Dental Materials 42 (2026) 586–595

Contents lists available at ScienceDirect

Dental Materials

journal homepage: www.elsevier.com/locate/dental

Cytotoxicity testing of dental materials: Establishment of a 3D-printed dentin barrier culture system

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ARTICLE INFO

Keywords:
Organotypic Cell Cultures
Dental Pulp
Dentin
3D Printing
Fibroblasts
Materials Testing

ABSTRACT

Introduction: Cytotoxicity testing is critical in assessing the biocompatibility of dental materials with regard to the pulp, however, conventional methods such as the extract test (ISO 10993–5) do not reflect the protective effects of dentin in the dentin-pulp complex. Traditional dentin barrier testing techniques (ISO 7405) address this problem, but face challenges due to their complex and expensive production and the difficult and error-prone handling. Therefore, the aim of this study was to develop a novel, user-friendly and easily accessible dentin barrier culture system (DBCS) to improve cytotoxicity testing of dental materials.

Method: The DBCS was designed and manufactured using stereolithography 3D printing. The components were assembled with dentin discs to create a barrier between an upper and lower compartment (pulp side/restoration side). Dentin discs were pretreated (EDTA or citric acid) and primary human pulp cells (HPC) or mouse fibroblasts (L-929) were cultured in the upper chamber, while a self-adhesive composite (Vertise Flow, Kerr; Scafati, Italy) was applied to the opposite side of the disc after 48 h. Scanning electron microscopy (SEM) was used to visualize dentin structure and cell attachment. Analyses included assessment of cell metabolism (MTT test), membrane integrity (LDH assay), and performing live/dead staining. To validate the model, various dentin disc parameters were evaluated, including species origin (human or bovine), location relative to the pulp (proximal or distal), thickness, and the effects of autoclaving. Statistical evaluation was performed using the Kruskal-Wallis or the Mann-Whitney test ($P \leq 0.05$).

Results: Both HPC and L-929 formed confluent cell layers in the DBCS setup. Pretreatment of dentin discs with EDTA or citric acid reliably removed the smear layer, with the latter having a positive effect on cell viability ($P \leq 0.03$). The self-adhesive composite had a similar effect on the viability of both cell types, and SEM revealed apoptotic cell morphology. Dentin provided a protection only at a thickness of greater than 400 µm. There were no significant differences in cellular responses to the material depending on dentin origin or autoclaving ($P > 0.9999$).

Conclusion: This 3D-printable culture system offers a readily available, user-friendly dentin barrier model for evaluating the cytotoxicity of dental materials and can be easily reproduced by anyone with access to a standard 3D printer.

1. Introduction

The pulp-dentin-complex describes the functional unit consisting of the soft tissue of the dental pulp and the surrounding tubular dentin. It is structurally unique in the human body and bears important functions, such as hard tissue formation, nutrient delivery, sensory perception, and defense against pathogens [39,54]. The dentinal tubules, which house the odontoblast processes, allow direct communication between the pulp and oral environment, and provide a pathway for bacteria that can significantly affect the pulp [29,9]. The permeability of dentin is particularly high in the immediate vicinity of the dental pulp, as the diameter and density of the tubules are increased [30,44]. However, tubular dentin provides protection against external stimuli as both a mechanical and a chemical barrier, as collagen and hydroxyapatite in

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https://doi.org/10.1016/j.dental.2025.11.016
Received 30 July 2025; Received in revised form 5 November 2025; Accepted 24 November 2025
Available online 2 December 2025

dentin can for instance neutralize acids and bind monomers [18,5].

Not only bacterial noxae, but also dental adhesives and filling materials pose a threat to the dental pulp [15,50]. Resin composites are known to exert cytotoxic effects through the release of monomers. Several *in vitro* studies show that, for example, HEMA (hydroxyethylmethacrylate) can induce mutagenic changes, negatively affect cell metabolism and lead to apoptosis, but also reduce the ability of cells to form tertiary dentin [11,35,49,50]. In addition, HEMA and TEGDMA (Triethylene Glycol Dimethacrylate) have been repeatedly shown to promote intracellular oxidative stress through the increased formation of reactive oxygen species, which can impair the immune capacity of the dental pulp [27,48,8]. The clinical consequences of such pulpal injury may include dentin sclerosis, tertiary dentin formation as a defensive response, or more severe outcomes such as pulpitis or pulp necrosis [13, 39,7]. With approximately 800 million composite restorations placed worldwide in 2015 alone, and the traditional alternative material amalgam being phased out in some parts of the world, mitigating these cytotoxic effects in clinical dentistry is critical [20,58].

Since most dental materials remain in the patient's mouth for an extended period of time, their safety is both a personally sensitive and medically important issue. This can be seen in the recurring discussions about amalgam and other dental materials. Therefore, cytotoxicity testing plays a crucial role in the development of materials to improve the safety, efficiency and predictability of dental treatment. In the European Union, as in most parts of the world, restorative dental materials are classified as medical devices and as such are subject to different types of evaluation. While the preclinical cytotoxicity testing methods defined in ISO 10993–5 include *in vitro* techniques such as agar diffusion and extract tests, ISO 7405 focuses on the requirements for use in the oral cavity and describes a "Dentin Barrier Test (DBT)" [23,24]. In this test, dentin discs are mounted in special stainless steel holders and placed outside the incubator on heating plates in a semi-sterile environment. The chambers are connected to a peristaltic pump that provides a continuous exchange of cell culture medium. In this setup, cells are grown three-dimensionally in a polystyrene mesh and placed in one chamber, while materials can be placed on the dentin disc in the other chamber. The use of bovine dentin and established cell lines is recommended [23]. Clearly, this test better reflects the *in vivo* situation compared to classical cell culture tests and has been a major achievement in reducing the need for animal testing [43]. However, several factors compromise the reliability of this system. When cultured outside of an incubator, the lack CO_2 buffering in the medium is a significant limitation, and maintaining sterility becomes more difficult. In addition, thermal stability during the assay is a concern. As a result, setting up and running the assay can be complicated and error-prone, potentially leading to large variations in test results. The need for specialized equipment adds another layer of complexity. All of these reasons have contributed to the relegation of the DBT to a less prominent position in current dental cytotoxicity research. At the same time, technological advances such as 3D printing, offer great potential to provide easily accessible, and potentially better standardized test conditions.

Therefore, the aim of this study was to develop a dentin barrier culture system (DBCS) using available 3D printing technology as a model to study material-induced effects on the dentin-pulp complex under laboratory conditions, allowing for organotypic cell culture and easy implementation. As ISO 7405 outlines guidelines for the traditional DBT, we extended our focus to systematically evaluate key variables in the DBCS, including cell type (fibroblasts and primary pulp cells), toxicity assessment methods, dentin origin (human and bovine), smear layer removal, dentin location (pulp-proximal and pulp-distal), thickness, and the impact of autoclaving.

2. Methods

2.1. Stereolithography (SLA) fabrication

The DBCS was designed in five parts (Fig. 1A) and 3D-printed using medical grade resin (BioMed Clear Resin, Formlabs; Berlin, Germany) on an SLA 3D printer (Form 4 L, Formlabs; Berlin, Germany). 3D-printable files are publicly available on GitHub to enable reproduction and further use by the research community. The devices were washed (Form Wash, Formlabs; Berlin, Germany) in 99 % isopropyl alcohol and post-cured (Form Cure, Formlabs; Berlin, Germany) at 60 °C for 60 min. The material used has been confirmed to be non-cytotoxic according to ISO 10993–5, with no evidence of cell lysis, as reported by the manufacturer [2] and supported by our own preliminary testing. The DBCS components were disinfected in 70 % isopropyl alcohol before use and were reused up to 6 times or discarded if damage or wear was observed.

When assembled, the dentin disc is secured between two gaskets (TrueSil Shore 60 A, Spectroplast; Zurich, Switzerland) by a spacer (Fig. 1B) using a screw mechanism (Fig. 1C), forming an upper compartment that simulates the pulp side of a cavity, with the disc at the bottom. It has the same dimensions as a 96-well and can be seeded with cells (Fig. 1A). The bottom is designed to allow materials to be placed directly on the dentin disc (Fig. 1D). The DBCS forms a device that fits into the wells of a 24-well plate and can be easily removed with tweezers (Fig. 1E).

2.2. Preparation of dentin discs

2.2.1. Sectioning

Extracted caries-free molars were collected and transported in 0.5 % chloramine T (Carl Roth; Karlsruhe, Germany) as approved by the Ethics Committee (19–1327-101; Faculty of Medicine, University of Regensburg, Regensburg, Germany). Before sectioning, they were embedded in polymethylmethacrylate resin (Paladur, Kulzer; Hanau, Germany) and fixed on a specimen holder. Dentin discs were prepared with a diamond saw blade using a saw microtome (RMS16G3, Reha Tec Engineering; Oostzaan, The Netherlands) by cutting perpendicular to the long axis, starting below the occlusal surface and ending at the cemento-enamel junction. The diameter of the slices was standardized to 8 mm using a hole punch (Paffrath; Wiesbaden, Germany) and the discs were stored in 0.5 % chloramine T at 4 °C. Prior to insertion into the DBCS, the dentin discs were washed in ultrapure water (EMD Millipore Corp.; Billerica MA, USA) under sterile conditions and placed in saline for 24 h.

2.2.2. Smear layer removal and dentin surface evaluation

Citric acid (CA, 40 %, Sigma-Aldrich; St. Louis, USA) or EDTA (ethylenediaminetetraacetic acid, 10 % disodium dihydrate in 1 N sodium hydroxide, UKR Pharmacy; Regensburg, Germany) was applied to optimize cell attachment and to remove the smear layer formed during dentin cutting. After the DBCS were fitted with 200 μm thick dentin discs and placed in a 24-well plate containing 400 μl of medium per well to prevent desiccation of the dentin, the upper compartment was filled with either 100 μl of 40 % citric acid solution for 30 s or 100 μl of EDTA for 10 min. After the treatment, the solution was removed, and the dentin surface was neutralized through multiple wash steps with cell culture medium to ensure biocompatibility. Untreated dentin discs, with the smear layer present were used as negative controls.

The micromorphologic changes of the dentin surface on both sides of the discs were visualized by SEM in low vacuum (LV) mode. For this purpose, the discs were gently removed from the DBCS, washed in distilled water and mounted on aluminium stubs using self-adhesive carbon discs (Leit-Tabs, PROVAC GmbH; Sprendlingen, Germany). SEM imaging was performed at 1.50 Torr, 4.0 kV, aperture 6, spot 3, with a working distance of approximately 10 mm (FEI Europe; Eindhoven, The Netherlands).

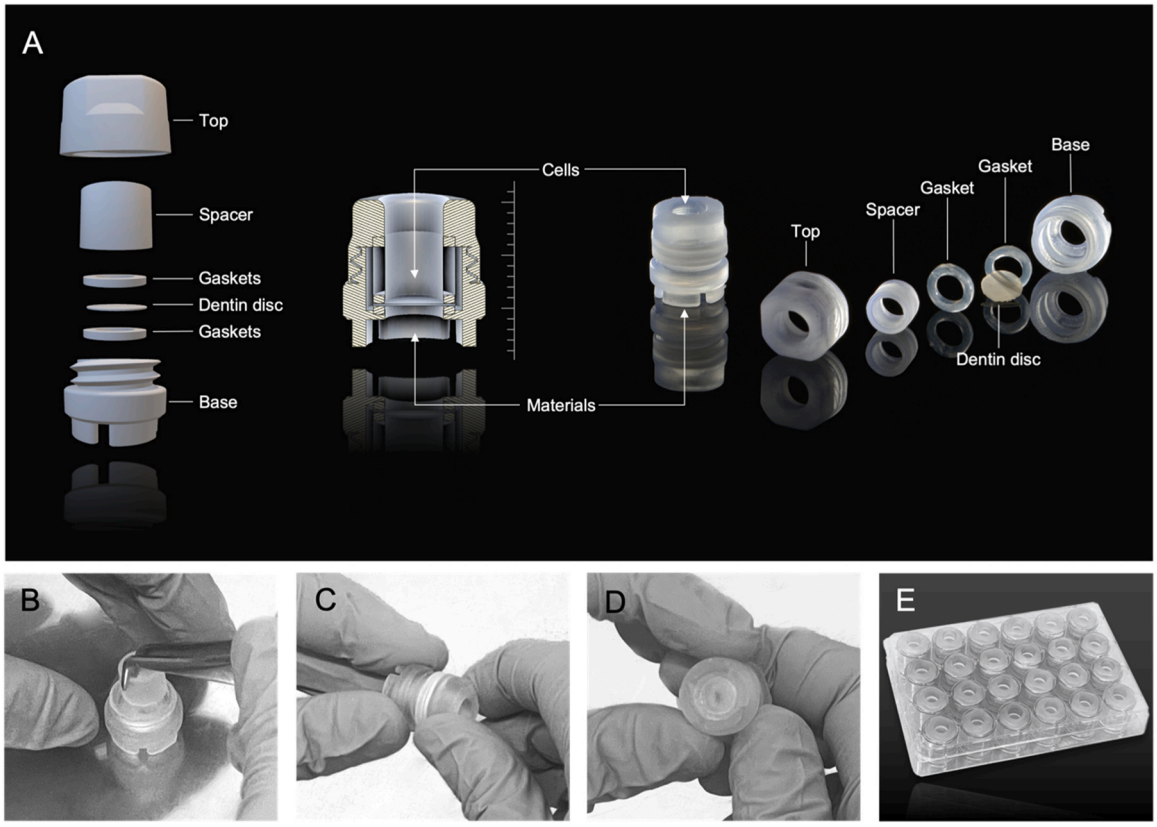

Fig. 1. Construction of DBCS. (A) From left to right: An exploded view of the virtually constructed DBCS, showing its individual components; a longitudinal section (technical drawing, scale bar: one tick = 1 mm); the fully assembled DBCS with two distinct compartments; and the individual 3D-printed components that make up the DBCS. (B-E) Assembly of the DBCS under sterile conditions: Placement of the dentin disc and spacer in the DBCS (B). Screwing the components together (C). Bottom view of the dentin disc (D). A 24-well cell culture plate equipped with DBK, allowing cells to be added to the upper compartment (E).

2.2.3. Assessing seal quality

To ensure the integrity of the DBCS seal and to confirm that penetration between the two compartments occurred exclusively through the dentin disc, DBCS were assembled with either a dentin disc (200 µm thick) that was etched with 40 % CA for 30 s or an impermeable glass cover slip (10 mm diameter, VWR; Darmstadt, Germany) as a negative control. Each DBCS was placed in a 24-well plate containing 400 µl of distilled water per well. Trypan blue solution (0.02 % in distilled water, Sigma-Aldrich; St. Louis, USA) was added to the upper chamber of each DBCS. After 72 h, 200 µl of fluid was collected from the lower chamber, and the absorbance was measured at 600 nm using a spectrophotometer (Tecan Infinite F200; Männedorf, Switzerland) (n = 9). The optical density (OD) of the trypan blue solution was measured as positive control (PC) and the OD of the distilled water was used as negative control (NC).

2.3. Cell culture

2.3.1. Effect of dentin pretreatment on cells

In order to test the effect of pretreatment modalities on cell attachment, 10,000 cells (L-929, mouse, ATCC CCL1) were seeded in 200 µl of Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, USA) supplemented with 10 % fetal bovine serum (Gibco; Billings, USA) and 1 % penicillin/streptomycin (Sigma-Aldrich; St. Louis, USA) into each DBCS containing dentin pretreated with either EDTA or CA. After 48 h of culture, cell viability was determined by the MTT test. Therefore, a 0.5 mg/ml MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide in DMEM without phenol red, Sigma-Aldrich; St. Louis, USA) was prepared and stored at −20 °C until use. For each measurement, the supernatant was exchanged for 100 µl of MTT solution and incubated at 37 °C for 2 h. The solution was then discarded and the DBCS units were disassembled. The dentin discs were transferred to 24-well plates containing 250 µl of DMSO to dissolve the dye. After shaking for 20 min, the solution was transferred to a 96-well plate, and the absorbance was measured at 540 nm. Experiments were carried out in triplicate and performed three times (n = 9).

2.3.2. Cell growth

Two different cell types were tested in the DBCS. The fibroblast-like cell line L-929 was used as the standard cell type and cultured in DMEM. 10,000 cells in 200 µl culture medium were seeded into each DBCS. Alternatively, primary human pulp cells (HPC) were used, which were isolated from patients aged 15–20 years according to an established protocol [12,17] with informed consent and as approved by the Ethics Committee (16–101-0022; Faculty of Medicine, University of Regensburg; Regensburg, Germany). A total of 10,000 pulp cells (passage lower than 5) in 200 µl medium were seeded into each DBCS for testing.

Cell growth was evaluated using 200 µm human dentin discs pretreated with CA as described above. Growth curves (3, 24, 48, and 72 h) were established for both cell types in the DBCS and cell viability was compared to that of cells cultured in a 96-well plate (51160, Greiner Bio-One; Kremsmünster, Austria). MTT tests were performed in DBCS as described above. For cultures in well plates, 250 µl DMSO was added directly to the well and treated in the same manner as DBCS. Experiments were performed three times in triplicate in the DBCS (n = 9) and in six replicates in culture plates (n = 18).

2.4. Toxicity evaluation

2.4.1. Toxicity control

To establish a toxicity control (TC) across the different variations of this experimental setup, cells were exposed to a self-adhesive resin composite with reported cytotoxic properties (Vertise Flow, Kerr; Scafati, Italy) 48 h after cell seeding into the DBCS, as described in Section 2.3. To do this, the supernatant was removed from the pulp side and the DBCS was gently inverted, ensuring that a film of liquid remained on the cells to prevent them from desiccation. The dentin disc was dried by vacuum suction and dabbed with sterile foam pellets (Henry Schein; Raesfeld, Germany). The material was applied in a thickness of 1.5 mm, as indicated by a height mark on the rim of the DBCS, and then light cured for 40 s with a mono-wave LED polymerization lamp (Bluephase C8, 800 mW/cm^2; Ivoclar Vivadent; Schaan, Liechtenstein) with a constant distance of 3 mm between the lamp and the material surface. Untreated controls were handled identically and were also exposed to light to ensure comparable conditions. The DBCS were returned into the 24-well-plates and fresh medium was added on the cells, which had remained moist throughout the procedure. The cells were transdentinally exposed to the self-adhesive resin for 24 h prior to analysis.

2.4.2. MTT test

L-929 cells were seeded into the DBCS as described above and untreated cells (untreated control, UC) were compared to cells with transdentinal exposure to the self-adhesive resin composite (TC). MTT tests were performed as described in Section 2.3 after 24 h. The experiment was performed in triplicate and conducted three times (n = 9).

2.4.3. LDH assay

To assess cell membrane integrity, a lactate dehydrogenase (LDH) release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega; Madison, USA) was performed in the DBCS. For reference, cells were lysed 45 min before the assay. 50 µl of supernatant was collected from the DBCS cultures and the lysed cells in technical duplicates and transferred to a 96-well plate (n = 9). The LDH assay reaction mix was added and the reaction was stopped after 15 min. Absorbance was measured at 450 nm using a photometer (Tecan Infinite F200; Männedorf, Switzerland). According to the manufacturer's protocol, cytotoxicity was expressed relative to the expected maximum LDH concentration determined from lysed cells.

2.4.4. Cell type comparison

To test the response of the two different cell types to transdentinal exposure to the self-adhesive resin composite (TC), L-929 and HPC were cultured in the DBCS and analyzed by the MTT test as described in Sections 2.3 and 2.4. The experiment was performed in triplicate three times (n = 9).

2.4.5. Scanning electron microscopy

SEM images were taken to show cells exposed to the TC transdentinally. Therefore, dentin discs were gently removed from the DBCS and cells were fixed in glutaraldehyde (2.5 % in 0.1 M Sørensen buffer, pH 7.4) for 30 min at room temperature. SEM images were taken in LV mode as described above.

2.4.6. Live-dead staining

Acridine Orange (AO) and Propidium Iodide (PI) live-dead staining was used to visually evaluate the proportion of viable cells. The AO/PI assay (DeNovix; Wilmington, USA) was added to the medium in the DBCS at a 1:10 dilution, followed by an incubation at room temperature for 3 min. After staining, the DBCS were directly disassembled, the dentin discs were placed cell-side-down on cover slips, and examined with a fluorescence microscope (Axio Observer Z1, Zeiss Microsystems; Wetzlar, Germany) equipped with appropriate filter sets for AO (excitation/emission ~ 488/530 nm) and PI (excitation/emission ~ 535/617 nm). Images were acquired using a 20 × objective under standardized exposure settings.

2.5. Variation of dentin parameters

2.5.1. Autoclaving

The effect of steam sterilization on dentin was investigated. Therefore, dentin discs were autoclaved at 121 °C and 9.6 MPa for 25 min in a 0.9 % sodium chloride solution. To test whether steam sterilization (SS) affected cell attachment and dentin permeability, DBCS were equipped with respective dentin discs, seeded with L-929 cells, and these were exposed to self-adhesive resin composite (see Sections 2.3 and 2.4). DBCS with dentin disc disinfected with Chloramine T only (non-SS) served as negative control. MTT tests were conducted and experiments were carried out in triplicate three times (n = 9).

2.5.2. Dentin thickness

To test the effect of dentin thickness on cell response to the material, dentin discs of different thicknesses (200, 300, 400, 600, 800, and 1000 µm) were cut as described in Section 2.2. DBCS were assembled and MTT tests were conducted as previously outlined (n = 9).

2.5.3. Dentin origin

The origin of the dentin disc was compared in terms of proximity to the pulp chamber. For the pulp-proximal dentin (PPD), a 200 µm thick disc was cut closest to the dental pulp horns, whereas for the pulp-distal dentin (PDD), another 200 µm disc was cut 800 µm further coronally. In addition to the MTT test as described above (n = 9), the dentin discs were imaged by SEM (see Section 2.4).

2.5.4. Dentin type

Bovine dentin was tested alongside human dentin to investigate any differences in characteristics. Bovine incisors were collected, stored and sectioned longitudinally using the same procedures as for human dentin (see Section 2.2), with the discs being stamped from the cervical region. They were etched with CA and, in addition to MTT testing (n = 9), SEM images were taken to visualize micromorphological differences (see Section 2.4).

2.6. Statistical analysis

The resulting data were tested for normal distribution using the D'Agostino & Pearson test. Statistical analysis was then performed using the Kruskal-Wallis test for multiple comparisons followed by Dunn's multiple comparisons test, or the Mann-Whitney test (when no more than two groups were compared) at a significance level of $\alpha = 0.05$. The Friedman test was used for paired data, such as comparisons of dentin location. All analyses were performed using Prism 10 (GraphPad, Version 10.4.0).

3. Results

3.1. Preparation of dentin discs and seal quality assessment

Removal of the smear layer was effective with both EDTA and CA treatments, as shown by the SEM images, which revealed exposed dentin tubules without compromising the smear layer on the opposite side of the dentin disc (Fig. 2A-D).

The integrity of the DBCS seal was verified and trypan blue solution was only able to penetrate through the dentin disc (Fig. 2E). When a glass disc was inserted into the DBCS instead, there was no significant penetration ($P = 0.1326$).

3.2. Cell culture

The effect of dentin pretreatment with either CA or EDTA was

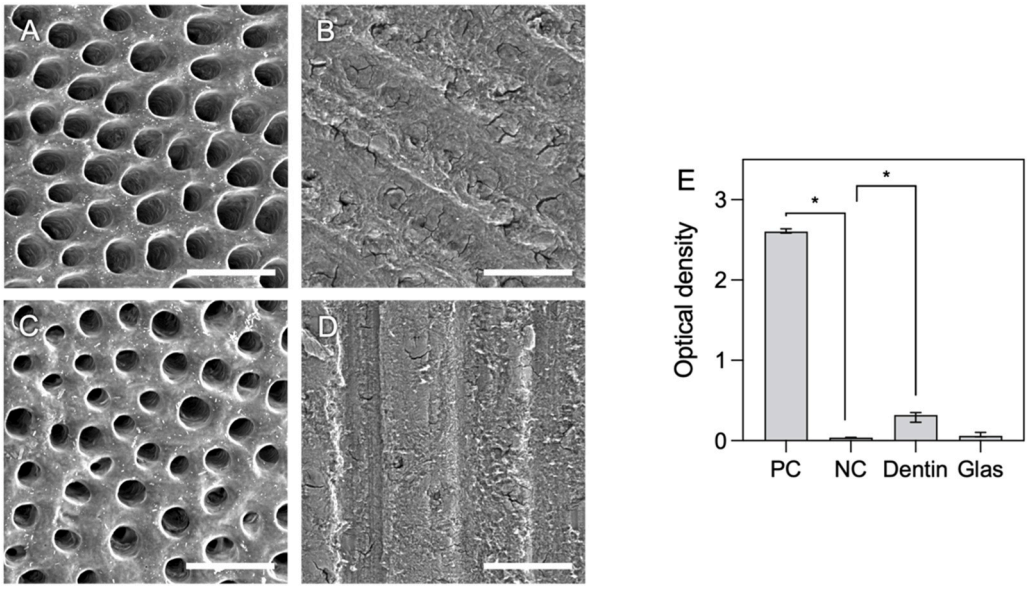

Fig. 2. (A-D) SEM images of dentin discs to observe micromorphological changes after smear layer removal: Top surface of dentin disc treated with CA for 30 s (A), while the untreated bottom surface shows no effect of treatment (B). Dentin surface after EDTA treatment (C) and untreated underside of the same disc (D). Scale bars: 10 μm. (E) Evaluation of the integrity of the DBCS seal: Positive control (PC) shows the optical density (OD) of the trypan blue solution used; negative control (NC) shows the initial OD of pure distilled water in the lower compartment. Trypan blue solution was able to moderately penetrate through dentin and cause a color change in the lower compartment, however, when the DBCS was fitted with a non-permeable glass disc, there was no significant color change when compared to the initial OD of the distilled water in the lower compartment (NC). Data are presented as median with interquartile range. Asterisks indicate significant differences ($P \leq 0.05$).

evaluated by MTT test after L-929 culture in the DBCS for 48 h. When cells were seeded in the DBCS, higher growth was observed on both pretreated dentin surfaces compared to the UC, where the smear layer remained. While no significant difference in cell viability was found between EDTA- and CA-treated surfaces ($P > 0.9999$), CA demonstrated a significant increase compared to untreated control ($P = 0.0289$) (Fig. 3A).

Furthermore, two cell types, primary HPC and immortalized L-929, were tested in the DBCS. Both exhibited continuous growth in standard 96-well plates and DBCS as shown by the MTT test. Initial attachment of L-929 appeared to be slightly reduced and showed a reduced growth rate on dentin discs (Fig. 3B). The overall growth rate of primary HPC was lower than that of L-929 in both DBCS and 96-well plates (Fig. 3C).

3.3. Toxicity evaluation

Various endpoint observation methods were evaluated in the DBCS to assess the functionality of the system in the context of toxic substance penetration.

The MTT test showed a significant decrease in OD ($P = 0.0042$), when comparing untreated L-929 cells and cells exposed to the self-adhesive resin composite (Fig. 4A), indicating reduced cell viability after treatment with the TC. Similarly, the LDH assay demonstrated a significant increase in toxicity for the TC ($P = 0.0059$).

The MTT test was further used to compare the UC and TC of L-929 and HPC. Both cell types exhibited a significant reduction in viability upon indirect exposure to the self-adhesive resin composite (Fig. 4B), with *P*-values of 0.0152 for HPC and 0.0096 for L-929. There were no significant statistical differences between the UC or the TC of either cell type ($P > 0.9999$).

The SEM images (Fig. 4C-F) revealed morphological changes and a reduction in cell number for both cell types after indirect exposure to the self-adhesive resin composite.

Live-dead staining using AO and PI representatively visualizes the difference in cell viability between UC (Fig. 4G) and TC (Fig. 4H) with a higher number of dead (PI-positive) cells in TC.

3.4. Variations of dentin parameters

Variations of dentin parameters included autoclaving of dentin discs, different dentin thicknesses, the comparison of pulp-distal dentin (PDD) and pulp-proximal dentin (PPD), as well as of bovine and human dentin.

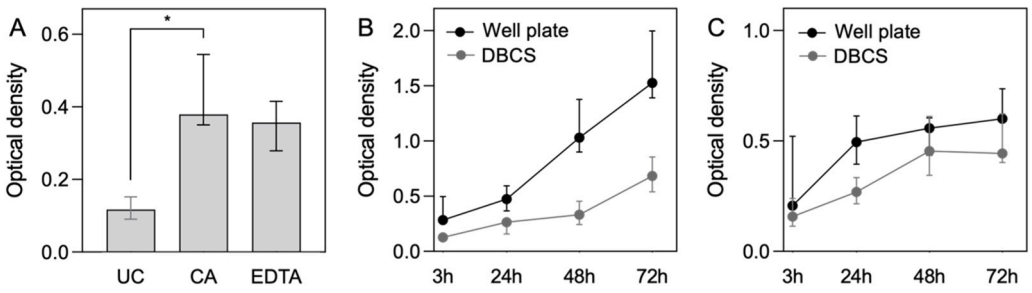

Fig. 3. (A) Different methods of smear layer removal were compared by measuring the viability of L-929 cells seeded on dentin discs. CA etching of dentin significantly ($P = 0.0289$) improved cell viability compared to the untreated control where the smear layer was not removed (UC). (B) L-929 and (C) HPC proliferation was assessed in both the DBCS and a 96-well plate using the MTT test. The graph shows the median and interquartile range.

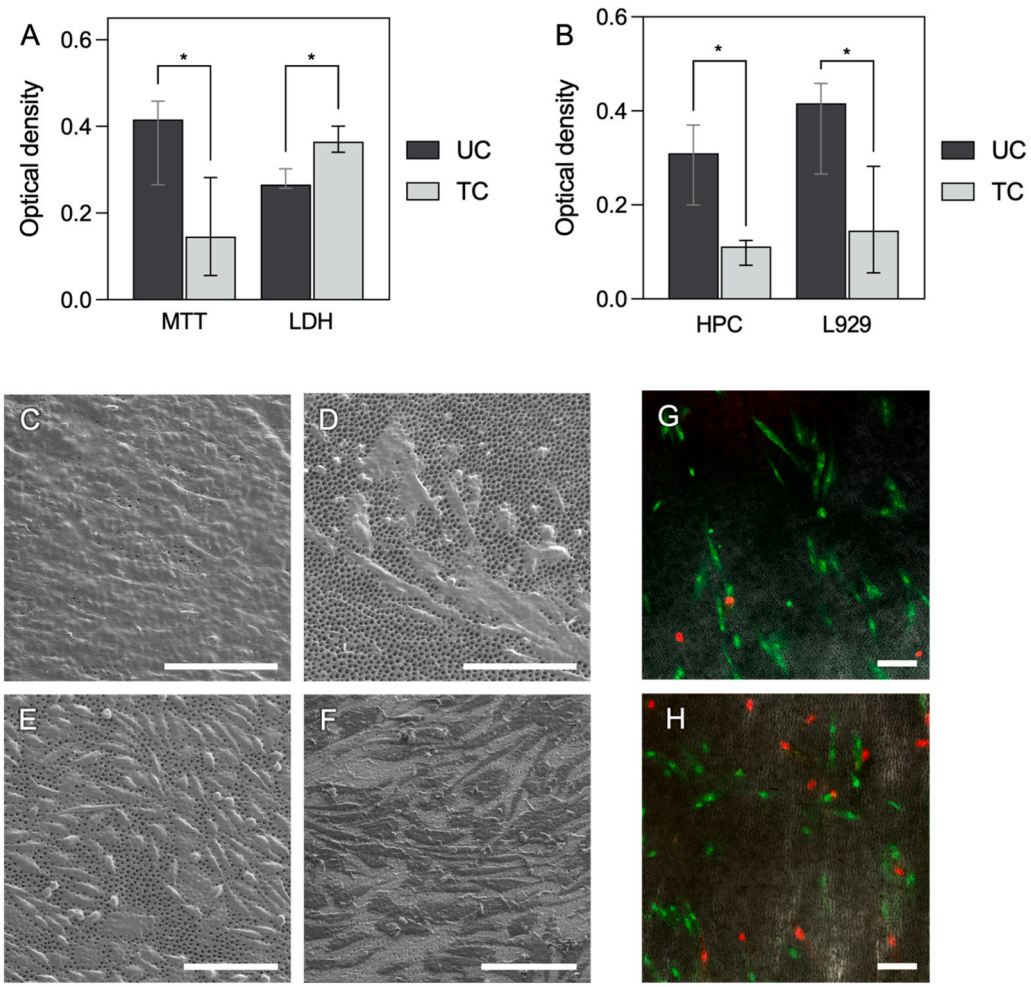

Fig. 4. (A-H) Results of toxicity evaluation with different endpoint observations. (A) MTT test vs. LDH assay: L-929 cells were either untreated or treated with the self-adhesive resin composite (TC) and the effects were recorded. In the MTT test, a high OD indicates a high viability/low cytotoxicity, while the LDH assay measures LDH release due to membrane disruption, with a high OD indicating a high toxicity. (B) Comparison of the responses to transdentinal exposure to a self-adhesive resin composite material of the two cell types in the DBCS over 24 h, as assessed by the MTT test. Data are shown as median and interquartile range. Asterisks indicate significant differences ($P \leq 0.05$). (C-F) SEM images of the two cell types: untreated HPC after 72 h in the DBCS (C) and HPC exposed to a self-adhesive resin composite through the dentin barrier for 24 h (D); untreated L-929 cells in the DBCS (E) and treated L-929 cells (F). (G-H) Representative images of live-dead staining on the dentin disc: UC (G) and TC (H). Images are taken from the center of the dentin disc, with live cells stained green (AO-positive) and dead cells stained red (PI-positive). Scale bars: 100 μm.

The effect of these parameters on the cell response to a self-adhesive resin composite (TC) was investigated.

The toxicity-related reduction in cell viability was not significantly different ($P > 0.9999$) between transdentinal exposure through a steam-sterilized dentin disc and a disc disinfected with chloramine T (Fig. 5A). Despite the absence of steam sterilization in the non-SS group, no contamination of the DBCS or culture medium was observed.

Different dentin thicknesses were tested (200, 300, 400, 600, 800 and 1000 μm), and cell viability was compared between untreated and transdentinally exposed cells. Exposed cells showed reduced metabolism, as determined by the MTT test, across all thicknesses (Fig. 5B). The viability of the UC was not reached in any DBCS exposed to the TC, however, this toxic effect was statistically significant only at thicknesses between 200 and 400 μm ($P \leq 0.0261$).

The origin of dentin discs cut close to the pulp and further distal was also tested. Cell viability remained similar between the two configurations; the MTT test showed no significant differences in viability when exposed to TC ($P < 0.9999$) (Fig. 5C). However, SEM images of dentin discs cut from pulp-proximal dentin showed enlarged tubule diameters and higher tubule density compared to pulp-distal dentin (Fig. 5D and E).

The use of human or bovine dentin discs did not significantly affect cell viability, either in untreated cells (UC) or in cells indirectly exposed to the self-adhesive resin composite through dentin (TC) ($P > 0.9999$) (Fig. 5F). However, human and bovine dentin showed structural differences as revealed by SEM imaging (Fig. 5G and H), with bovine dentin showing a slight variation in the angle of the dentinal tubules due to its cervical origin.

4. Discussion

The DBCS represents an innovative approach to the cytotoxicity testing of dental materials and the development of organotypic cell cultures, offering considerable advantages over extract tests and conventional dentin barrier models. Our study demonstrates as a proof of concept the feasibility of using the newly developed DBCS to evaluate cytotoxic responses under dentin barrier conditions, effectively simulating a more clinical scenario compared to classical cell culture tests and avoiding the problems of the conventional DBT as described in ISO 7405 [23].

Although numerous modifications of the traditional dentin barrier test have been described in the literature, a universally accepted model

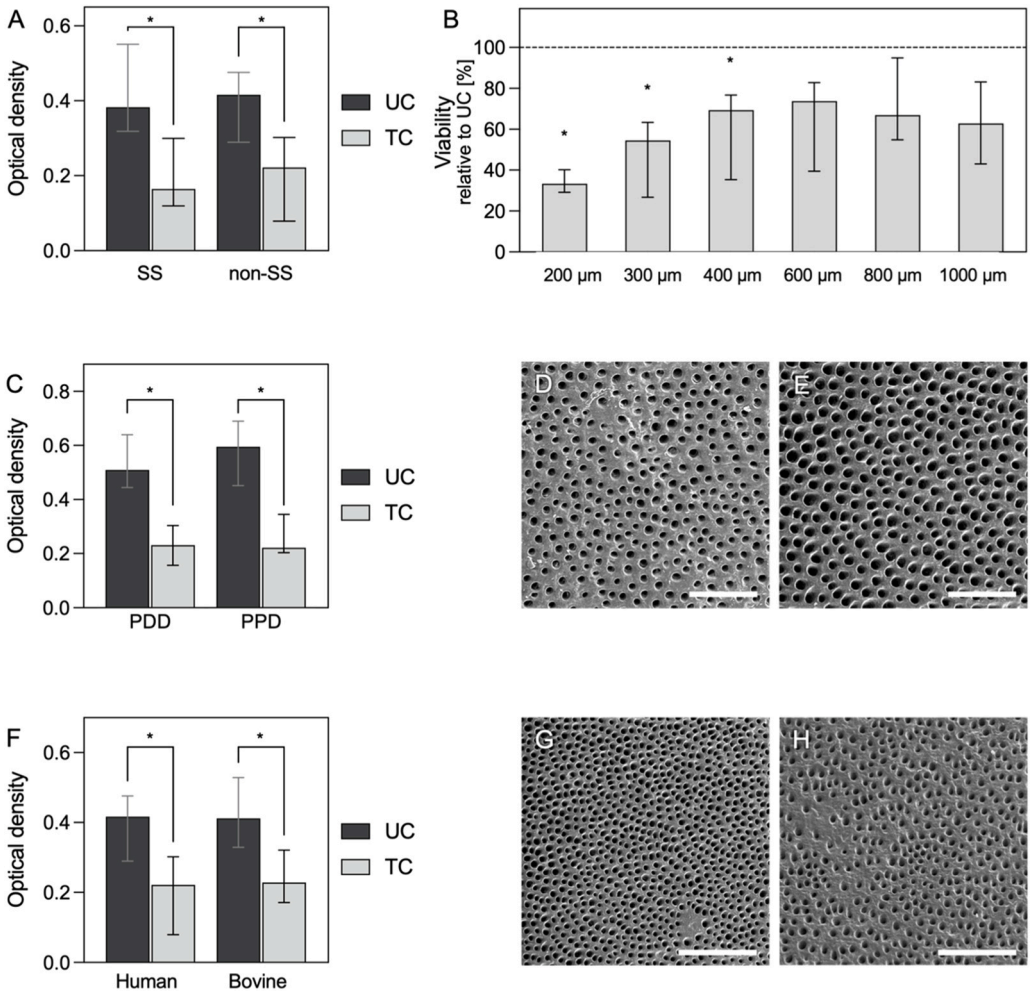

Fig. 5. (A) Results showing the effect of SS on the permeability of dentin discs to TC, as determined by the MTT test. No statistically significant differences were observed between SS and non-SS treated dentin. (B) Comparison of cell viability after indirect exposure to a self-adhesive resin composite through dentin of different thicknesses. Transdentinal exposure to TC resulted in significantly lower cell metabolism in dentin between 200 and 400 µm thickness compared to the UC. No differences were observed in discs with a thickness of 600 µm or more. (C) Comparison of dentin from different origins. No statistically significant differences were found between the two dentin locations, distal (PDD) or proximal (PPD) to the pulp (D-E) SEM images showing variations in the diameter and frequency of dentinal tubules in dentin located distal to the pulp (D) and proximal to the pulp (E). Scale bars: 25 µm. (F) Comparison between human and bovine dentin. Cell viability did not differ significantly between human and bovine dentin, either in untreated controls (UC) or in cells exposed to TC. (G-H) SEM images of human dentin (G) and bovine dentin (H), showing a slight difference in the density of tubules in bovine dentin. Scale bars: 50 µm. The graph displays the median and interquartile range. Asterisks indicate significant differences ($P \leq 0.05$).

for standardized transdentinal cytotoxicity testing has not yet been established [19,26,4,51]. For instance, Jiang et al. adapted the classic DBT by incorporating a 3D polystyrene scaffold within a split-chamber device. A dentin disc was placed on top of the scaffold, creating two compartments. The lower compartment, containing cells, was perfused, while test materials were applied to the upper compartment [26]. In their 2020 study, Hadjichristou et al. introduced the "DentCytoTool," a 3D tissue-engineered model designed to replicate the dentin-pulp complex for biocompatibility testing. This model consists of a Merck Millipore cell culture insert with odontoblast-like cells on the microporous membrane covered by a disc-shaped human treated dentin matrix, upon which materials can be placed, and a lower pulp compartment containing umbilical vein endothelial cells and stem cells from the apical papilla embedded in a collagen I/fibrin hydrogel to simulate vascularized pulp tissue [19]. Caldas et al. developed a Teflon 'fitting device' that holds a 500 µm-thick human dentin disc. In this model, human pulp fibroblasts are cultured at the bottom of a 24-well plate. Materials are first applied to a dentin disc that is then clipped into the device and placed into the well plate [4]. Şişmanoğlu et al. use a custom two-part screwable device, though the material of the device is unspecified. The dentin disc is placed into the outer part of the device, with the inner part screwed into place to create a seal. This is then suspended above cells cultured in a 24-well plate using metal rods, allowing indirect exposure via the culture medium [51]. Despite their novelty, these models present several limitations. They, for example, require specialized equipment [26]. Additionally, establishing stable 3D cultures remains challenging [19,26]. In many of these models, cells are cultured at the bottom of the well rather than in direct contact with the dentin, which limits their ability to accurately simulate the biological response at the dentin-pulp interface [19,26,51].

Another limitation of these models is that seal integrity is not demonstrated, leaving open the possibility that stimulants such as monomers and toxins may bypass the dentin barrier. To accurately simulate *in vivo* conditions, it is critical to ensure that cell exposure occurs only through the dentin tubules. Dentin acts not only as a mechanical diffusion barrier but also as a chemical barrier, with collagen and albumin binding certain composite components and hydroxyapatite neutralizing acids [18,22,45]. Therefore, recreating the dentin barrier within the DBCS is essential for realistic testing of dental materials. In our study, we confirmed the integrity of the DBCS seal, ensuring that

dentin remains the exclusive pathway for material diffusion.

The traditional DBT [23], which is currently part of the ISO guidelines, incorporates media perfusion to better mimic *in vivo* conditions. It is described to play a critical role in improving physiological relevance and assay reliability. Perfusion supports nutrient and gas exchange, prevents waste accumulation, and facilitates the continuous removal of leachates from test materials – factors that help distinguish true material-induced cytotoxicity from artefactual cell death. Moreover, by standardizing exposure conditions, perfusion enhances reproducibility and aligns with ISO 10993–5 recommendations for more accurate cytotoxicity assessment. Nevertheless, the perfusion has been discussed controversially in previous studies: While a perfusion of 2 ml/h showed reduced cytotoxicity for certain materials, higher flow rates have also been reported to harm cells in the system [10,46,47]. A static approach as made in this study facilitates easy placement in a cell culture incubator, requires no specialized equipment, and aligns with widely accepted protocols for initial cytotoxicity testing [23]. Although perfusion was not implemented in the presented DBCS setup, it can be readily integrated into the model design. Respective approaches are underway, as this may allow for improved comparability with the traditional system described by Schmalz et al. [47].

The outward fluid pressure within dentinal tubules is widely recognized as a natural protective mechanism of the dental pulp [31,32]. In the DBCS, this phenomenon is partially mimicked by the hydrostatic pressure exerted by the culture medium in the upper compartment. Although physiological pulpal pressures have been estimated at approximately 14.1 cm H_2O in healthy teeth [6], and various experimental models apply pressures between 5 and 36 cm H_2O, the pressure generated in our system remains significantly lower. Consequently, the model only provides a limited approximation of *in vivo* fluid dynamics. Nonetheless, the presence of a moist surface in the lower compartment, along with the observed dye distribution, clearly indicates a direction of fluid movement – whether due to hydrostatic flow or capillary action through dentinal tubules [1]. Regardless of the underlying mechanism, this fluid presence is critical, as it facilitates solubility and diffusion processes that are essential for the realistic simulation of transdentinal interactions and for evaluating material-associated effects on pulp tissue.

Regarding cell types, the International Organization for Standardization (ISO 10993–5) recommends the use of standardized cell lines (e.g. immortalized cells) for cytotoxicity testing to ensure comparability and reproducibility of results [23]. Therefore, in addition to primary HPC isolated from human pulp tissue, immortalized L-929 fibroblasts were also evaluated. Both cell types successfully adhered and proliferated in the DBCS, forming confluent cell layers, with primary cells reaching the plateau phase more rapidly. No significant differences in cytotoxic responses were observed between the two cell types, however, previous studies suggest that different endpoint observation methods may yield different results [21,35]. Primary cells are considered to have superior repair mechanisms [37] and more closely mimic *in vivo* conditions, particularly since they are the direct target cells for dental filling materials. In contrast, immortalized cells often exhibit increased sensitivity to cytotoxic agents [56] and are easier to handle [42,56]. In summary, primary cells provide a more physiologically relevant model, while immortalized cells offer practical advantages in handling and experimental consistency. This underscores the importance of selecting the appropriate cell model based on specific testing objectives.

In this study, a monolayer culture was selected for its robustness, ease of handling, suitability for high-throughput testing. However, this format presents certain limitations, as it lacks the architectural complexity and the cell-cell and cell-matrix interactions characteristic of native tissue [36,52]. Monolayer cultures may also respond differently to cytotoxic stimuli compared to more physiologically relevant 3D models [36,47]. To address this, future developments of the DBCS will aim to incorporate 3D culture systems to enhance biological relevance and predictive value.

In order to ensure cell growth within the DBCS, it is necessary to condition the dentin by removing the smear layer and exposing the tubular dentin surface. While other models use etching of dentin discs from both sides [26], this approach does not accurately reflect clinical scenarios where the smear layer remains in the cavity and provides a protective effect [33]. Although research suggests that EDTA conditioning of dentin enhances the adhesion, migration, and differentiation of pulp stem cells to or onto dentin [14], other studies suggest that CA treatment may offer comparable benefits with similar cell behavior and viability [40]. 40 % CA was selected for this setup, because it promotes slightly higher cell viability and faster processing time. In addition, CA etching within the DBCS offered a useful indication of proper assembly and sealing. Any leakage through the DBCS resulted in discoloration of the phenol red-containing medium in the lower compartment, serving as a visual marker of inadequate sealing.

For the TC, we chose Vertise Flow, a self-adhesive resin composite, due to its ease of handling, elimination of the need for adhesives, and previously established cytotoxicity in extract tests [35]. Although ISO 7405 Annex B4 provides an example of a positive control material, alternative substances with a validated history and reproducible toxicity data are also permitted [23].

In this study, cytotoxic effects were primarily assessed using the MTT test, which remains a widely accepted and robust method for evaluating cell viability [28]. While this assay indirectly reflects cellular metabolism through the enzymatic reduction of MTT to formazan, its results closely correlate with the number of viable cells. Accordingly, we use the term ‘viability’ throughout this manuscript to describe the outcomes, without attributing them to specific underlying mechanisms such as cell type, metabolic activity or proliferation rate. Nonetheless, we also demonstrated that complimentary assessments of the cellular integrity and health, such as the LDH assay, live-dead staining, and even imaging via SEM, can be successfully implemented within the DBCS.

Depending on availability and ethical regulations, either human or bovine teeth may be more accessible. Human dentin discs more closely mimic *in vivo* tissue properties [26], but variability arises due to differences in dentin origin relative to the pulp and regional sclerosis-related permeability reductions [30,55], In contrast, young bovine dentin has more uniform properties, although, human dentin has a significantly higher tubule density, as confirmed by SEM [30]. Despite the structural differences, studies support bovine dentin as a viable alternative to human dentin [44]. Importantly, the permeability to saline of bovine discs sectioned near the cementoenamel junction is not significantly different from that of human dentin [44] and no significant differences in toxin permeability were observed between human or bovine dentin discs in DPSC applications. In human dentin, permeability is known to be lowest at the enamel-dentin junction and highest near the pulpal interface [16]. However, no significant differences in toxin permeability were observed between proximally and distally sectioned discs within the DBCS application, allowing the use of multiple discs from a single tooth.

Decontamination of both materials and dentin is an important factor in cytotoxicity testing [42]. In terms of the dentin, ISO 7405 recommends steam sterilization for dentin discs [23]. While earlier studies suggested that autoclaving of healthy teeth does not affect dentin permeability [38], more recent findings indicate significant changes in the organic components of dentin. Autoclaving has been reported to reduce dentin permeability by 66 % due to denaturation of type I collagen [25]. Given the interaction with the organic matrix of dentin and the potential release of signaling molecules [57], harsh heat or chemical treatment may affect material-dentin interactions and cytotoxicity. In our study, no significant difference in toxin penetration from the resin composite was observed between the steam-sterilized group and the group treated with chloramine T alone. Given the lack of contamination in our experiments and the established use of chloramine T as a storage and disinfection solution, that can be completely removed from dentin [41], its use for disinfection was considered appropriate.

Dentin thickness plays a critical role in modulating cytotoxic responses by shielding the pulp from irritants through an increased diffusion barrier [26]. Galler et al. demonstrated that cytotoxicity varies with dentin thickness for certain dental materials, a finding we confirm in this study, where a protective effect was observed at dentin thicknesses greater than 400 μm [10]. Notably, *in vivo* pulp irritation becomes significant when residual dentin thickness falls below 0.5 mm, consistent with *in vitro* observations in the DBCS [34,53]. The evaluation of dental materials at different dentin thicknesses provides valuable insights, as it allows precise determination of cytotoxic effect ranges and facilitates objective comparisons with clinically used materials. In addition, future studies should focus on the interactions between irritants and dentin, as these may significantly influence permeation and subsequent biological responses [57].

Overall, the DBCS presented in this study, offers several practical advantages over traditional methods like the DBT, including good potential for standardization, easy repeatability, cost effectiveness, and a controlled cell culture set-up with ease of handling and application. It is compatible with standard cell culture dishes and incubators, ensuring stable temperatures and CO_2 buffering of the culture medium, which maintains reproducible conditions through physiological buffer systems such as bicarbonate, proteins and phosphates [3]. In addition, the use of the incubator and a laminar flow bench minimizes the risk of contamination. Technically, the DBCS design accommodates 24 units in a standard 24-well plate, with the dimensions of upper/pulpal chamber matching those of a 96-well plate. This facilitates comparisons with results from other testing methods, such as extract tests, as comparability with established data remains important [21]. Placing the cells on the upper surface improves accessibility for supernatant analysis and presumed cell attachment, while also allowing the simulation of pulpal pressure, promoting the external flow of dentinal fluid as observed *in vivo*.

By incorporating human dental pulp cells and human dentin, the model may capture essential interactions between dentin matrix proteins and cells, offering a more accurate representation of *in vivo* conditions. However, it also supports the use of established cell lines and bovine dentin for broader applicability. Beyond cytotoxicity, the DBCS allows for the assessment of physiological factors, such as temperature changes caused by the exothermic polymerization of materials. Furthermore, the integration of 3D printing technology and customizable parameters increases the flexibility of the model at a moderate cost, allowing for tailored investigations. Together, these features establish the DBCS as a valuable tool for advancing biological dental material testing, ultimately improving the quality and safety of dental materials and supporting more robust research in the field. To further validate the method and demonstrate its reliability in a range of applications, additional studies will be conducted in the future using a wider range of test materials.

5. Conclusion

The described DBCS provides user-friendly model for evaluating the cytotoxicity of dental materials. Its 3D-printable design allows for easy reproduction on any standard 3D printer, eliminating the need for specialized fabrication or proprietary components. Its accessibility makes it ideal for widespread adoption and standardization across research groups. Despite its simplicity, the DBCS retains critical physiological relevance by allowing the use of variable dentin types and thicknesses. Its modular design supports high-throughput testing with multiple cell types, making it a versatile and practical tool for reproducible and physiologically meaningful *in vitro* testing of dental materials. Further studies with a larger number of dental restorative materials are, however, warranted.

Funding

This research was supported by the DGR2Z-Kulzer-Start Grant and the IZKF ELAN Grant. The funding source had no role in the design of the study; collection, analysis, and interpretation of the data; writing of the report; or decision to submit the article for publication.

CRediT authorship contribution statement

Ella Ohlsson: Conceptualization, Methodology, Formal analysis, Writing - original draft, Investigation, Visualization, Funding acquisition. **Sandra Pohl:** Writing - Review & Editing. **Carola Bolay:** Investigation. **Kerstin M. Galler:** Writing - Review & Editing. **Wolfgang Buchalla:** Writing - Review & Editing, Resources. **Gottfried Schmalz:** Conceptualization, Writing - Review & Editing. **Matthias Widbiller:** Conceptualization, Methodology, Formal analysis, Resources, Writing - original draft, Visualization, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research was supported by the DGR^{2}Z-Kulzer-Start Grant and the IZKF ELAN Grant. The funding source had no role in the design of the study; collection, analysis, and interpretation of the data; writing of the report; or decision to submit the article for publication.

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