Biomaterial and biofilm interactions with the pulp-dentin complex on-a-chip

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Appendix

Fabrication of the tooth-on-a-chip

Third molars extracted for orthodontic reasons according to the institutional ethics committee guidelines were sectioned perpendicular to the dentin tubules using an automated saw (Accutom 5, Struers, Cleveland, OH, USA) to obtain dentin slabs (500 μm W x 1 mm H x 4.5 mm L). These dentin pieces were stored in sodium azide 0.25% at 4ºC for no more than 2 weeks until further use. Next, the tooth-on-chip device was designed using Computer Aided Design (CAD) software (Autodesk Fusion 360, Autodesk Inc, San Rafael, CA, USA) and positive molds fabricated using a laser cutter (Boss LS1416, Boss laser, Sandorf, FL, US) on a 1mm-thick polymethylmethacrylate (PMMA) board. The templates were molded with polydimethylsiloxane (PDMS) cured at 80ºC overnight. After curing, the PDMS was peeled off from the template, and four 8-mm reservoirs were prepared with an 8-mm punch (**Figure 1D**). The device is comprised of two parallel channels, two perfusable chambers (300 μm W x 1 mm L x 1 mm H) and containing a central groove (**Figure 1 D, E**). Subsequently, a PDMS positive mold and PDMS-spin coated coverslips were plasma cleaned (Plasma Cleaner, PDC-32G, Harrick Plasma, Ithaca, NY, US) and a dentin fragment was carefully inserted with tweezers into the groove of the PDMS mold. The system was assembled onto the PDMS-coated coverslip using slight pressure, thus forming a sealed and leak-proof microdevice (**Figure 1E**) with two chambers separated by a semi-permeable membrane (dentin) creating distinct microenvironments for each chamber. The fully assembled microdevice replicates the interface of dentin with the dental pulp on one side and the dental material with dentin on the other, thus forming two accessible chambers representing the "pulp side" and the "cavity side", respectively (**Figure 1E**). Of note, dentin was not submitted to plasma treatment to avoid any chemical or biological change to its structure. The devices were sterilized with ultraviolet light (EXFO Acticure 4000, 365 nm, 45 mW/cm^2) for 40 min prior to use. Each device was then filled with sterile distilled water (DIW) and kept inside sealed petri dishes at 4ºC to prevent dentin dehydration.

hDPSC culture and device seeding

Human dental pulp stem cells (hDPSC, P3-6) (cat # PT5025, Lonza) were cultured in odontogenic medium (alpha Minimal Eagle's medium (α-MEM*)* (Gibco, Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin, 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/mL ascorbic acid (all from Sigma–Aldrich) (Bakopoulou et al. 2011; Jones et al. 2015; Kim et al. 2014; Sabbagh et al. 2020).

Cells were maintained in an incubator (100% humidity, 37ºC, 5% CO_2) for 7 days and cell medium was changed every two days. After reaching 80% confluence, cells were washed with Dulbecco's phosphate buffered saline solution, trypsinized (0.05% Trypsin, Sigma) and counted using a hemocytometer. On day -2 each device had both sides of the dentin treated with 17% ethylenediaminetetraacetic acid (EDTA) for 45 s to remove the smear layer, then rinsed thoroughly with DIW and a suspension of 20 µL with 10^5 hDPSCs was seeded into the 'pulp side' chamber. The device was flipped 90 degrees and incubated for 1 h at 37ºC to promote cell contact and attachment onto the dentin wall. Afterward, reservoirs were filled with medium and the device was incubated (100% humidity, 37 ºC, 5% CO_2) to allow an intact cell layer formation onto the dentin surface for 24 h (**Figure 1F-H**). To better emulate the 3D extracellular matrix environment of the dental pulp, on day 1, we prepared a solution of 1.5 mg/mL collagen I (Collagen I, rat tail, Gibco) following the manufacturer's instructions, then the cell medium from the 'pulp side' was gently removed with a micropipette and 6 µm of the collagen solution was slowly inserted into the 'pulp channel' until the collagen was visibly filling the 'pulp chamber' but not blocking the channels (**Figure 1I-K**). The device was flipped 90 degrees and incubated at 37ºC for 15 minutes to let the collagen self-assemble, then the reservoirs were filled with osteogenic medium and the device was returned to the incubator (100% humidity, 37ºC, 5% CO_2) for another 24 h before the insertion of the biomaterial on the 'cavity side' (**Figure 1L-N**).

Preparation of biomaterials

To evaluate the effect of calcium silicate cements on dentin and pulp cells at the biomaterial-dentin-pulp interface, on Day 0, we treated the opposite side of the dentin of cell-laden devices with ProRoot (MTA), Biodentine, or Theracal LC (**Figure 1L-N**). Devices with no cement placed on the dentin were used as controls. All groups were done in quadruplicate.

Each calcium silicate cement was prepared according to the manufacturer's instructions. Briefly, ProRoot (ProRoot MTA Gray, Dentsply, Tulsa Dental, Tulsa, OK) was prepared by gradually mixing 1 mg powder with the liquid within 1 minute until forming a thick paste with creamy consistency. Subsequently, the device had the cell medium gently removed from the cavity channel using a micropipette, and the MTA was gently inserted with a dental curette until the paste was completely covering the dentin and filling the 'cavity side'. For Biodentine (Septodont, Saint Maur des Fosses, France) the capsules of the cement were mixed using an amalgamator, then the cement was gently applied as described for MTA. For Theracal (Bisco Inc, Schaumburg, IL), the material was inserted into the 'cavity side' of device, a photomask was placed on the 'pulp side' to protect the cells from light injuries and a dental light (395–480 nm, 10.5 mm curing tip, 1650 mW/cm^2 Valo Ultradent Products Inc, South Jordan, UT, USA) was used to polymerize Theracal for 20 s, shining perpendicular to the interface, from the bottom of the device. Of note, during the whole process, the 'pulp side' was filled with cell medium.

Live and dead assay

The effect of the biomaterials on the viability of hDPSCs at the biomaterial-dentin-pulp interface on days 1 and 7 was determined using a Live/Dead assay kit (Molecular Probes, cat # R37609) under a fluorescence microscope (EVOS FL Auto, Life Technologies). The stain was added to the cell medium, the devices were incubated for 15 min (37ºC, 5% CO_2). All groups (control, ProRoot, Biodentine and Theracal) were done in quadruplicate, and at least 3 locations of each samples were imaged. Live and dead cells were counted using ImageJ software (Fiji, NIH, Maryland, USA). Since the NucBlue® live reagent binds to the DNA of all cells, live and dead, we calculated the number of live cells by subtracting the number of cells stained in blue by the number of cells stained in green (**Supplementary Figure 1**).

hDPSC staining and imaging

On day 1 and 7, at least four chips from each group were rinsed with PBS, fixed with 4% paraformaldehyde (v/v) for 30 min, rinsed with PBS, and cell membranes permeabilized with 0.1% (w/v) Triton X-100 for 15 min under gentle agitation. Unspecific biding sites were blocked with 1.5 % (w/v) bovine serum albumin (BSA) for 1 h and after washing with PBS, chips were incubated in Actin Red 555 (cat. # R37112, Molecular Probes, ThermoFisher) for 1h, rinsed with PBS and then incubated with NucBlue (cat. # R37606, Molecular Probes, ThermoFisher) for 30 min at 37 °C, rinsed with PBS and immediately imaged. Imaging was conducted in a confocal microscope (Zeiss, LSM 880, Germany) with a 20X objective (Zeiss, Plan-Apochromat 20x/0.8 M-27) and an imaging depth of 100-200 µm, split into at least 20 Z-stacks. Zen or Imaris software (v9.1, Bitplane – Oxford Instruments, Zurich, Switzerland) were used to convert 3D (XYZ) Z-stacks into TIFF files. The whole cell layer in contact with dentin was photographed in 3 consecutive images and analyzed using ImageJ (Fiji, NIH, Maryland, USA).

Measurement of pH and TGFβ release

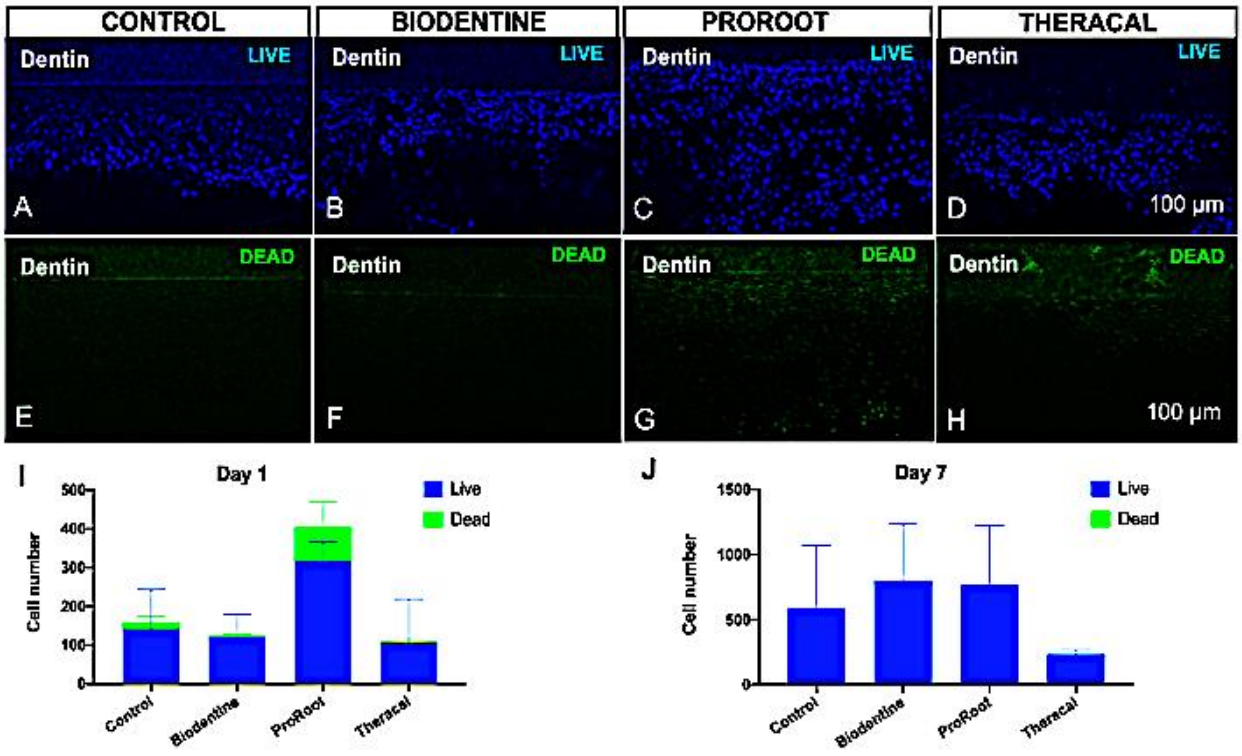
To evaluate the potential influence of each cement on the cumulative release of growth factors and other bioactive molecules from the dentin matrix, we used TGFβ as a target surrogate protein marker. We prepared the devices, treated the dentin with 45 s of 17% EDTA and filled the pulp chamber with DIW. The cements were applied as described above. The reservoirs were filled with DIW and incubated for up 7 days at 100% humidity and 37ºC. After 6, 24, 48 and 72 h, a 15 µl aliquot of the DIW from the 'pulp side' was collected and pH was measured with a pH tape. Additionally, we removed 200 µl of DIW from each chip and stored it at 80ºC for ELISA at these same time points, with the addition of a 7-day sample. An ELISA assay was performed following the manufacturer's directions (cat # ELH-TGFb1, Raybiotech, Peachtree Corners, GA, USA). In short, high binding 96-well plates were pre-coated with anti-human TGFβ and subsequently blocked with BSA. TGFβ standards and supernatant were activated, loaded and detected with biotin-conjugated anti-human TGFβ. Four replicates were used for each condition. The optical density was read at 450 nm using a spectrophotometer (Epoch Microplate Spectrophotometer, BioTek Instruments Inc, Winooski, VT, USA)

Biofilm confocal imaging and analysis

We developed a biofilm of *S. mutans* onto the 'cavity side' of the dentin for 19h (**Figure 4A**). The three-dimensional architecture/bacterial viability of the biofilm and the impact of biomaterial treatment were examined by high-resolution confocal microscopy using optimized protocols with modifications (Ren et al. 2019). Briefly, the device was incubated for 1h at 37°C after applying CSC before assessing live/dead of *S. mutans*. Next, biofilms were pre-stained with 5 μM SYTO9 and 30 μM propidium iodide (Molecular Probes) to label live and dead bacteria after removal of the culture medium. ProRoot was mixed with DIW supplemented with 5 μM SYTO9 and 30 μM propidium iodide and gently applied into the cavity side until the biofilms on the dentin surface were covered. For the control group, DIW with 5 μM SYTO9 and 30 μM propidium iodide was added. The device was incubated for 1h at 37°C before multi-channel images were acquired using an LSM800 confocal microscope (Zeiss) equipped with a 40× water immersion objective lens (numerical aperture = 1.0). Biofilms on the dentin surface were sequentially scanned (488nm laser for SYTO9, 561nm laser for propidium iodide and 405nm with reflection mode for the biomaterial) with optimum emission filters. At least 4 positions of each group were imaged. Computational analysis of biofilm confocal images was conducted using BiofilmQ to determine the percentage of dead bacteria in the residual biofilm (Hartmann et al. 2021).

Supplementary Figures

Supplementary Figure 1 – Representative images of cells cultured with different CSC on day 1



Supplementary Figure 1: **Representative images of cells cultured with different CSC on day 1**. Groups cultured with ProRoot (C) had significantly more cells per mm^2 than the other groups (A-I) ($p<0.05$) (Two-way ANOVA with Tukey post-hoc, $\alpha=0.05$).

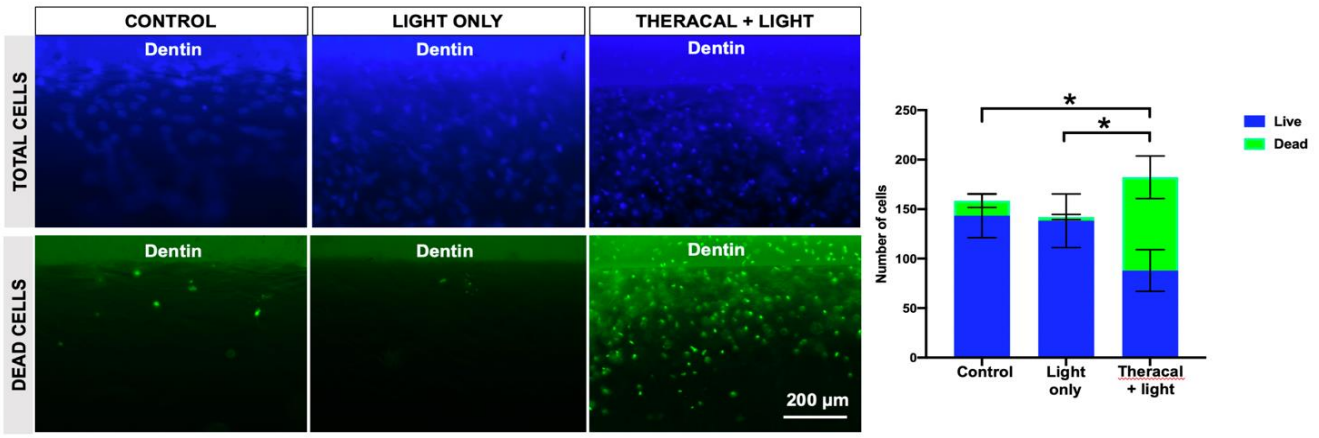
Supplementary Figure 2 – Comparison of the effect of Theracal and light only on cell viability

To clarify whether light exposure would have a deleterious effect on the cells, we performed new experiments in which we fabricated cell layer with dental pulp stem cells on-chip, then added the collagen and after 24 h we tested:

- Control group: no treatment in the 'cavity chamber'.

- Theracal group: addition of Theracal and polymerization with dental light for 20 s. For the polymerization, a photomask was placed on the 'pulp side' to protect the cells from light injuries and a dental light was used for 20 s, shining perpendicular to the interface, from the bottom of the device.

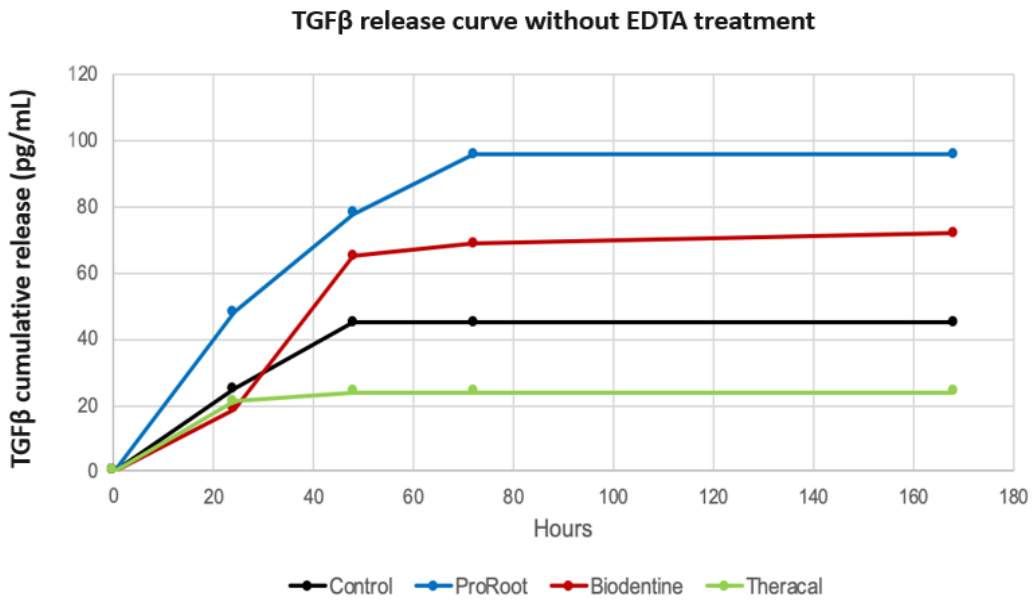
- Light group: to evaluate the effect of light on cell viability, this group was not treated with Theracal, but received the same irradiation as described above.



Supplementary Figure 2: A live and dead assay was performed after 4 h showing that the group which received only the light irradiation had a similar number of live and dead cells as the control, while cells cultured with Theracal showed around 50% of cell death in all samples (n=3, one-way ANOVA with Tukey post-hoc, $p < 0.05$).

Supplementary Figure 3 - Detection of TGFβ after 7 days of treatment with biomaterials

This graph shows the concentration of TGFβ in the pulp chamber of the devices in different experimental time points when the dentin was not treated with EDTA. ProRoot and Biodentin elicited the highest levels of TGFβ release at 24 and 48 h. (ANOVA with Tukey post-hoc).



Supplementary Figure 3 – Detection of TGFβ after 7 days of treatment with biomaterials. This graph shows the concentration of TGFβ in the pulp chamber of the devices in different time points when the dentin was not treated with EDTA. ProRoot and Biodentin elicited the highest levels of TGFβ release at 24 and 48 h. (ANOVA with Tukey post-hoc).

Supplementary References

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