

Survival of human dental pulp cells after 4-week culture in human tooth model

Mariano S. Pedano^{a,1}, Xin Li^{a,1}, Charlotte Jeanneau^b, Manosij Ghosh^c, Kumiko Yoshihara^{d,e}, Kirsten Van Landuyt^a, Imad About^b, Bart Van Meerbeek^{a,*}

^a KU Leuven (University of Leuven), Department of Oral Health Sciences, BIOMAT & UZ Leuven (University Hospitals Leuven), Dentistry, Kapucijnenvoer 7, 3000 Leuven, Belgium

^b Aix Marseille Univ, CNRS, IMS, Inst Movement Sci, Marseille, France

^c KU Leuven, Department of Public Health and Primary Care, Centre Environment and Health, Kapucijnenvoer 35, 3000 Leuven, Belgium

^d Okayama University Hospital, Center for Innovative Clinical Medicine, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan

^e National Institute of Advanced Industrial Science and Technology (AIST), Health Research Institute 2217-14 Hayashi-cho Takamatsu Kagawa 761-0395 JAPAN

ARTICLE INFO

Keywords:

Pulp biology
Vital pulp therapy
Pulp capping
Tissue engineering
Biomaterials
Calcium-silicate cements

ABSTRACT

Objectives: This study aimed to validate the human tooth model by investigating the growth efficiency, expression of mesenchymal stem cell (MSC) markers and differentiation ability of human dental pulp cells (hDPCs) harvested from extracted immature third molars and cultured for different periods. Moreover, the effect of exposure and capping with a hydraulic calcium-silicate cement on pulp tissue after 4-week culture in the tooth model was investigated.

Methods: Primary hDPCs were collected from 18 molars from six individuals (15–19 years). One tooth of each patient was immediately cultured (control), while the other teeth were exposed to culture medium for 1, 2 or 4 weeks. After different culture periods, cells were harvested using the explant method, upon which cells were evaluated for cell-doubling time, colony-forming efficiency and expression of cell surface markers. The osteogenic, adipogenic and chondrogenic differentiation efficacy was also determined. Two teeth from three different patients (n = 6) were used for the pulp-capping assay. Three teeth were capped with ProRoot MTA (Dentsply Sirona), while three other exposed teeth remained uncapped (control).

Results: Cells were found to grow, express MSC markers and showed osteogenic, adipogenic and chondrogenic differentiation potential at all time periods. Histology of the teeth subjected to the pulp-capping assay showed the formation of mineralized tissue after 4-week exposure to ProRoot MTA (Dentsply Sirona) and normal histological features in the control teeth.

Conclusions: This study confirmed that hDPCs of teeth cultured for up to 4 weeks in a human tooth model are viable, express MSC markers and show differentiation ability.

Clinical Significance: The human tooth model can be seen as an advanced cell-culture model that makes use of the original 3D pulp-chamber structure. It can serve as a screening tool to evaluate new pulp-capping formulations in a relatively cheap and fast manner.

1. Introduction

Hydraulic calcium-silicate cements (hCSCs) are becoming the material of choice for pulp-capping procedures [1,2]. However, the body of evidence to support their clinical use is coming mostly from *in-vitro* tests or *in-vivo* animal studies, instead of well-controlled randomized clinical trials [3,4].

While cell-culture laboratory studies are very useful for investigating some of the basic cell-interaction/response requirements of the materials, they do not provide clinical evidence of actual pulp-capping effectiveness. Moreover, most of the cell studies involve 2D-cell cultures, which have limitations because these cultures do not represent cell behavior in 3D structures [5–7]. On the other hand, animal studies are good models for testing pulp-capping agents, but the pulp-tissue

* Corresponding author at: KU Leuven (University of Leuven), Department of Oral Health Sciences, BIOMAT & University Hospitals Leuven (UZ Leuven), Dentistry, Campus Sint-Raphaël, block A - box 7001, Kapucijnenvoer 7, BE-3000 Leuven, Belgium.

E-mail addresses: bart.vanmeerbeek@kuleuven.be, bart.vanmeerbeek@uzleuven.be (B. Van Meerbeek).

¹ Equal first-author contribution.

reaction when exposed to materials is not necessarily the same as that recorded in humans [8]. In this way, an *ex-vivo* tooth-culture model has been introduced [9–11], hereby also providing means to reduce animal experimentation. The pulp-capping agents can be applied as done clinically, while the pulp cells remain within their natural structural arrangement and can function in nearly similar physiological conditions as those in the human body. However, the application of this model to evaluate pulp-capping effectiveness can also be criticized as it lacks the immune and vascular systems of the patient. Nevertheless, the *ex-vivo* tooth-culture model can be seen as an improved cell-culture model, where the original 3D scaffold is used and tooth pulps are capped following common clinical procedures. Even if the tooth model cannot fully replace animal models, which probably will remain as ultimate test prior to *in-vivo* human use, it can, when validated, serve as a screening tool to evaluate new pulp-capping formulations in a cheaper and faster manner with a reproducibility that may be higher than that of animal models. However, the survival and characterization of the human dental pulp cells (hDPCs) in the *ex-vivo* tooth model at different time points has not sufficiently been investigated to validate the experimentation model.

The main goal of a pulp-capping treatment is to maintain pulp vitality of the affected tooth [12]. To achieve this, a cascade of physiological biochemical events needs to occur, starting from a slight inflammatory reaction generating chemical signals that will lead dental pulp progenitor cells to migrate to the affected area [13]. Moreover, these cells have been shown to proliferate and differentiate to form a mineralized bridge that will separate the pulp tissue from the noxious stimuli to avoid further damage and eventually to enable the pulp to heal. In this way, a successful *in-vitro* pulp-capping model should imitate the initial steps of reparative dentinogenesis of human teeth in a similar time frame.

Therefore, the objective of this work was to characterize hDPCs harvested from teeth that were freshly extracted and cultured for up to four weeks. Moreover, we aimed to evaluate the histological reaction of teeth capped with calcium-silicate cement upon culture for 4 weeks, in comparison with teeth with the pulp exposed but not capped. The null hypothesis tested was that the cells harvested from pulp tissue of teeth cultured for 1, 2 or 4 weeks were not able to grow, differentiate and express mesenchymal/medicinal stem/signaling-cell (MSC) markers as hDPCs from teeth that were cultured immediately upon extraction. Our secondary null hypothesis was that the teeth capped for 4 weeks do not show any histological reaction to the exposure or capping material.

2. Materials and methods

2.1. Sample collection and human dental pulp cells (hDPCs) isolation

The samples were gathered after approval by the Commission for Medical Ethics of KU Leuven (file number S54254) and following informed consent from the donors. Fully impacted human third molars with their roots only partially formed, were collected immediately after extraction from six healthy young patients (15–19 years). A total of 18 teeth were used in this experiment. In the surgery room, immediately after extraction, the teeth were placed in 50 ml centrifuge tubes (VWR, Leuven, Belgium), containing Dulbecco's Modified Eagle Medium (DMEM; Gibco, Merelbeke, Belgium) supplemented with 10% Fetal Bovine Serum (FBS; Gibco), 1% penicillin-streptomycin (Gibco) and 1% amphotericin B (Gibco) (tooth-culture medium). The samples were brought to the cell-culture room within 4 h to proceed with the culture experiments. From each patient, one tooth was immediately processed ($n = 6$; control), while the other teeth were cultured for 1, 2 or 4 wks, as described below (Fig. 1: one control and three experimental groups).

All teeth were rinsed in 70% ethanol (Hydral 70, VWR, Leuven, Belgium) for 1 min, followed by sterile Phosphate Buffered Saline (PBS; Sigma Aldrich, St. Louis, MO, USA) for 1 min and then placed in tooth-

culture medium until they were used. The periodontal ligament was removed with a sterile #15 scalpel blade (Swann Morton, Sheffield, UK). The immediately processed teeth were mechanically split using a sterile high-speed diamond bur under water cooling in the flow hood, enabling to gently collect the pulp tissue using sterile tweezers. The isolated pulp tissue was next cut with a new sterile blade into approximately 1-mm³ fragments. These pulp-tissue fragments were seeded on the surface of 25-cm² cell-culture flasks (Costar, Cambridge, MA, USA) filled with 1 ml culture medium consisting of DMEM (Gibco) supplemented with 10% FBS (Gibco) (cell-culture medium). When the hDPCs reached 70–80% confluence, the cells were harvested using 0.25% Trypsin/EDTA (Gibco) and observed as passage 0. The hDPCs were cultured in 175-cm² cell-culture flasks (Costar) at 37 °C, 5% CO₂ and 95% humidity. Cells at passages 2–5 were further used in this study.

2.2. Ex-vivo human tooth-culture model

The teeth that were not immediately processed, were placed in tooth-culture medium for 1, 2 or 4 weeks. After cleaning, the teeth were handled with sterile gauzes (Yibon Medical, Kuurne, Belgium) soaked in tooth-culture medium to avoid desiccation. The tooth crown was gently dried with a sterile cotton swab, upon which the occlusal surface was locally etched for 30 s with phosphoric acid (Scotchbond Universal Etchant, 3 M Oral Care, Seefeld, Germany), rinsed with distilled water for 20 s and again gently dried with another sterile cotton swab. Next, flowable composite (G-aenial Flo, GC, Tokyo, Japan) was applied on the occlusal surface, in which a sterilized stainless steel orthodontic wire (M form; ORMCO, Orange, CA, USA) was seated, followed by 40-sec light-curing of the flowable composite using a light-curing unit with a light output of 1000–1200 W/cm² (Bluephase 20i, Ivoclar Vivadent, Schaan, Liechtenstein). The teeth were immediately hanged using the wire in separate wells of 24-well culture plates (Costar, Cambridge, MA, USA), each containing 1.5 ml of tooth-culture medium to ensure generous exposure of the pulp tissue to the medium. The medium was refreshed every day. The teeth were retrieved from culture for hDPC isolation after respectively 1-wk ($n = 5$), 2-wk ($n = 4$) and 4-wk ($n = 3$).

2.3. Cell characterization as mesenchymal stem cells (MSC)

The cultured cells were characterized to evaluate their mesenchymal stem cells (MSC) properties, this employing the criteria of the International Society for Cellular Therapy [14].

2.3.1. Cell-doubling time assay

2×10^5 hDPCs were seeded in four 75-cm² cell-culture flasks (Costar) with the cell-culture medium changed three times per week. The cells were trypsinized after 2, 4, 7 and 10 days, upon which the cell number was counted to produce a cell-growth curve. The experiment was conducted in triplicate with cells from three different patients.

2.3.2. Colony-forming unit assay (CFU)

500 and 2000 cells were seeded in 6-well plates (Costar) with the cell-culture medium changed three times per week. After 14 days, the medium was removed, upon which the cells were fixed with 4% paraformaldehyde (PFA; VWR) for 20 min at room temperature (RT: 21 °C) and stained with 1% crystal violet (Sigma-Aldrich) for 1 h. After washing with PBS, cell colonies containing more than 50 cells were counted under the light microscope (Primo Vert, Carl Zeiss, Jena, Germany). The experiment was conducted in triplicate with cells from three different patients.

2.3.3. Flow cytometry analysis of surface markers

5×10^5 hDPCs were seeded in T-175 culture flasks (Costar) and harvested with 0.25% EDTA-free trypsin (Gibco) when 80% confluence

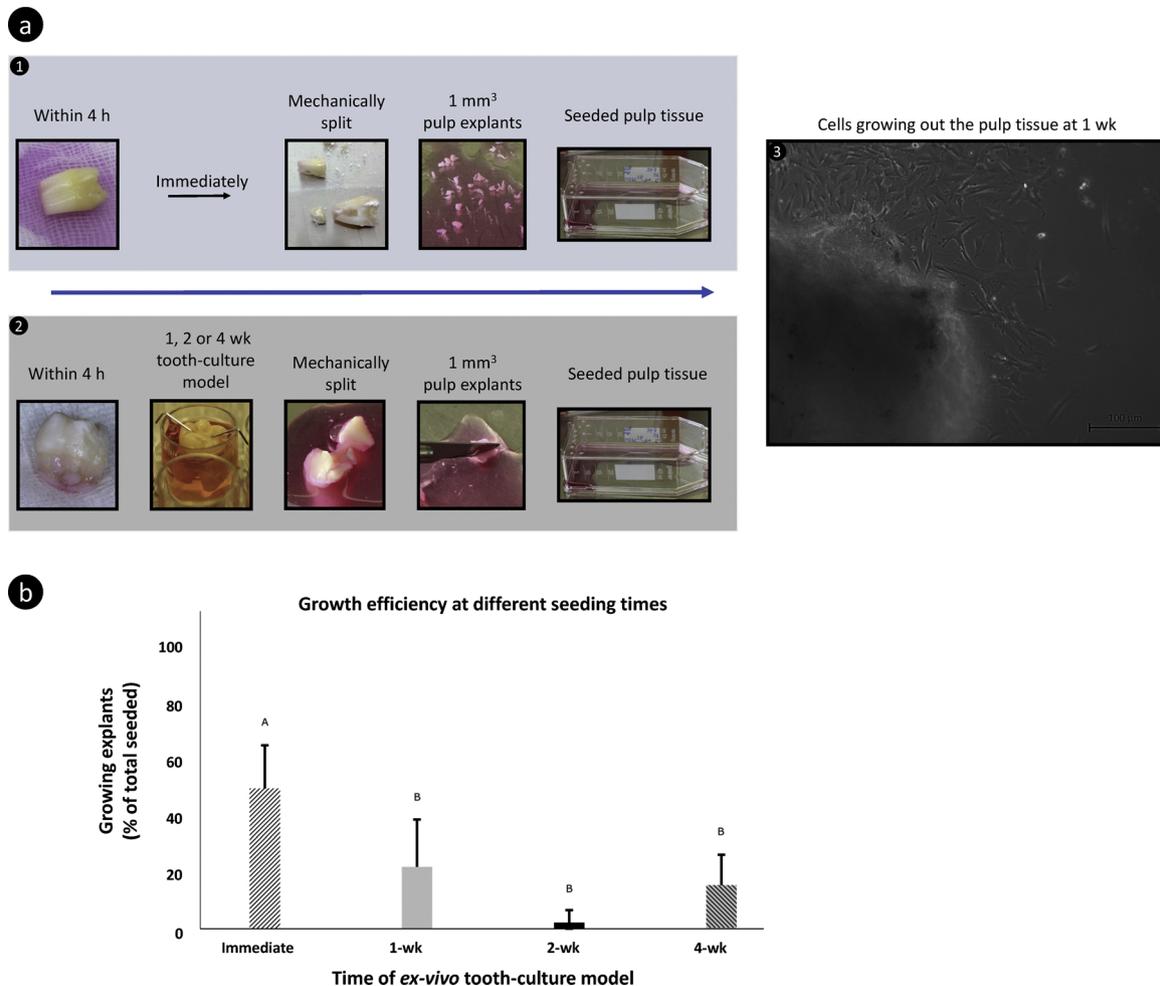


Fig. 1. Experimental set-up of the study in (a) and cell-growth in (b). (a) Four teeth were gathered from the same patient. (a-1) Immediately after collecting them from the surgery room (within 4 h), the teeth were cleaned and the pulp tissue was mechanically exposed and cut with a sterile blade into approximately 1-mm³ explants; 10 to 15 pulp explants per tooth were gently placed with the help of a sterile pipette tip at the bottom of a T-25 cell-culture flask containing 1 ml cell-culture medium (DMEM + 10% FBS). At the beginning, the flask was turned upright to avoid excess contact of the cells with the medium. After 12 h, the flask was placed in its normal position to enable good contact with the medium; (a-2) Immediately after collecting the teeth from the surgery room (within 4 h), the teeth were cleaned and placed in tooth-culture medium (DMEM supplemented with 10% FBS, 1% Penicillin-streptomycin and 1% amphotericin B) for 1, 2, and 4 wk; hDPCs isolation and culture was done as described in a-1; (a-3) Representative light-microscopy image showing the outgrowth of pulp fibroblasts from the seeded tissue at 1 wk. (b) Cell-growth efficiency in percentage of the number of pulp explants with outgrowing cells at the different seeding times. The bar height represents the mean with the line extensions representing the standard deviation. For each seeding time, different letters indicate statistically significant cell-growth efficiency ($p < 0.05$).

was reached. After washing with PBS, groups of 5×10^5 cells in PBS with 1% Bovine Serum Albumina (BSA; Sigma Aldrich) were deposited in Eppendorf tubes (VWR) protected from light for each antibody. The cells were subsequently incubated for 1 h at 37 °C with the primary antibodies CD34, CD73, CD105, CD45, and CD90 (all from BD Biosciences, Aalst, Belgium), and Stro-1 (Santa Cruz Biotechnology, San Francisco, CA, USA). As a negative control for non-specific background staining, appropriate isotype controls were also included. Thereafter, the cells were washed twice with PBS and re-suspended in PBS with 1% BSA followed by flow cytometry analysis using a FACSVerser flow cytometer (BD Biosciences) equipped with CellQuest Pro Software (BD Biosciences). The experiment was repeated three times with cells from three different patients.

2.3.4. Osteogenic, adipogenic and chondrogenic assay

For osteogenic differentiation, hDPCs were seeded at a density of 2×10^5 cells in a 6-well plate (Costar). After 24 h, the seeded cells were exposed to osteogenic differentiation medium consisting of cell-culture medium supplemented with 50 µg/ml ascorbic acid, 10 mmol/L β-glycerophosphate and 10 nmol/L dexamethasone (all from Sigma-Aldrich). The differentiation medium was changed three times per week. After 3-

wk exposure, the cells were fixed with 4% PFA for 30 min. After washing with PBS, the calcium deposits were detected by 2% Alizarin red S staining (pH 4.1–4.3; Sigma-Aldrich) for 10 min at RT.

For adipogenic induction, hDPCs were seeded at a density of 2×10^5 cells on a 6-well plate (Costar). After 24 h, the cells were incubated in adipogenic medium (Adipogenic Differentiation Kit, Gibco) for 6 wk following the manufacturer's instructions. After fixation in 4% PFA for 30 min, the cells were washed and stained with 0.5% (w/v) Oil Red O reagent (Sigma-Aldrich) for 15 min to reveal lipid droplets.

For chondrogenic differentiation, hDPCs were seeded at a density of 2×10^5 cells on a 6-well plate (Costar). After 24 h, the cells were incubated in chondrogenic medium (Chondrogenic Differentiation Kit, Gibco) for 3 wk following the manufacturer's instructions. After fixation in 4% PFA for 30 min, the cells were washed and stained with 1% (w/v) Safranin O reagent (VWR) for 5 min to reveal chondrogenic deposits.

2.4. *Ex-vivo* human tooth-culture pulp-capping assay

Six teeth from three different patients were gathered and handled as described above. Once the teeth were cleaned, a pulp-capping procedure was performed in sterile conditions and with the aid of 2.8x

magnification. For the pulp-capping assay, we followed the protocol described by Télclès et al. 2005 [9]. Briefly, a class-I cavity (approx. 4 × 4 × 4 mm) was cut using a sterile bur (1.1 mm in diameter; Endo Access Bur Size 1, A 0164 300 001 00, Dentsply Sirona, Ballaigues, Switzerland) at high speed under copious irrigation with sterile saline (Fresenius Kabi, Bad Homburg, Germany). The pulp was exposed with a round carbide bur (1.0 mm in diameter; H1SE.205.010, Komet, Lemgo, Germany) at low speed with abundant irrigation. Afterwards, the cavity was cleaned with sterile saline and gently dried with sterile cotton pellets.

The teeth were divided into two groups depending on the pulp-capping procedure carried out: (1) Application of the commercial calcium-silicate cement ProRoot MTA ('MTA'; n = 3; Dentsply Sirona), or (2) without capping as negative control (n = 3). MTA was applied in a 2–3 mm layer with a calcium-hydroxide applicator on the exposed pulp and gently compacted with sterile cotton pellets. The cavity was restored with glass-ionomer cement (Fuji II LC Capsules, GC, Tokyo, Japan). A sterile wire (M-form; ORMCO) was used to hold the teeth in the wells of a 24-well plate, containing 1.5 ml tooth-culture medium. The negative control teeth, which were not capped, were completely immersed in the medium. The medium was refreshed every day and after four weeks, the wire was removed and the teeth were immediately placed in 4% PFA for two weeks to properly fix the tissue.

2.4.1. Histology

Upon fixation, the teeth were decalcified (4–6 weeks) with 10% formic acid (Chem-Lab Analytical, Zedelgem, Belgium) and subsequently processed in a routine way for histology, as described previously [15,16]. Serial sectioning was performed using a microtome (Microm HM 360, Hyland Scientific, Stanwood, WA, USA) set at 5–7 µm until the whole pulp tissue was cut. Every six sequential sections, two were randomly selected. One section was stained with Gill's III hematoxylin (Leica Microsystems, Diegem, Belgium) and 1% aqueous eosin solution (Leica Microsystems) (H&E); the other section was processed for Gram-Twort bacteria staining. These sections were examined using light microscopy (Axio Imager M2, Carl Zeiss).

2.5. Statistical analysis

For the cell-doubling time, CFU assay and the antibody staining capacity of cells, the Kruskal-Wallis test was applied with Mann-Whitney-U test as post-hoc analysis when necessary ($p < 0.05$).

3. Results

3.1. Isolation of hDPCs

After 1-wk culture following the outgrowth method, hDPCs started to grow out of pulp tissue from the immediately processed teeth (control), as well as from the teeth that were cultured for 1, 2 and 4 wk (Fig. 1). However, from the teeth that were immediately processed, the cells grew out after 1 week from significantly more pulp explants ($p < 0.05$) than from the teeth that were cultured for 1–4 wk (Fig. 1b). In addition, seeded cells from the immediately processed teeth could be trypsinized and start passaging already after 14 days, while it took 21 days (1 wk longer) before the cells from the 1–4-wk cultured teeth could be trypsinized and start passaging. No significant difference ($p \geq 0.05$) in growth efficiency was found between the teeth that were cultured for 1–4 wk.

3.2. Cell characterization as mesenchymal stem cells (MSC)

3.2.1. Cell-doubling time assay

The cell-growth curves measured for the control and three experimental groups are shown in Fig. 2a. No significant difference ($p \geq 0.05$) in cell-doubling time was found between the cells originating from the

control immediately processed teeth and the teeth that were cultured for 1–4 wk.

3.2.2. Colony-forming unit (CFU) assay

No significant difference ($p \geq 0.05$) in colony forming ability was found between the cells originating from the control immediately processed teeth and the teeth that were cultured for 1–4 wk and this for the 2000 and 500 cells seeded (Fig. 2b).

3.2.3. Flow cytometry analysis

Flow cytometry analysis showed a negative staining for the hematopoietic stem-cell markers CD34 and CD45, and a positive staining for the MSC markers STRO-1, CD90, CD105 and CD73. No significant difference ($p \geq 0.05$) in surface marker staining was found between the cells originating from the control immediately processed teeth and the teeth that were cultured for 1–4 wk (Fig. 3 a,b).

3.2.4. Osteogenic, adipogenic and chondrogenic differentiation

The cells isolated in the control and all experimental groups grew, forming alizarin red-positive mineral deposits, oil red O-positive lipid clusters and red chondrogenic staining upon induction in osteogenic, adipogenic or chondrogenic culture medium, respectively (Fig. 4).

All abovementioned findings combined confirm that the cells originating from the extracted teeth in the control and experimental groups meet the MSC characteristics as per guidelines by the International Society for Cellular Therapy [14].

3.3. Ex-Vivo human tooth-culture pulp-capping assay

All MTA-capped teeth showed the formation of a continuous band of collagen at the contact area between pulp and material (Fig. 5b). Moreover, mineral foci could be distinguished all around the exposure area (Fig. 5a–c). When the pulp was not capped (negative control), the pulp tissue showed a normal architecture without eosinophilic area and the cell distribution in the exposed area did not differ from the rest of the pulp tissue (Fig. 5).

4. Discussion

This study showed that the cells growing out from pulp tissue of teeth processed immediately after extraction have a better growth efficiency than the cells originating from teeth that were cultured for 1, 2 or 4 wk. However, there was no difference in cell-doubling time, colony-forming efficiency, expression of MSC markers, nor osteogenic/adipogenic/chondrogenic differentiation. Therefore, the first null hypothesis was partially rejected.

Our secondary null hypothesis that the teeth capped for 4 weeks do not show any histological reaction to the capping materials was also rejected.

The cells growing out from teeth cultured for 1, 2 and 4 wk were able to grow and express mesenchymal stem-cell characteristics in a similar way as the cells originating from the control immediately processed teeth. Pulp tissue from teeth kept in medium for up to 4 weeks still contained cells that behaved in a similar way as cells from immediately seeded pulp tissue. The cells were vital since they demonstrated MSC characteristics, which may serve as potential progenitor cells to induce pulpal repair.

Histology of the teeth capped with MTA showed tissue reaction with signs of initial mineralization in contrast to the teeth that were not capped. The histology of the three teeth capped with MTA revealed the formation of a continuous collagen band and the formation of mineral foci around the exposed area (Fig. 5a–c). These histological features have been confirmed in our study by H&E staining and also by immunostaining by previous research by other groups (positive staining for Nestin, Collagen I and Dentin Sialoprophosphoprotein) [9,10,17]. On the other hand, the teeth exposed but not capped with any material, did

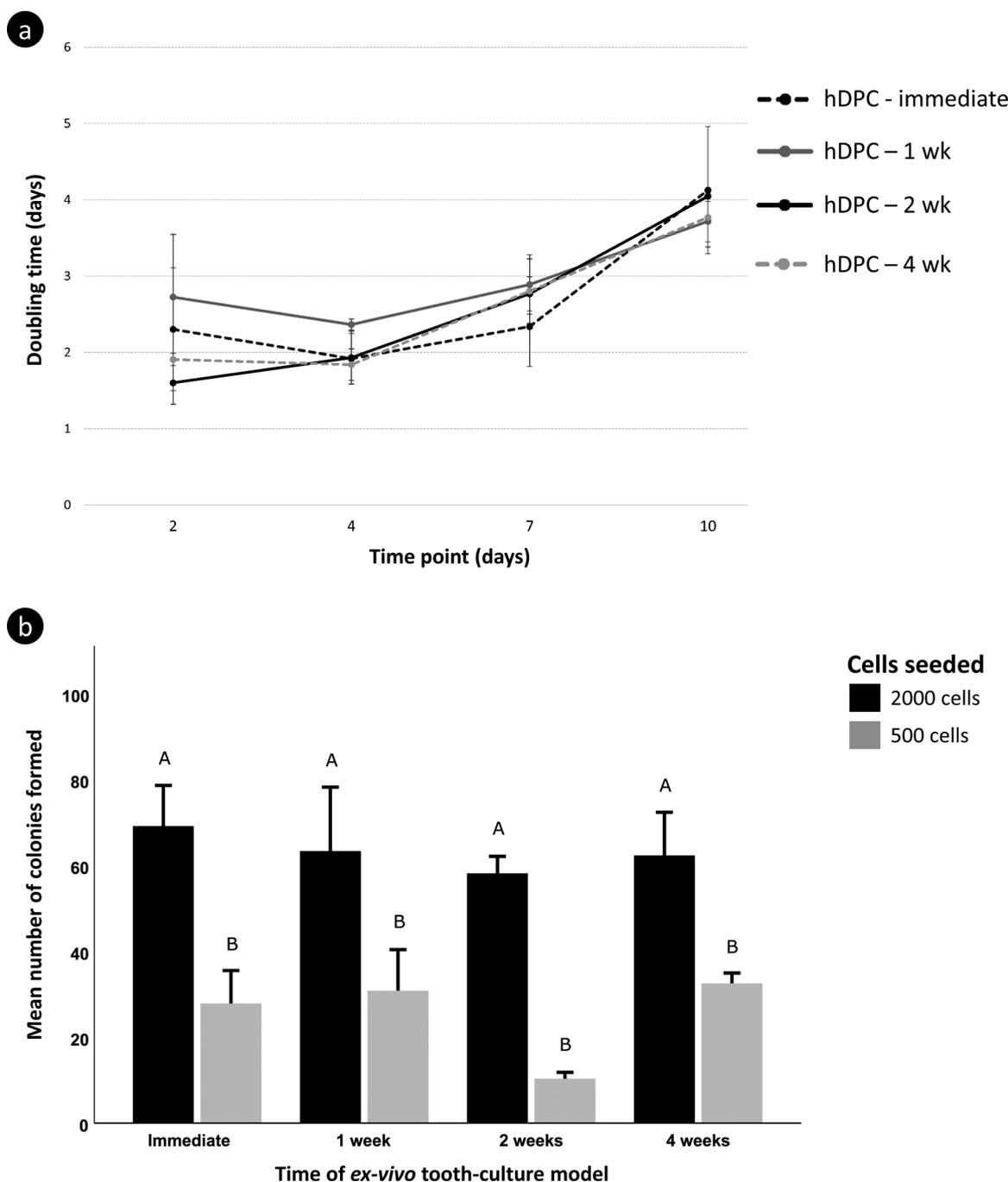


Fig. 2. Cell-growth curves in (a) and colony forming ability in (b) for the control and three experimental groups. (a) Cell-doubling time at different time points (2, 4, 7 and 10 days) for the four seeding times (hDPC-immediately/1wk/2wk/4wk). The dots represent the mean cell-doubling time (in days) with the line extensions representing the standard deviation. No statistically significantly different cell-doubling time was recorded among the different seeding times ($p \geq 0.05$). (b) Colony forming unit (CFU) assay at different seeding times for two different cell densities (500 and 2000 cells). The bar height represents the mean with the line extensions representing the standard deviation. For each seeding time, different letters indicate statistically significant difference between the two cell densities ($p < 0.05$). No significant difference in colony forming ability was found among the different seeding times ($p \geq 0.05$).

not show any kind of tissue reaction. These results confirm the previous findings using the human tooth culture model [9–11,18] and also the histological characteristics of human teeth capped with calcium hydroxide or calcium-silicate cements [19,20].

A well-orchestrated series of events occur in the process of pulpal repair upon exposure of pulp tissue [21]. Among these events, proliferation and migration of multipotent cells and their differentiation into odontoblast-like cells are of paramount importance in order to form a mineralized bridge to protect the pulp tissue against further damage and/or bacterial invasion [13,21]. However, most of the

research done in order to understand the pulp biology and (patho) physiology behind pulp-capping procedures has been performed in animal studies, while the pulp biology/physiology in animal models does not necessarily reflect that in humans [22,23]; animal experimentation is additionally not exempt from ethical and economical restrictions. For these reasons, the *ex-vivo* human dental pulp model might be useful to elucidate the cellular processes occurring during pulp inflammation and healing, in particular to record the events occurring at an early stage after exposure and pulp capping with different pulp-capping materials [18]. These results confirm the previous

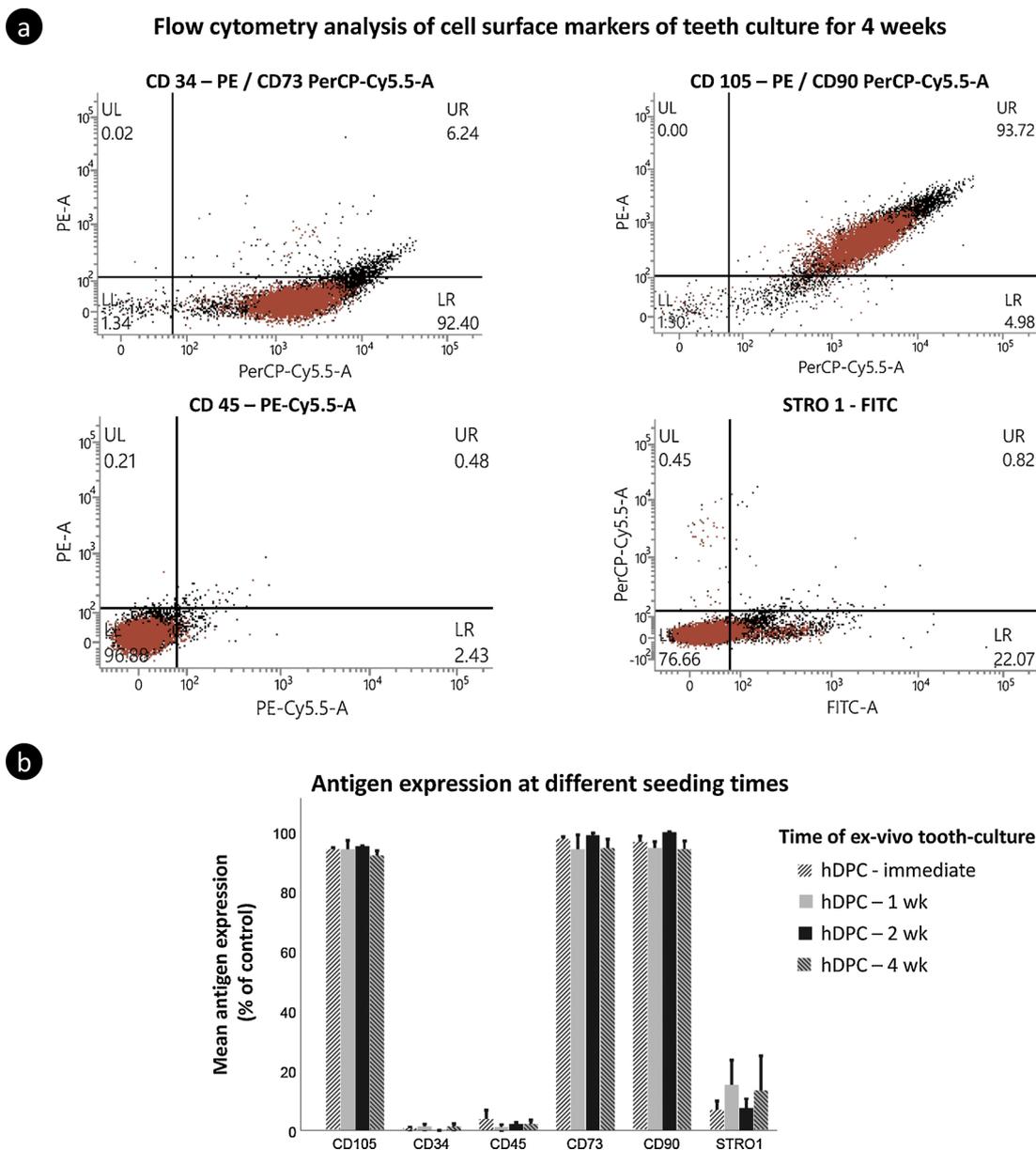


Fig. 3. Flow cytometry analysis in (a) and expression of specific surface markers in (b) as part of the characterization of cells as mesenchymal stem cells. (a) Representative gate plots for the cells of a tooth culture for 4 weeks in tooth culture model showing the expression of the different antibodies tested (CD34, CD45, CD73, CD90, CD105 and Stro1). (b) Antibody expression at the different seeding times. The bar height represents the mean with the line extensions representing the standard deviation. No significant difference was found for the expression of any of the surface markers tested for pulp tissue seeded at the different time points ($p \geq 0.05$).

findings with the tooth culture model [9–11].

Some doubts may arise regarding the viability of the cells after the teeth were cultured for several weeks. However, the use of dental pulp (stem) cells for *in-vitro* studies is widely accepted and the cells are able to proliferate, migrate and differentiate *in vitro* when they are cultured in fresh culture medium [17,23,24]. The concept behind this *ex-vivo* human dental pulp model is very similar: the pulp remained vital and the hDCPs were found to respond to the pulp exposure and capping because they received a continuous supply of medium from the culture plate [24,25]. The data obtained in this study confirmed that pulp tissue, even up to 4 weeks after tooth extraction, still contain cells that are able to behave and express markers in a similar way as pulpal cells originating from freshly extracted teeth. Our findings validate the usefulness and applicability of the *ex-vivo* human dental pulp model to screen initial pulpal healing.

Naturally, the cultured teeth are not supplied with oxygen and

nutrients as transported *in vivo* as part of a continuous blood circulation. In our study, the growth efficiency of the cells cultured for up to 4 wk was lower than that of freshly cultured pulp tissue. This can be explained by the fact that the amount of medium reaching the coronal part of the pulp may not have been sufficient to supply nutrients leading to apoptosis of the cells. However, apoptosis has not been assessed in this study, by which further research is warranted. Nevertheless, we were able to harvest sufficient viable cells at all study periods to perform our tests and the cells behave similarly as those originating from pulp tissue of teeth processed immediately.

5. Conclusions

Based on the findings of this study, we can conclude that the pulp tissue from teeth cultured for up to 4 weeks still have viable cells that are able to behave and express MSC markers. In this sense, we believe

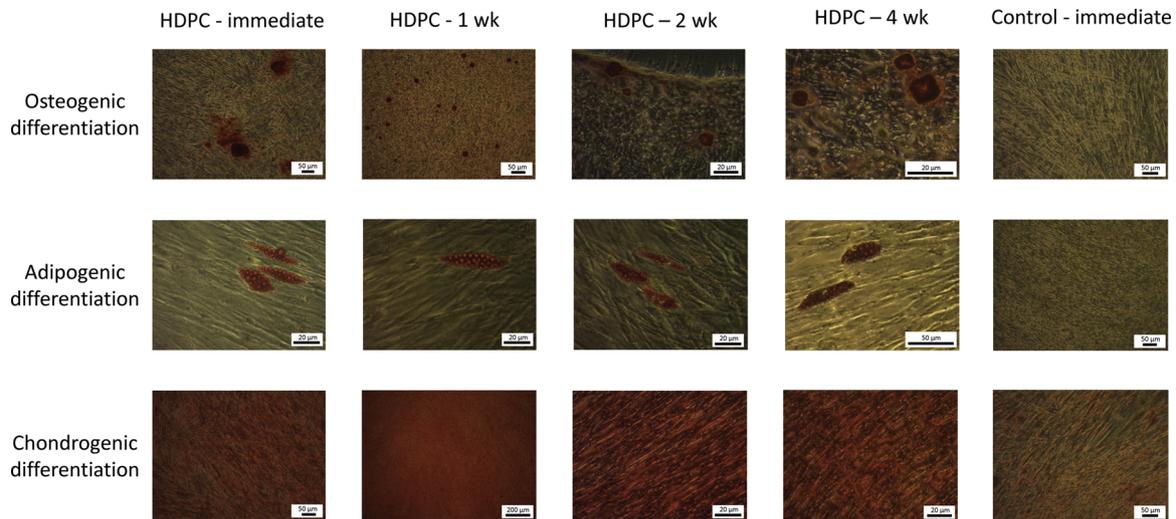


Fig. 4. Osteogenic, adipogenic and chondrogenic differentiation assay for the pulp tissue cultured at the different time points. Alizarin Red S staining showed the formation of calcified nodules for all the different groups tested after exposure to osteogenic differentiation medium for 2–3 weeks. Oil Red O staining showed the formation of lipid droplets after exposure to adipogenic differentiation medium for 3–4 weeks. Safranin O staining revealed chondrogenic staining after 3 weeks exposure to chondrogenic medium. The cells exposed to cell-culture medium did not reveal any differentiation capacity ('control-immEDIATE').

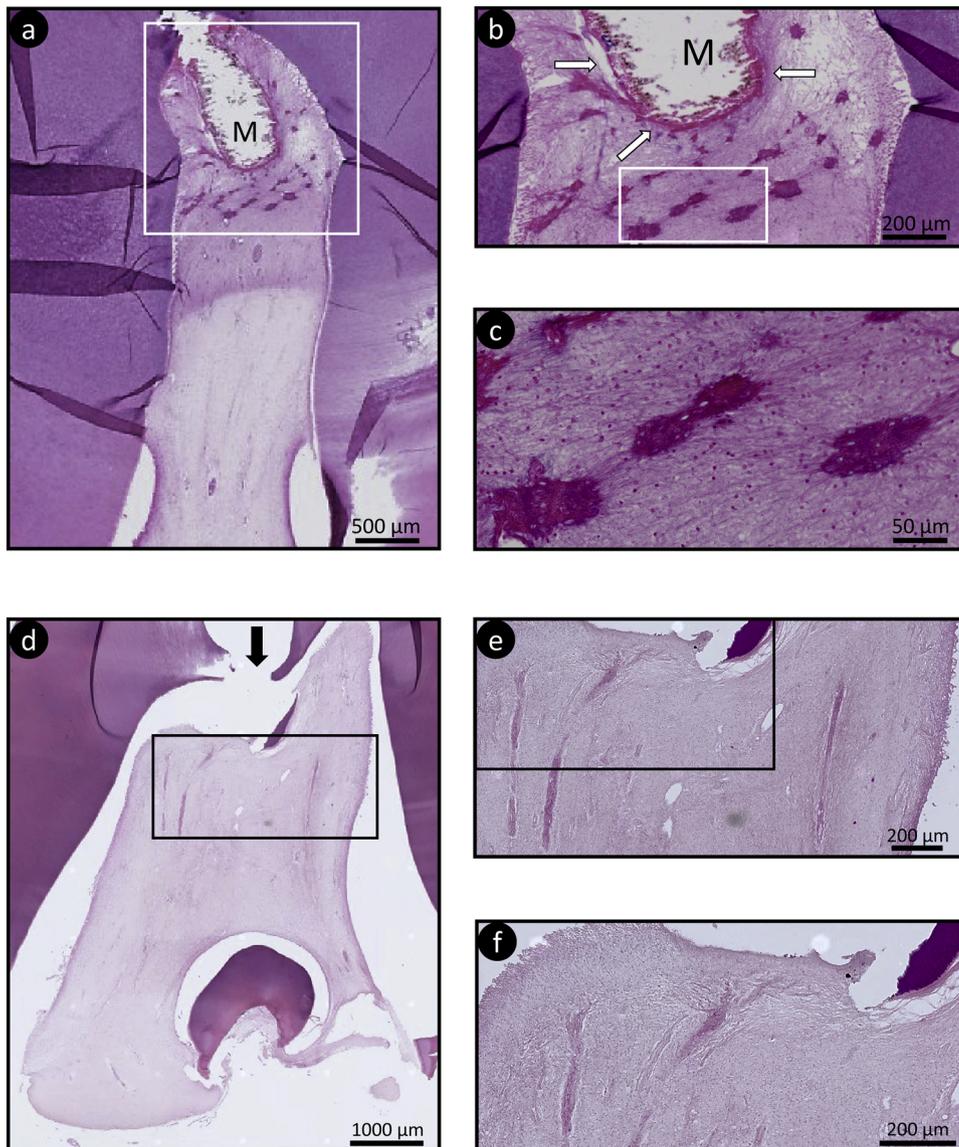


Fig. 5. Representative histological images of the teeth used for the pulp-capping assay. (a) Tooth capped with ProRoot MTA (Dentsply Maillefer), showing an intense eosinophilic area rich in cells and capillaries beneath the area in contact with the material (M). (b) Higher magnification of the white rectangle in (a). A collagen band (white arrows in b), lining the contact surface between the material and the pulp tissue, can be observed. Moreover, many mineral foci can be seen around the exposure area. (c) Higher magnification of the white rectangle in (b). A detailed view of some of the mineral foci is depicted. The mineral foci contain many holes, which are supposed to be generated by the presence of cells during the formation of this mineralized tissue. (d) Tooth with pulp exposure but without pulp capping, showing a normal pulp-tissue architecture. No difference in tissue histology was observed between the exposure area (black arrow) and the rest of the pulp tissue. (e) Higher magnification of the black rectangle in (d). Closer image at the exposure area. No collagen tissue was formed at the exposure site. The number of cells appeared not different from the rest of the pulp tissue. (f) Higher magnification of the black rectangle in (e). Closer look at the exposure site. At the right side, a dentin chip can be seen. Neither mineral foci, nor signs of inflammation were detected.

that the *ex-vivo* tooth-culture model might be a valid tool to study the early pulp-tissue events occurring after pulp exposure and capping with different pulp-capping agents.

Declaration of interests

None.

Acknowledgments

MSP is appointed as PhD fellow ('Aspirant') of the Research Foundation – Flanders (FWO). This research was supported by the FWO (research grant G.0893.15). We would like to thank all staff members of the Oral and Maxillofacial Surgery (MKA) of UZ Leuven (University Hospitals Leuven) for their help to collect freshly extracted human third molars. We would also like to acknowledge Dr. Patrick Laurent and Dr. Charlotte Rombouts from the University of Marseille for their invaluable help and assistance with the *ex-vivo* human dental pulp model. We want to thank the dental manufacturers Dentsply Sirona and GC for providing the commercial materials.

References

- [1] T.J. Hilton, J.L. Ferracane, L. Mancl, Northwest practice-based research collaborative in evidence-based dentistry (NWP), comparison of CaOH with MTA for direct pulp capping: a PBRN randomized clinical trial, *J. Dent. Res.* 92 (2013) 16S–22S.
- [2] J. Mente, S. Hufnagel, M. Leo, A. Michel, H. Gehrig, D. Panagidis, D. Saure, T. Pfeifferle, Treatment outcome of mineral trioxide aggregate or calcium hydroxide direct pulp capping: long-term results, *J. Endod.* 40 (2014) 1746–1751.
- [3] F. Schwendicke, F. Brouwer, A. Schwendicke, S. Paris, Different materials for direct pulp capping: systematic review and meta-analysis and trial sequential analysis, *Clin. Oral Investig.* 20 (2016) 1121–1132.
- [4] A. Schmidt, E. Schäfer, T. Dammaschke, Shear bond strength of lining materials to calcium-silicate cements at different time intervals, *J. Adhes. Dent.* 19 (2017) 129–135.
- [5] F. Pampaloni, E.G. Reynaud, E.H.K. Stelzer, The third dimension bridges the gap between cell culture and live tissue, *Nat. Rev. Mol. Cell Biol.* 8 (2007) 839–845.
- [6] M. Widbiller, S.R. Lindner, W. Buchalla, A. Eidt, K.-A. Hiller, G. Schmalz, K.M. Galler, Three-dimensional culture of dental pulp stem cells in direct contact to tricalcium silicate cements, *Clin. Oral Investig.* 20 (2016) 237–246.
- [7] M.S. Pedano, X. Li, S. Li, Z. Sun, S.M. Cokic, E. Putzeys, K. Yoshihara, Y. Yoshida, Z. Chen, K. Van Landuyt, B. Van Meerbeek, Freshly-mixed and setting calcium-silicate cements stimulate human dental pulp cells, *Dent. Mater.* 34 (2018) 797–808.
- [8] R. Emara, K. Elhennawy, F. Schwendicke, Effects of calcium silicate cements on dental pulp cells: a systematic review, *J. Dent.* 77 (2018) 18–36.
- [9] O. Tévelès, P. Laurent, S. Zygouritsas, A.S. Burger, J. Camps, J. Dejou, I. About, Activation of human dental pulp progenitor/stem cells in response to odontoblast injury, *Arch. Oral Biol.* 50 (2005) 103–108.
- [10] O. Tévelès, P. Laurent, V. Aubut, I. About, Human tooth culture: a study model for reparative dentinogenesis and direct pulp capping materials biocompatibility, *J. Biomed. Mater. Res. - Part B Appl. Biomater.* 85 (2008) 180–187.
- [11] P. Laurent, J. Camps, I. About, Biodentine(TM) induces TGF-β1 release from human pulp cells and early dental pulp mineralization, *Int. Endod. J.* 45 (2012) 439–448.
- [12] H.F. Duncan, K.M. Galler, P.L. Tomson, S. Simon, I. El-Karim, R. Kundzina, G. Krastl, T. Dammaschke, H. Fransson, M. Markvart, M. Zehnder, L. Björndal, P.M.H. Dummer, European Society of Endodontology position statement: Management of deep caries and the exposed pulp, *Int. Endod. J.* (2019).
- [13] U. Schröder, Effects of calcium hydroxide-containing pulp-capping agents on pulp cell migration, proliferation, and differentiation, *J. Dent. Res.* 64 (1985) 541–548.
- [14] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop, E. Horwitz, Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement, *Cytotherapy.* 8 (2006) 315–317.
- [15] D. Ricucci, S. Loghin, L.M. Lin, L.S.W. Spångberg, F.R. Tay, Is hard tissue formation in the dental pulp after the death of the primary odontoblasts a regenerative or a reparative process, *J. Dent.* 42 (2014) 1156–1170.
- [16] X. Li, M.S. Pedano, B. Camargo, E. Hauben, S. De Vleeschauwer, Z. Chen, J. De Munck, K. Vandamme, K. Van Landuyt, B. Van Meerbeek, Experimental tricalcium silicate cement induces reparative dentinogenesis, *Dent. Mater.* 34 (2018) 1410–1423.
- [17] I. About, D. Laurent-Maquin, U. Lendahl, T.A. Mitsiadis, Nestin expression in embryonic and adult human teeth under normal and pathological conditions, *Am. J. Pathol.* 157 (2000) 287–295.
- [18] P. Laurent, J. Camps, M. De Méo, J. Déjou, I. About, Induction of specific cell responses to a Ca3SiO5-based posterior restorative material, *Dent. Mater.* 24 (2008) 1486–1494.
- [19] D. Ricucci, S. Loghin, L.M. Lin, L.S.W. Spångberg, F.R. Tay, Is hard tissue formation in the dental pulp after the death of the primary odontoblasts a regenerative or a reparative process, *J. Dent.* 42 (2014) 1156–1170.
- [20] T. Dammaschke, A. Nowicka, M. Lipski, D. Ricucci, Histological evaluation of hard tissue formation after direct pulp capping with a fast-setting mineral trioxide aggregate (RetroMTA) in humans, *Clin. Oral Investig.* (2019).
- [21] I. About, Dentin-pulp regeneration: the primordial role of the microenvironment and its modification by traumatic injuries and bioactive materials, *Endod. Top* 28 (2013) 61–89.
- [22] S. Kakehashi, H.R. Stanley, R.J. Fitzgerald, The effects of surgical exposures of dental pulps in germfree and conventional laboratory rats, *J. South. Calif. Dent. Assoc.* 34 (1966) 449–451.
- [23] M. Fitzgerald, Cellular Mechanics of Dentinal Bridge Repair Using 3H-Thymidine, *J. Dent. Res.* 58 (1979) 2198–2206.
- [24] S. Gronthos, M. Mankani, J. Brahimi, P.G. Robey, S. Shi, Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 13625–13630.
- [25] J. Camilleri, P. Laurent, I. About, Hydration of Biodentine, Theracal LC, and a prototype tricalcium silicate-based dentin replacement material after pulp capping in entire tooth cultures, *J. Endod.* 40 (2014) 1846–1854.