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ARTICLE

DENTIN SLICE MODEL OF DENTAL STEM CELLS IN A FIBRIN-AGAROSE CONSTRUCT FOR DENTAL PULP REGENERATION.

Modelo de trozo de dentina de células madre dentales en una construcción de fibrina-agarosa para la regeneración de la pulpa dental.

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ABSTRACT:

Objectives: To implement a dentin slice model of mesenchymal stem cells derived from dental tissues in a fibrin-agarose construct for dental pulp regeneration.

Material and Methods: MSCs derived from different oral cavity tissues were combined with a fibrin-agarose construct at standard culture conditions. Cell viability and proliferation tests were assayed using a fluorescent cell dye Calcein/Am and WST-1 kit. The proliferation assay was evaluated at 24, 48, 72, and 96 hours. Also, we assessed the dental pulp stem cells (DPSCs) cell morphology inside the construct with histological stains such as Hematoxylin and Eosin, Masson's trichrome, and Periodic acid–Schiff. In addition, we elaborated a tooth dentin slice model using a culture of DPSC in the fibrin–agarose constructs co-adhered to dentin walls.

Results: The fibrin-agarose construct was a biocompatible material for MSCs derived from dental tissues. It provided good conditions for MSCs' viability and proliferation. DPSCs proliferated better than the other MSCs, but the data did not show significant differences. The morphology of DPSCs inside the construct was like free cells. The dentin slice model was suitable for DPSCs in the fibrin-agarose construct.

Conclusion: Our findings support the dentin slice model for future biological use of fibrin-agarose matrix in combination with DPSCs and their potential use in dental regeneration. The multipotency, high proliferation rates, and easy obtaining of the DPSCs make them an attractive source of MSCs for tissue regeneration.

KEYWORDS:

Stem cells; Agarose; Tissue scaffold; Dentin; Dental pulp; Regeneration.

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RESUMEN:

Objetivos: Implementar un modelo de dentina con células madre mesenquimales derivadas de tejidos dentales en una constructo de fibrina-agarosa para la regeneración de la pulpa dental.

Material y Métodos: Las MSC derivadas de diferentes tejidos de la cavidad oral se combinaron con una construcción de fibrina-agarosa en condiciones de cultivo estándar. Las pruebas de viabilidad y proliferación celular se ensayaron utilizando un kit de colorante celular fluorescente Calcein/ Am y WST-1. El ensayo de proliferación se evaluó a las 24, 48, 72 y 96 horas. Además, evaluamos la morfología celular de las células madre de la pulpa dental (DPSC) dentro de la construcción con tinciones histológicas como hematoxilina y eosina, tricrómico de Masson y ácido peryódico de Schiff. Además, elaboramos un modelo de rebanadas de dentina dental utilizando un cultivo de DPSC en las construcciones de fibrina-agarosa coadheridas a las paredes de la dentina.

Resultados: La construcción de fibrina-agarosa fue un material biocompatible para las MSC derivadas de tejidos dentales. Proporcionó buenas condiciones para la viabilidad y proliferación de las MSC. Las DPSC proliferaron mejor que las otras MSC, pero los datos no mostraron diferencias significativas. La morfología de las DPSC dentro de la construcción era como la de las células libres. El modelo de corte de dentina fue adecuado para DPSC en la construcción de fibrina-agarosa.

Conclusión: Nuestros hallazgos respaldan el modelo de corte de dentina para el futuro uso biológico de la matriz de fibrina-agarosa en combinación con DPSC y su uso potencial en la regeneración dental. El multipotencial, las altas tasas de proliferación y la fácil obtención de las DPSC las convierten en una fuente atractiva de MSC para la regeneración de tejidos.

PALABRAS CLAVE:

Células Madre; Agarosa; Andamios del tejido; Dentina; Pulpa dental; Regeneración.

INTRODUCTION.

Tissue engineering technologies involve the successful interaction between three components: 1. The matrix that holds cells to create the physical shape of the fabric;

2. The cells that create the tissue; and 3.- biological signaling molecules, such as growth factors, that guide cells to express the desired tissue phenotype.[1,2] Several biomaterials are used as a matrix for delivering cells and drugs. For example, natural polymers include alginate, gelatin, and fibers, among others,[2] and synthetic polymers are classified as biodegradable and non-biodegradable.[3] Biomaterials maximize the chances of a successful repair, including biodegradability or biocompatibility, which help the growth of functional tissue;

3. Among hydrogels, alginate, and agarose-derived algae are inert because they lack native ligands interacting with mammalian cells.[2,3] Peptide injectable hydrogels such as Puramatrix TM might be helpful to scaffolds for stem cell-based regenerative endodontic procedures;

4. Additionally, these hydrogels provide several advantages for tissue engineering, including injecting minimally invasive hydrogel/cell constructs.

Researchers focused on alginate, agarose, and gelatin hydrogels to act as the matrix material to regenerate cartilage tissue.[5] Many studies showed that alginate and agarose promote the chondrocyte phenotype *in vitro*;

5. Nevertheless, little is known about agarose biomaterial's functional and mechanical properties, although its ability to support chondrogenic differentiation of adult stem cells was demonstrated .[5] These stem cells were self-renewing clonogenic progenitor cells that generated one or more specialized cell

types. The gold standard for cell-based therapies in tissue engineering is the mesenchymal stem cells (MSCs). MSCs are multipotential adult stem cells easily isolated from bone marrow, adipocytes, umbilical cord, and dental tissues, among others.[6,7] MSCs have differentiation potential to multiple lineages; also, they produce growth factors involved in the regeneration of damaged tissues.[8] We can isolate them from the pulp (DPSCs), dental follicle, and gingival connective tissue in the oral cavity.[7] The pulp has undifferentiated MSCs that derive from the ectoderm of the neural crest, making up a proper cellular reserve with differentiation potential.[9,10] DPSCs have immunomodulatory properties, show a high colony formation rate, and differentiate into odontoblasts, adipocytes, chondrocytes, osteoblasts, and neurons.[5,11] Dental tissues as sources of MSCs could be a practical advantage in cell-based therapy and the eventual development of techniques for its use in regenerative endodontics and degenerative diseases.[12] Actual reports described autologous blood derivatives such as Platelet-Rich Plasma (PRP), a natural biomaterial concentrated with growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF-b1, TGF-b2), insulin (IGF-1, IGF-2), and vascular endothelial growth factor (VEGF).[13] These factors stimulate chondrocyte proliferation, among other cellular functions.[14]

Previous reports showed that in combination with agarose (PRP-agarose) gel, PRP works as flexible gelatin with three-dimensional geometry, non-toxic with good mechanical properties, and appropriate structure for tissue regeneration.[14] Also, reports described the MSCs' functionality in PRP-agarose as a bioengineered oral mucosa.[15,16] For dental pulp regeneration, two approaches were described. One consists of host cells from the apical papilla that migrate into the root canal and differentiate into a vascularized pulp tissue.[17] The other following therapy uses MSCs in a biocompatible material in a tooth model in vitro. The differentiation potential of MSCs works into the root model for the neoformation of dental pulp tissue.[18-20] MSCs play a significant role in pulp tissue regeneration in both cases.

Few researchers address the study of MSCs properties extracted from the oral cavity with natural scaffolds for dental pulp regeneration. Nevertheless, the materials used in many studies were synthetic materials such as Puramatrix TM and Matrigel BD. They generate cytotoxic acidic breakdown pro-ducts for cell cultures, suitable for in vivo tissue regeneration and repair studies.[19]

The researchers in pulp regeneration used the *in vitro* tooth slice/scaffold model that allowed the generation of a dental-pulp-like tissue with DPSCs seeded in a biodegradable construct within the dental pulp lumen of a human tooth slice.[20] However, dentin irrigation must be considered in a regeneration model because it is crucial for the disinfection of the root canal and the MSCs' survival and proliferation.[21]

This study aimed to implement a dentin slice model with MSCs derived from dental tissues in a fibrin-agarose construct for dental pulp regeneration. This model would be a new option for future clinical practice with potential application in regenerative medicine.

MATERIALS AND METHODS.

Study participants

The Ethics Committee Ethics Committee of the Health Service Metropolitan East (SSMO), Santiago de Chile (03-2017). Also, this research was approved by the Committee of Ethics in Research (CER) of the International University of Catalunya (UIC), Spain. CBAS 2018-06 study code. This protocol was designed according to the principles expressed in the Declaration of Helsinki. Dental tissues were obtained from the permanent teeth of patients 18-60 years old who attended the health center (CESA, San Bernardo Hospital of the Universidad de Los Andes) and agreed to participate in this study and signed the informed consent. Dental pulp, gingiva, peri coronary pocket, and apical papilla tissues were collected from 5 patients undergoing third molar extraction by indication of orthodontics based on clinical practice; teeth were free of a carious lesion.

For processing, samples were immediately transferred to the CIBRO (Investigation Center in Biology

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and Oral Regeneration) at the Dental School, Universidad de Los Andes (Supplemental material).

Isolation and culture of MSCs

All dental tissues were rinsed in PBS 1X and vortexed twice. Tissues were cultured for two weeks in α-MEM (Minimum Eagle Medium Corning®,) 10% Fetal Bovine Serum (FBS, Corning®), and 1% Penicillin-Streptomycin (Pen-Strep, Corning®) in an incubator (Sanyo, MCO-17AC) at 37°C and 5% CO2 until the observation of the first cells with fibroblastic morphology and adhered to the plate. MSCs derived from dental tissues (apical papilla, gingiva, dental pulp, and peri coronary pocket) were cultured as previously described.[6] Briefly, MSCs from the 4th to 8th passage were grown in α-MEM complete media at an incubator (Sanyo, MCO-17AC) at 37°C and 5% CO_2. The MSCs immunophenotype profile was previously reported.[8-11] (Supplemental material).

MSCs and fibrin-agarose constructs

Briefly, molten suspensions type VII-agarose (A9045, Sigma, St. Louis, MO) dissolved in PBS 1X (Hy Clone®) were prepared at 0.1% w/v, 0.25% w/v, and 0.5% w/v final concentrations of type VII-agarose (A9045, Sigma, St. Louis, MO). The hydrogels were supplemented with human plasma from the same patients who donated the dental tissues (plasma was prepared with standard protocol and stored at -80°C until use). Also, added α-MEM and tranexamic acid (Amchafibrin, Fides-Ecofarma, Valencia, Spain) avoided spontaneous fibrinolysis.

In addition, 1% $CaCl_2$ (Sigma, St. Louis, MO) was added to the solution to precipitate the polymerization reaction of the hydrogels, and 1mL of mixture previous was added rapidly to fill the wells of the six-well plate. The mixture was polymerized at 37°C in a humidified atmosphere with 5% CO_2. Cryopreserved cells were thawed and plated in supplemented complete media until the cell cultures reached 80% confluency. Cells were harvested using trypsin/EDTA (Triple Select, Corning®), and then $5x10^5$ cells were injected with a tuberculin syringe onto the set fibrin agarose construct. They were cultured in 6-well plates with 5 ml of complete medium in humidified air at 37°C and 5% CO_2. The control of the experiment was a fibrin-agarose matrix without cells. An optical microscope (Olympus® CKX41, Tokyo, Japan) was used for monitoring cells. Also, digital images were taken with a Nikon camera D7100 under visible light.

Assessment of cell viability and proliferation

Cell proliferation assay was determined using WST-1 TM (Roche) and MTT TM (Promega) kits according to suppliers' instructions, tests were assessed at 24, 48, 72, and 96 hours and the absorbance at 570 nm was measured using a microplate reader (Rayto RT 2100C, USA). Cell viability was assayed with a LIVE/DEAD® kit assay (Molecular Probes, Life Technologies, Dublin, Ireland). 100µL of LIVE/DEAD® reagent was added to the coverslip, and cells were covered by solution and were incubated at room temperature for 30 minutes. Fluorescence images were taken using a fluorescence microscope (Zeiss Germany, Axioskop).

Histology analysis

A punch of 3 mm of the cell construct was fixed in 10% buffered formalin (Sigma). The selected constructs were dehydrated in a gradient of alcohols and then embedded in paraffin. Sections of 3 um thickness were stained with hematoxylin-eosin (H-E, Sigma Aldrich) to provide morphological context to the sample. This is routinely used as the first stain confirming the condition of the tissue after processing.

Masson-trichrome (M-T, Sigma Aldrich), is a technique used to show collagen fibers (primary) of a different color from the rest of the basic structures present in the tissue due to the difference in molecular weight of both dyes and by the permeability of the tissues; Periodic Acid Schiff (PAS) (Sigma Aldrich) uses Schiff's reagent as a stain, which requires being in contact with aldehydes present in glycogen and polysaccharides in general, including agarose, exposed by oxidizing the tissue, the Schiff's reagent causes electronic resonance and takes on a magenta coloration.

All samples were mounted with Entellan R (Merck Millipore). Images were captured with the inverted light microscope Olympus CKX41 (Tokyo, Japan).

Dentin slice model

Human molar teeth extracted from healthy

individuals (same patients who gave dental tissues for stem cells extraction) were sliced horizontally with a thickness of 3mm and length of 1 mm using a Dremel saw (100-N/6 220-Volt Single Speed Rotary Tool Kit). We prepared 1-mm-thick human tooth coronal slices (cross-sections). The dentine slice was disinfected with 2 ml sodium hypochlorite at 1.5%, shaken in a vortex for 1 minute, then centrifuged at 1,700 rpm for 2 minutes. Samples were immersed into another sterile tube containing 1 ml of sodium hypochlorite at 1.5% and 1 mL of 1x PBS; after that, they were vortexed and centrifuged again at 1700 rpm for 2 minutes. Finally, dentin blocks were added in a new sterile tube with 2 ml of PBS 1X, vortexed, and centrifuged at 1700 rpm for 2 minutes, then dried in a 10mm plate in a flow chamber until use. Macroscopic images were taken with a Nikon D7100.

Injection of MSCs and fibrin-agarose construct into the dentin slice.

Dentin slices previously prepared were cultured in a plate of 24 wells with complete media α-MEM, 10% FBS, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Then hydrogels of fibrin-agarose were prepared at 0.25%w/v according to previously described methods. Twenty milliliters of the molten mix were injected with a pipette tip (AxygenTM) inside the lumen of the dentin slice. Once the hydrogel hardened, $5x10^5$ MSCSs were injected with a tuberculin syringe. The dentin slice was cultured for seven days in 24-well plates with 1ml of a complete medium at 37°C and 5% CO_2. Fibrin-agarose without cells was used as a negative control. An optical light microscope Olympus CKX41 (Tokyo, Japan), was used for cell monitoring. Macroscopic digital images of the fibrin-agarose hydrogel were taken with a Nikon camera D7100.

Statistical Analysis

Data were analyzed using the Kruskal-Wallis one-way ANOVA, which accounts for non-normal distributions with small sample sizes and multiple groups. Also, analyses were performed with Mann–Whitney test and GraphPad Prism (GraphPad, San Diego, CA, USA) statistical software. Data were presented as means ± standard deviation (SD).

A $p< 0.05$ was considered statistically significant with a 95% confidence interval.

RESULTS.

Fibrin-agarose hydrogel supports MSCS proliferation.

The hydrogel of fibrin-agarose at 0.25%w/v was the hydrogel concentration of most accessible manipulation, with an appropriate consistency that allows management in culture and *in vitro* analysis.

Also, the hydrogel of fibrin agarose 0.25%w/v with MSCs derived from the gingiva, apical papilla, peri coronary pocket, and dental pulp stem cells proliferated under culture conditions. It was incorporated inside the construct (Figure 1). Qualitative observations of the hydrogels showed that DPSCs increased better than the other MSCs. This result was confirmed in the MTT proliferation assay described in Figure 2.

We illustrated DPSCs proliferation over time at different hydrogel fibrin–agarose concentrations. This result showed that the fibrin–agarose at 0.25%w/v would correspond to the appropriate concentration of biological material that allowed cell proliferation due to increased rigidity with a more stable gel than a thinner and fragile one with lower concentrations (0.1%). Of note, DPSCs survived in all concentrations of fibrin-agarose hydrogel but with lower proliferation at 0.5%w/v. Indeed, no significant differences were observed in DPSCs proliferation in the different concentrations of the hydrogel tested, over time, and at the cell density with a *p*-value >0,05 (Figure 2).

DPSCS morphology in the fibrin-agarose

DPSCs stained with calcein/AM showed cell viability at day seven in culture. Live DPSCs were seeded at 20000 cells/ml in fibrin agarose 0.25%w/v and death DPSCs in fibrin agarose 0.25%w/v; it was possible to observe some nuclei of DPSCS (Figure 3). Histological cuts of fibrin-agarose 0.25% w/v with DPSCs stained with hematoxylin-eosin (H-E), Masson trichrome (M-T), and PAS demonstrated some cells inside the construct with a spindle shape even more characteristic like free MSCs.

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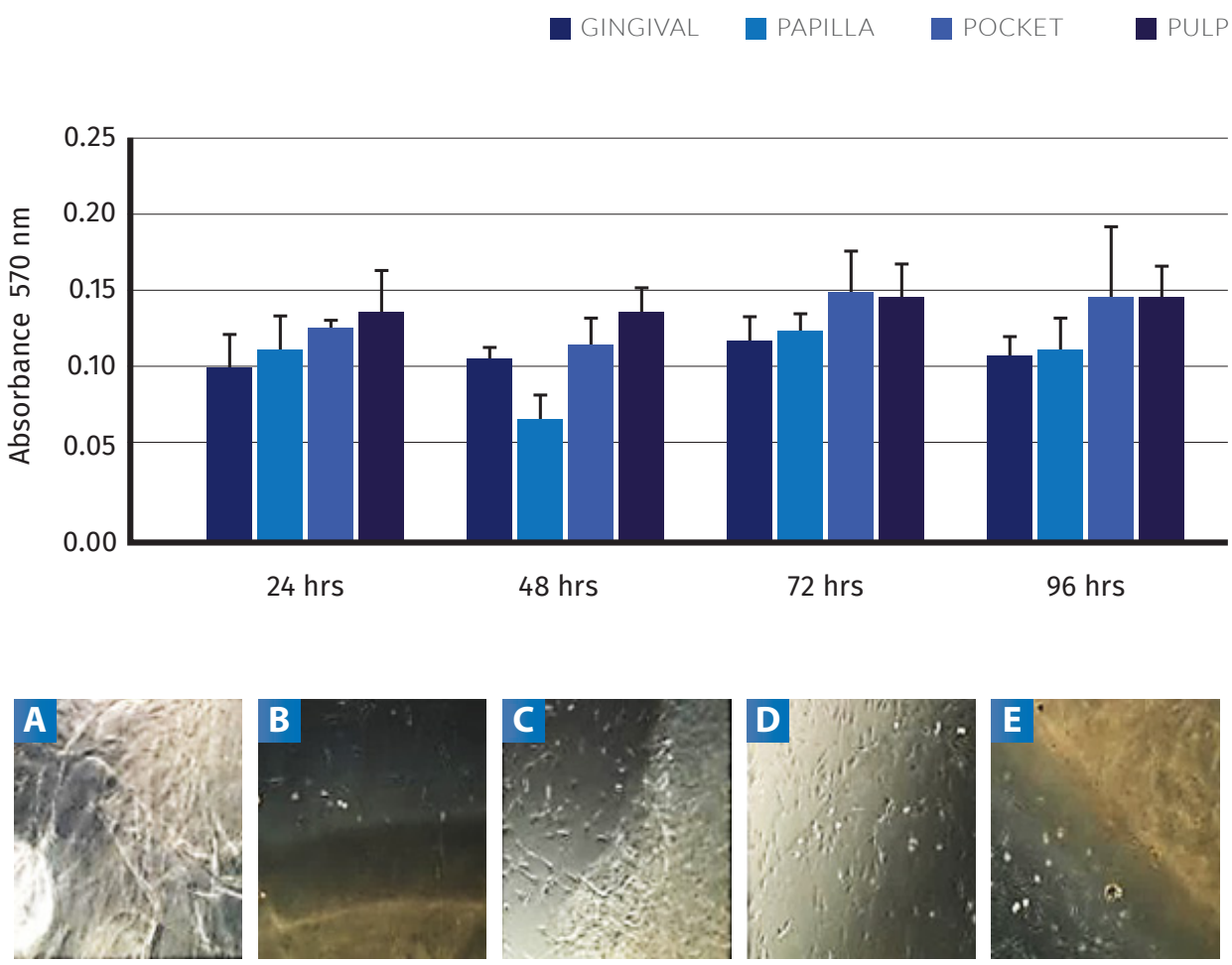
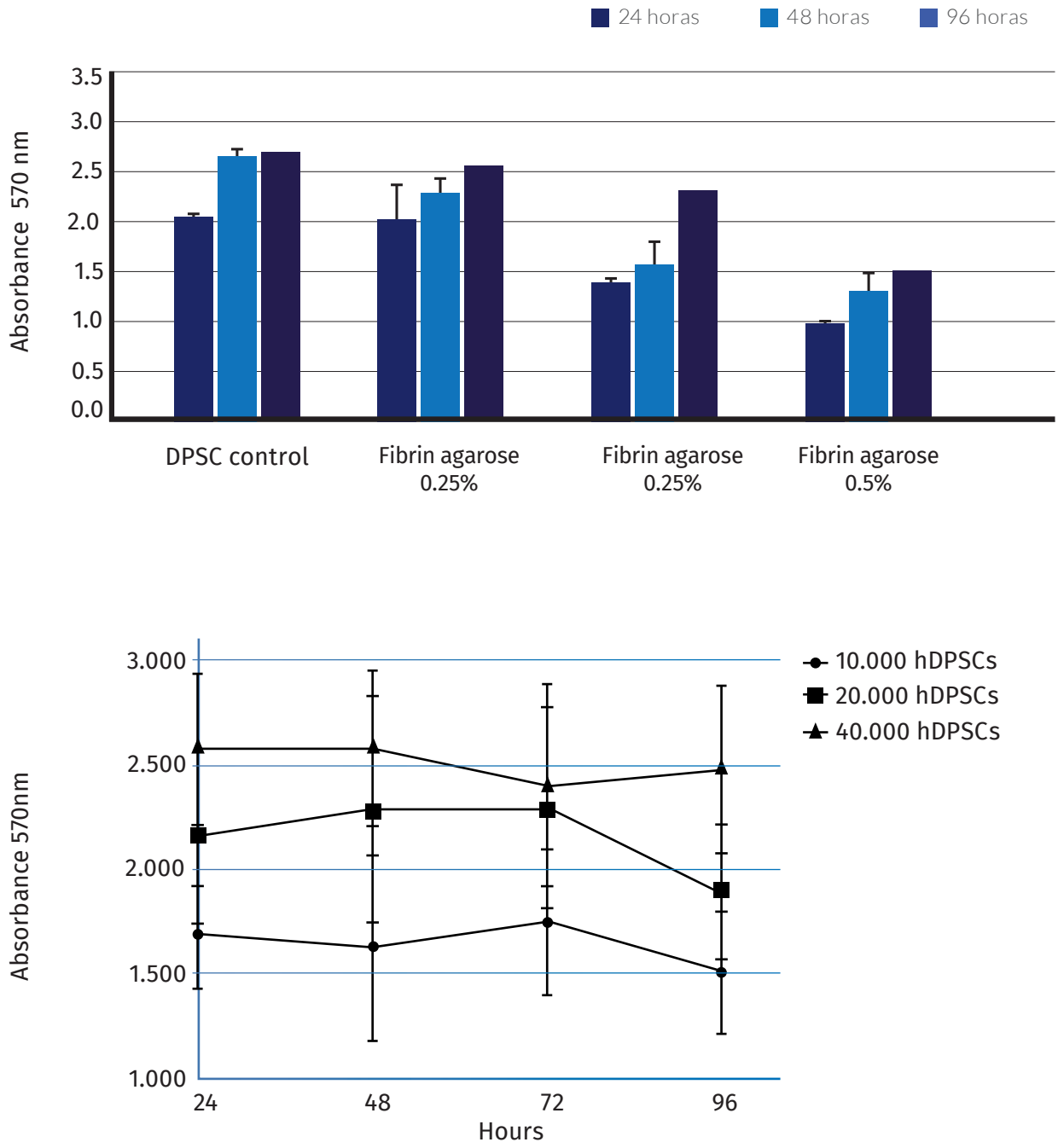
Figure 1. Distribution of patients according to the types of maxillofacial fractures and the consumption of alcoholic beverages.



A: Fibrin-agarose constructs 0.25% w/v without stem cells. B: Fibrin agarose constructs 0.25% w/v with MSCs derived from the gingiva. C: Apical papilla. D: Peri coronary pocket. E: Dental pulp stem cells.
Magnification 10x. Viability and proliferation assay of MSCs in the fibrin-agarose 0.25%w/v. Stem cells derived from gingiva tissue, apical papilla, peri coronary pocket, and dental pulp were analyzed at 24, 48, 72, and 96 hours. Data expressed as means of quintuplicate (n=5) with standard deviation. No statistical differences were found, p=0.8029.

Figure 2. WST-1 proliferation assay of DPSCs according to the concentration of Fibrin-agarose and cell density over time.



DPSCs cells were plated in 0.1, 0.25, and 0.5% fibrin-agarose. Also, DPSCs cells were plated in 0.25% Fibrin agarose at a cell density of 1, 2, and 4×10^4 cells/ml. Results are expressed as means of absorbance in arbitrary optical density units (OD) with standard deviations. Experiments were done in triplicate; results are expressed as means with standard deviation. No statistical differences were found. ANOVA results, p=0,3096 and p=0.9334, respectively.

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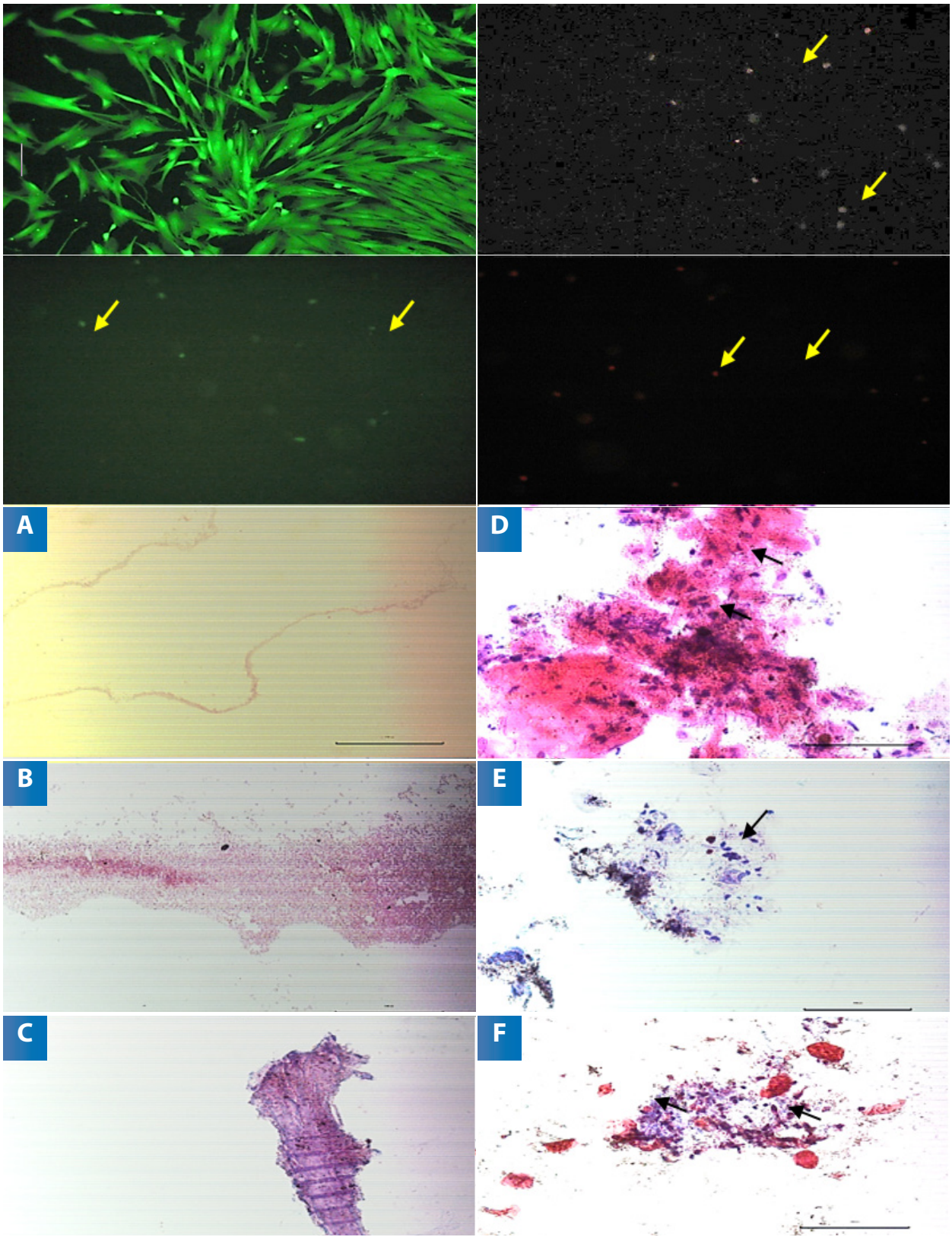
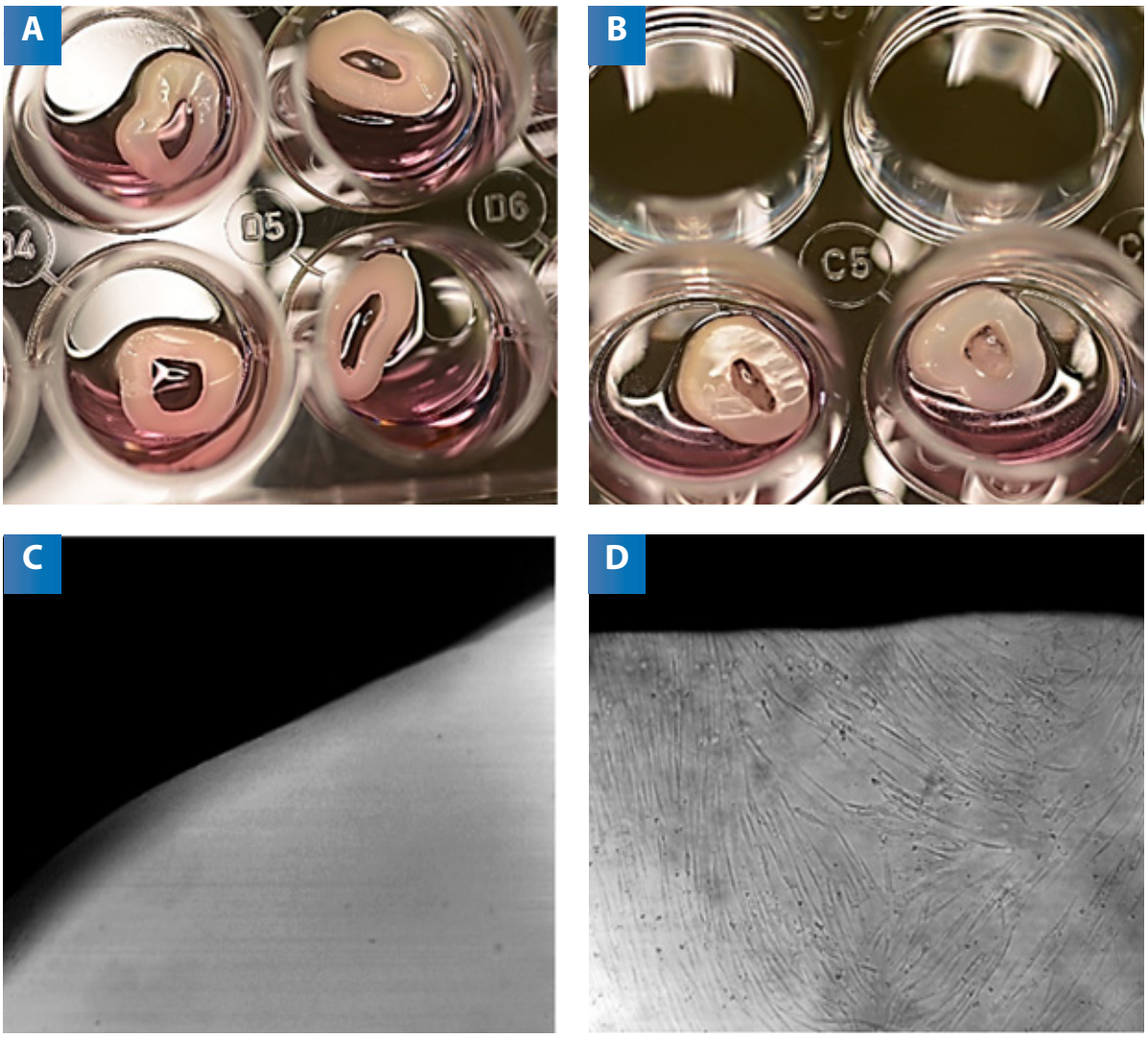
Figure 3. DPSCs in fibrin-agarose 0,25% w/v stained with Calcein/AM and in histological cuts.



Live DPSCs (1), death DPSCs (2), live DPSCs in fibrin-agarose 0,25% w/v (3), death DPSCs in fibrin agarose 0,25% w/v (4). Fibrin-agarose 0,25%w/v without cells stained with H-E (A), M-T (B), and PAS (C). Fibrin-agarose 0,25%w/v with DPSCs stained with H-E, cytoplasm in pink and nucleus in purple (D), M-T shows cytoplasm in dark red and black nuclei (E) PAS showed agarose full of polysaccharides in pink (F), yellow indicates show cells. Magnification 10X. Bar 100µm.

Figure 4. Transversal cuts of the dentin tooth slice in culture conditions.



Dentin slice empty in culture media (A). Dentin slice in culture with the fibrin agarose constructs 0.25% with DPSCs after 15 days (B), macroscopic photos. The lumen of the tooth with fibrin agarose 2.5% w/v without cells (C) and the tooth with fibrin agarose 0.25%w/v with DPSCS (D), microscopic images magnification 10x.

Microscopy images allowed us to discriminate between cytoplasm-stained pink in H-E and dark red in M-T. The DPSCs nucleus was shown in purple in H-E and dark brown in M-T, and agarose has a high content of polysaccharides in pink color in PAS (Figure 3).

DPSCs in the fibrin-agarose seeded in the dentin slice model

DPSCs seeded in the fibrin-agarose construct and cultured in dentin slices showed that cells proliferated inside the fibrin-agarose construct and the dentin slice during 15 days in culture. Macroscopic and microscope images of the lumen of the dentin slice showed adherence of the fibrin-agarose construct to the dentin walls (Figure 4).

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DISCUSSION.

In the present study, we implemented a dentin slice model with DPSCs in fibrin-agarose construct and its potential use in dental pulp regeneration. The fibrin-agarose was a hydrogel of easy manipulation, with an appropriate consistency that allowed analysis in culture and *in vitro* characterization. According to previous reports, cell viability is a determinant factor for the functionality of an artificial tissue; therefore, proliferation assays are critical tests for stem cell viability.[22] Mesenchymal stem cells (MSCs) derived from dental tissue have gained popularity for tissue engineering and regenerative medicine applications because the highly proliferative and self-renewing expands their applicability for the regeneration of tissues. In our study, we elaborated a construct of fibrin-agarose with stem cells derived from different dental tissues for choosing the cell type with better results in proliferation rate.

Previous studies reported fibrin-agarose as a biological substitute with applications on artificial tissues such as cornea,[23] among others. This study showed that the fibrin-agarose construct formed a three-dimensional structure of such consistency that allowed easy manipulation and consistency that facilitated the incorporation of MSCs. Agarose is a biocompatible polysaccharide adequate without growth factors. For that reason, we built a construct of the agarose and included the rich components of the PRF or PRP widely described growth factors such as PDGF, TGF-b, IGF, VEGF, epidermal growth factor, and epithelial cell growth factor that stimulate bone and soft-tissue healing.[13]

The fibrin-agarose, combined with MSCs, was monitored in culture, and our results suggested that the MSCs were incorporated within it. Notably, DPSCs were observed in greater quantities, which offered a more significant proliferation according to the WST-1 and MTT assay. Also, the construct of fibrin-agarose supported the proliferation and survival of DPSCs. Similar studies were done with the same tests with Puramatrix™ hydrogel in combination with DPSCs4; We want to highlight that it is not a natural material.

Our results described that the fibrin–agarose at 0.25%w/v corresponded to the appropriate concentration of natural biological material that allowed cell proliferation over time. Also, this hydrogel was a moldable construct that sustained cell viability and proliferation and made the agarose-fibrin and DPSCs, a suitable model for dental pulp regeneration. Therefore, we demonstrated with the fluorescent dye that the DPSCs could survive and proliferate inside the construct; the histological analysis confirmed it.

H-E staining is the most common technique in histopathology; combining the two stains allowed for the visualization of tissues, nuclei, and cytoplasmic structures. Microscopic images of the cells inside the construct presented morphological features like free cells in culture with a defined nucleus and typical spindle shape. PAS is a staining method for detecting polysaccharides, glycogen, glycoproteins, glycolipids, and mucins in tissues, and it was used to demonstrate the high content of that in the agarose construct, an important characteristic for bonding with stem cells and its biocompatibility in the first stage and then their ability to exchange gas and nutrients. For implementing an *in vitro* model for dental pulp regeneration, we tested the compatibility of the construct of fibrin-agarose with DPSCs that show great potential for producing large volumes of the mineralized matrix, which opens a window of hope for being used in regenerative dental treatments.

For that reason, we designed easy handling enriched natural constructs in a dentin slice model that could be injected into a root canal by using a protocol of activation of dentin factors proven to contribute to regenerative endodontics.[21] Similar proposals described a commercial non-natural hydrogel Puramatrix TM 4, platelet-rich plasma or fibrin, collagen, polyester, chitosan, or hydroxyapatite.[19,22] among others, for diverse purposes.

Our model included the fibrin-agarose and the DPSCs in the dentin slice model for regenerative endodontic procedures. The construct would polymerize inside the pulp chamber, facilitating the

regenerative endodontic strategies based on MSCs. We want to highlight the DPSCs proliferation and adherence of the construct to the dentin walls. These cells are expected to adhere, proliferate and differentiate to give rise to new tissue; thus, the physiological tissue condition of the dental pulp is mimicked. It is essential to address the variability between patients and, therefore, between stem cells and plasma components.

Thus, the reproducibility of the study results is limited. Further, studies are required with a larger sample and other culture conditions like hypoxia or cellular stress to mimic a disease state better. It will also be interesting to evaluate the growth factors present in the construct and analyze the morphology of the latest dental tissue formed inside the tooth slice to achieve a clinical translation of pulp regeneration with this model strategy.

CONCLUSION.

We have implemented a construct of fibrin-agarose that was easy to manipulate, allowed the inclusion of stem cells derived from dental tissues, and incorporated PRP factors that stimulate cell proliferation,[24] hence creating a suitable environment for the potential use in tissue reparation or regeneration.

We described three essential elements in tissue engineering: stem cells derived from the intra-oral sources, growth factors in the PRF, and the agarose scaffold. From the perspective of dental pulp regeneration, this innovative model could be used in dental practice as a new strategy based on MSCs and bioartificial substitutes.

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Conflict of interests:

The authors disclaim any conflicts of interest in this study.

Ethics approval:

This protocol was approved by the Ethics Committee of the Health Service Metropolitan East (SSMO), Santiago de Chile (03-2017). Also, this research was approved by the Committee of Ethics in Research (CER) of the International University of Catalunya (UIC), Spain. CBAS 2018-06 study code.

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Authors' contributions:

Inostroza C: Was responsible for this research, made substantial contributions to this research's conception or design; worked in the acquisition, analysis, and complete interpretation of data. Wrote the final version of the paper.

Brizuela C and Hernández M: Contributed to all the dental processes. Collaborated in writing and reviewing the paper and approved the final version of the manuscript.

Vega-Letter A and Ortiz J: Contributed to the construct histology analysis, reviewed the draft, revised the documents, and approved the final version.

Carrión F: contributed to writing and reviewing the manuscript, revising the draft critically for important scientific content, and approving the final version of the paper.

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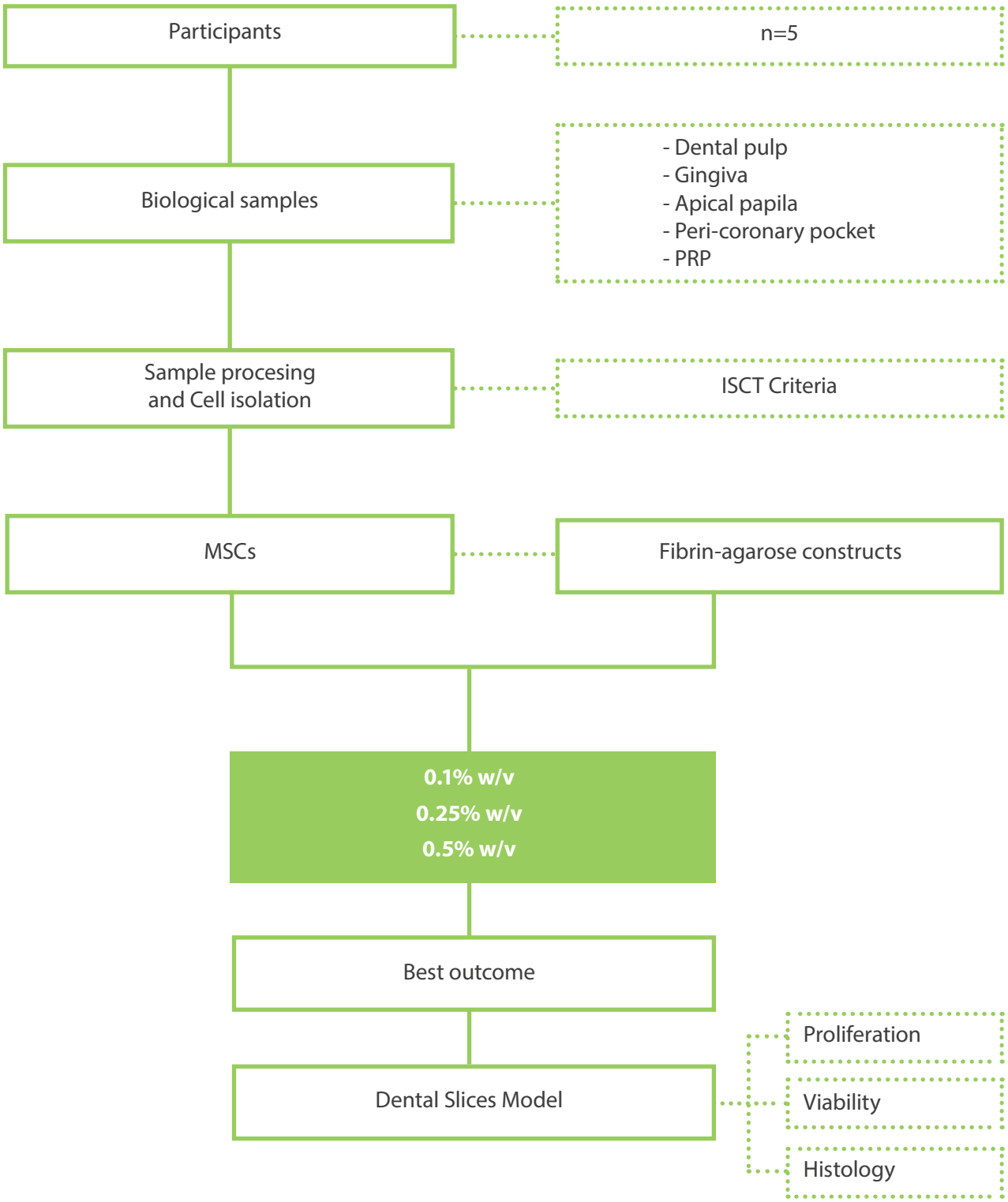
ANNEXES: SUPPLEMENTAL MATERIAL



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