al. Full-thickness human skin-on-chip with enhanced epidermal morphogenesis and barrier function. Mater Today 2018;21:326–40.

[20] Franca CM, Tahayeri A, Rodrigues NS, Ferdosian S, Puppin Rontani RM, Sereda G, et al. The tooth on-a-chip: a microphysiologic model system mimicking the biologic interface of the tooth with biomaterials. Lab Chip 2020;20:405–13.

[21] Rodrigues NS, Franca CM, Tahayeri A, Ren Z, Saboia VPA, Smith AJ, et al. Biomaterial and biofilm interactions with the pulp-dentin complex-on-a-chip. J Dent Res 2021;100:1136–43.

[22] Rosa V, Sriram G, McDonald N, Cavalcanti BN. A critical analysis of research methods and biological experimental models to study pulp regeneration. Int Endod J 2022;55(Suppl 2):446–55.

[23] Alberti M, Dancik Y, Sriram G, Wu B, Teo Y, Feng Z, et al. Multi-chamber microfluidic platform for high-precision skin permeation testing. Lab Chip 2017;17:1625–34.

[24] Jiang RD, Lin H, Zheng G, Zhang XM, Du Q, Yang M. In vitro dentin barrier cytotoxicity testing of some dental restorative materials. J Dent 2017;58:28–33.

[25] Hadjichristou C, About I, Koidis P, Bakopoulou A. Advanced in vitro experimental models for tissue engineering-based reconstruction of a 3D dentin/pulp complex: a literature review. Stem Cell Rev Rep 2021;17:785–802.

[26] Modena KC, Casas-Apayco LC, Atta MT, Costa CA, Hebling J, Sipert CR, et al. Cytotoxicity and biocompatibility of direct and indirect pulp capping materials. J Appl Oral Sci 2009;17:544–54.

[27] Atkuru S, Muniraj G, Sudhaharan T, Chiam KH, Wright GD, Sriram G. Cellular ageing of oral fibroblasts differentially modulates extracellular matrix organization. J Periodontal Res 2021;56:108–20.

[28] Xie H, Chua M, Islam I, Bentini R, Cao T, Viana-Gomes JC, et al. CVD-grown monolayer graphene induces osteogenic but not odontoblastic differentiation of dental pulp stem cells. Dent Mater 2017;33:e13–21.

[29] Sriram G, Sudhaharan T, Wright GD. Multiphoton microscopy for noninvasive and label-free imaging of human skin and oral mucosa equivalents. Methods Mol Biol 2020;2150:195–212.

[30] OECD. Test No. 431: in vitro skin corrosion: reconstructed human epidermis (RHE) test method, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. ⟨https://doi.org/10.1787/9789264264618-en⟩.

[31] Hamid A, Hume WR. The effect of dentine thickness on diffusion of resin monomers in vitro. J Oral Rehabil 1997;24:20–5.

[32] Hanks CT, Craig RG, Diehl ML, Pashley DH. Cytotoxicity of dental composites and other materials in a new in vitro device. J Oral Pathol 1988;17:396–403.

[33] Pashley DH, Andringa H, Derkson G, Derkson M, Kalathoor S. Regional variability in the permeability of human dentine. Arch Oral Biol 1987;32:519–23.

[34] Hanks CT, Wataha JC, Parsell R, Strawn SE, Fat J. Permeability of biological and synthetic molecules through dentine. J Oral Rehabil 1994;21:475–87.

[35] Schmalz G, Schuster U, Nuetzel K, Schweikl H. An in vitro pulp chamber with three-dimensional cell cultures. J Endod 1999;25:24–9.

[36] da Fonseca Roberti Garcia L, Pontes EC, Basso FG, Hebling J, de Souza Costa CA, Soares DG. Transdentinal cytotoxicity of resin-based luting cements to pulp cells. Clin Oral Investig 2016;20:1559–66.

[37] Hadjichristou C, Papachristou E, Bonovolias I, Bakopoulou A. Three-dimensional tissue engineering-based Dentin/Pulp tissue analogue as advanced biocompatibility evaluation tool of dental restorative materials. Dent Mater 2020;36:229–48.

[38] Soares DG, Bordini EAF, Swanson WB, de Souza Costa CA, Bottino MC. Platform technologies for regenerative endodontics from multifunctional biomaterials to tooth-ona-chip strategies. Clin Oral Investig 2021;25:4749–79.

[39] Pashley EL, Talman R, Horner JA, Pashley DH. Permeability of normal versus carious dentin. Endod Dent Traumatol 1991;7:207–11.

[40] Korwar A, Sharma S, Logani A, Shah N. Pulp response to high fluoride releasing glass ionomer, silver diamine fluoride, and calcium hydroxide used for indirect pulp treatment: an in-vivo comparative study. Contemp Clin Dent 2015;6:288–92.

[41] Lau L, Quock RL, Wu DI, Harrington DA, Patel SA, Barros JA. Effect of surface preparation and light curing on penetration of silver particles from 38% silver diamine fluoride in dentin of primary teeth: an in vitro evaluation. Am J Dent 2021;34:44–8.

[42] Puapichartdumrong P, Ikeda H, Suda H. Facilitation of iontophoretic drug delivery through intact and caries-affected dentine. Int Endod J 2003;36:674–81.

[43] Kruse CR, Singh M, Targosinski S, Sinha I, Sørensen JA, Eriksson E, et al. The effect of pH on cell viability, cell migration, cell proliferation, wound closure, and wound reepithelialization: in vitro and in vivo study. Wound Repair Regen 2017;25:260–9.

[44] Hachem JP, Crumrine D, Fluhr J, Brown BE, Feingold KR, Elias PM. pH directly regulates epidermal permeability barrier homeostasis, and stratum corneum integrity/cohesion. J Invest Dermatol 2003;121:345–53.

[45] Ishida-Yamamoto A, Igawa S. The biology and regulation of corneodesmosomes. Cell Tissue Res 2015;360:477–82.