Effect of human dental pulp stem cell conditioned medium in the dentin-pulp complex regeneration: A pilot in vivo study

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1. Introduction

Dental trauma, restorative operative procedures and/or caries lesions can expose the dental pulp (Olsburgh et al., 2002). Facing this clinical condition, where the maintenance of the dentin-pulp complex vitality is imperative, is challenging in Dentistry. When the exposure of dental pulp occurs in immature teeth, without complete root formation, this problem is even worst since the rhizogenesis process is interrupted (da Rosa et al., 2018). In the presence of a localized injury, there is the death of the odontoblast layer and the dentin-pulp complex responds by inducing the migration, proliferation and differentiation of dental pulp stem cells into odontoblast-like cells, which will secrete reparative dentin matrix. This process occurs in pathological conditions in order to form a mineralized barrier to protect the pulp tissue (Hashemi-Beni et al., 2017; Smith et al., 2016).

The balance between inflammation and regeneration has a significant relevance in this process of the dentin-pulp complex. Although inflammation is considered as one of the most important prerequisites for healing and regeneration, it can also be harmful if it has prolonged persistence. The pulp tissue is located within an inflexible environment formed by rigid dentinal walls. On the top of it, its terminal blood circulation also collaborates for the particular dental pulp vulnerability impairing local circulation. Thus, a severe and prolonged inflammation may hamper the regenerative processes and lead to pulp necrosis (Cooper et al., 2014; Goldberg et al., 2015; Giraud et al., 2019).

The direct pulp capping, based on the placement of a material directly on the top of the dental pulp exposure, helps to protect the pulp tissue and to avoid contamination, allowing the repair process (Hashemi-Beni et al., 2017). Among the biomaterials available, the Mineral Trioxide Aggregate (MTA) has been highly recommended for direct pulp capping (Parirokh and Torabinejad, 2010). Despite its high rates of clinical and radiographic success, some of the drawbacks of MTA are the formation of a disorganized and amorphous tissue with the presence of

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https://doi.org/10.1016/j.tice.2021.101536
Received 26 November 2020; Received in revised form 19 March 2021; Accepted 22 March 2021
Available online 1 April 2021
0040-8166/© 2021 Published by Elsevier Ltd.
pulp remains trapped inside the mineralized tissue (Simon et al., 2008; Dammenschke et al., 2019).

Nowadays, there is a growing interest in developing new strategies capable of overcoming the drawbacks of the current materials available (Al-Hezaimi et al., 2013; Mehrvarzfar et al., 2018). Among them, the use of growth factors has shown promising results. This because some growth factors may reduce inflammatory cell responses and accelerate the injured pulp tissue to regenerate (Yoshida et al., 2016; Wu et al., 2019; Imura et al., 2019). A known source of growth factor is the stem cells. The regenerative capability of these cells are not only based in their proliferation, integration and differentiation properties, but mainly in their potential for secreting proteins, such as trophic and growth factors, cytokines and chemokines which have a beneficial impact on damaged tissue (Yang et al., 2013). These secreted factors, when used alone without the stem cell itself, can cause tissue repair in various conditions of damage to organs and tissues (Pawitan, 2014). These factors can be found in the medium in which the stem cells are grown in culture named conditioned medium (CM) (Kim et al., 2013).

The human dental pulp stem cells (hDPSC) conditioned medium (hDPSC-CM) has been described as an attractive, non-invasive and acellular tool for therapeutic approaches in regenerative medicine (Kichenbrand et al., 2019). This CM is able to promote angiogenesis (de Cara et al., 2019), neurogenesis, dental tissue repair, treatment of cardiovascular diseases, organ failure, among others (Kichenbrand et al., 2019). Thus, the hDPSC-CM is a promising substance for regenerative therapies with excellent results in tissue engineering (Kichenbrand et al., 2019) and should be further explored for clinical applications in dentistry.

Based on the above, our experimental hypothesis was that exposed pulps could better respond to pulp capping if growth factors would be added to a dental pulp capping material. This alternative pulp capping material could improve the regeneration of the damaged pulp tissue. To test this hypothesis, MTA was associated with medium conditioned by hDPSCs (hDPSC-CM) and then applied to exposed dental pulps of rats.

2. Materials and methods

2.1. In vitro assessment

2.1.1. Cell culture

Human dental pulp stem cells (hDPSCs) that were previously isolated and characterized (Pedroni et al., 2019) were thawed and cultured in clonogenic culture medium composed by alpha-modified minimum Eagle’s medium (α-MEM) (Gibco Life Technologies, Grand Island, NY, US) supplemented with 15 % mesenchymal stem cell-qualified fetal bovine serum (MSC-FBS, Gibco); 100 μM penicillin (Invi-trogen/Gibco, Grand Island NY; US); 100 μg/mL streptomycin (Invi-trogen/Gibco,); 0.1 mM ascorbic acid (Sigma-Aldrich) and 2 mM L-glutamine (Gibco). Cells were maintained in an incubator at 37°C in a humid atmosphere of 5% CO2 and 95% of air. All the cells used in this study were assayed at the fourth passage. This study was approved by the Local Human Research Ethics Committee (CAAE: 89912318.3.0000.007).

2.1.2. Characterization of cells

The immunoprofile of the surface molecules of the cells was evaluated by using flow cytometry. Aliquots of the cells (1 x 10⁵ cells) were washed and resuspended into phosphate buffered saline (PBS) containing saturating concentrations (1:200) of the following panel of primary antibodies, conjugated with allophycocyanin (APC), fluorescein (FITC) or phycoerythrin (PE), against human surface molecules: mesenchymal stem cell (MSC)-associated markers [CD44-APC (BD Biosciences, CA, US), CD105-FITC (BD Biosciences) CD45PE (BD Biosciences, CA, US)] and non-associated markers [CD34-APC, CD34-FITC, and CD14-FITC (BD Biosciences)]. Matched isotypic controls were used in this experiment. Cells were classified on a flow cytometer (FACS Calibur, BD Biosciences), and 50,000 events were analyzed using FlowJo software version 9.6.2 (Tree Star, Ashland, OR, US).

2.1.3. hDPSC-CM preparation and concentration

The hDPSCs were cultured in 75 cm² culture flasks and grown until it could be observed approximately 70–80 % of cell confluence. Cells were then washed 3 times with PBS and each flask was incubated with 8 mL of α-MEM free of fetal bovine serum and other supplements. After 24 h in contact with the hDPSCs, 96 mL of conditioned medium was collected. hDPSC-CM was concentrated 120 times by centrifugation in ultrafiltration units (Amicon Ultra-PL 10, Millipore, Bedford, MA, US) at 4,000 g during 15 min at 15 °C. A total of 800 μL of concentrated hDPSC-CM were obtained. After collected and filtered in 0.22 μm filters (Milli-pore), the hDPSC-CM was divided in 40 μL aliquots that were stored at -80 °C until use, avoiding freezing/thawing cycles (Gama et al., 2018).

2.2. In vivo assessment

2.2.1. Animals

This study was approved by the Local Ethics in the Use of Animals in Research Committee (CEUA # 005/2019) and was carried out in accordance with the relevant guidelines. Twelve 3-months-old male Wistar rats (Rattus norvegicus: var. albinus, Rodentia, Mammalia) weighting an average of 303.2 ± 15.2 g were used in this study. The animals were housed in ventilated polypropylene cages with bedding, and kept under a 12 h/12 h light / dark reversed cycles, at a temperature ranging from 22 °C to 27 °C, humidity from 45 to 65 % and an organized and sanitized environment. They were fed with rodent food and ad libitum water.

2.2.2. Mechanical pulp injury model

After weighing, the animals were anesthetized with an intramuscular injection of Ketamine hydrochloride (50 mg/kg; Ketalar, Cristalia, Ita-pira, SP, Brazil) associated with Diazepam (5 mg/kg; Valium, Cristalia). Animals were fixed on an operative board in a dorsal position with their heads immobilized by the upper and lower incisors to maintain an opened mouth. To access the two first upper molars, a device made of orthodontic wire was developed, moving the cheeks away. Oral cavity and dental surface were disinfected with 0.12 % and 2 % chlorhexidine solution. Both first upper molars (right and left) were used in each animal. In these teeth, a Class I occlusal cavity was performed with a 1/4 spherical carbide bur (bur head diameter 0.5 mm) (Kavo, São Paulo, SP, Brazil) at low rotation and constant irrigation with sterile saline solution (Fig. 1a). When pulp was visible through the transparency of the dentine at the cavity floor, which corresponded to approximately half the size of the bur in depth, a pulp exposure was created mechanically using a #15 K-File (Dentsply, Tulsa, OK, US) with light pressure, in order to standardize the exposure diameter to 0.15 mm (size of the tip of the file) (Simon et al., 2008) (Fig. 1b). The exposed area was irrigated with 3 mL sterile saline (Fig. 2a) and gently pressed with sterile paper points (Tanari Indústria, Manacapuru, AM, Brazil) for 2 min to contain the bleeding (Fig. 2b).

2.2.3. Direct pulp capping

The animals were randomized allocated into 2 experimental groups, as follows: MTA: ProRoot MTA (Dentsply) and MTA-CM: ProRoot MTA + hDPSC-CM.

The MTA was prepared according to the manufacturer’s recommendations for the MTA group. For the MTA-CM group, the distilled water routinely used to prepare this material was replaced by the hDPSC-CM, as follows: MTA powder (0.003 g) was mixed with 5 μL of hDPSC-CM freshly thawed. Pulp capping materials were carefully carried onto the exposure site using a ball-point probe (Millenium, Pinda-monhangaba, SP, Brazil), with an amount of material corresponding to the tip of the probe. The material was then adapted and accommodated at the cavity with the aid of sterile paper points with gentle pressure.
The coronary sealing was performed according to the following steps: the cavity surface was conditioned with 37% phosphoric acid (Dentsply) applied for 15 s and then washed with distilled water for the same period. The cavity was gently dried with sterile paper points and the Adper Single Bond 2 Adhesive System (3 M ESPE, St. Paul, MN, USA) was applied and polymerized for 20 s after gently agitating for

Fig. 1. Illustrative photographs of class I occlusal cavity being prepared with a carbide bur at low rotation (a). Pulp exposed (arrow) in the upper molar surface (b).

Fig. 2. Photographs illustrating the procedures for pulp capping and coronary sealing in the rat molar. (a) Irrigating with sterile saline after pulp exposure. (b) Absorbing the excess liquid with paper points. (c) Capping material deposited on exposed pulp. (d) Conditioning with phosphoric acid. (e) Placing the Bulk Fill composite. (f) Molar tooth with final coronary sealing (arrow).
solvent evaporation. The Bulk Fill Surefil SDR Flow (Dentsply) composite was then deposited and adapted onto the cavity with the tip of an explorer and polymerized for 20 s (Fig. 2d–f). The animals received a restricted diet of crushed and sieved feed.

2.2.4. Histological analysis

The animals were euthanized 4 or 8 weeks after pulp capping procedure. After removal of soft tissue from specimens, the maxillae were fixed in 10 % paraformaldehyde solution buffered in PBS. Samples were submitted to 3 cycles of 5 min in microwave with controlled heating (37°C; Pelco 3400, Ted Pella, Redding, CA, US) (Massa and Arana-Chavez, 2000). After 48 h, decalcification was accomplished using a 10 % EDTA solution under constant agitation. Solution was substituted every other day. Decalcification process occurred until 70 days. Samples were washed in water, dehydrated and embedded into paraffin. Tissue specimens were sagittally sectioned at 5 μm until reach the pulp exposure location and then stained with hematoxylin and eosin (H&E).

The slides were randomly renamed and distributed to a blind evaluator (experienced pathologist). They were qualitatively evaluated under light microscopy at up to 100x magnification and classified according to the following criteria:

Dentin bridge formation: presence (+) or absence (–) of dentin bridge formation at the pulp exposure site. When dentin bridges were identified, the degree of coverage with dentin bridge was evaluated, as follows: complete (+) or incomplete (–) bridges. Samples in which dentin bridge did not fully covered the exposed pulp area were considered incomplete;

Pulp tissue condition: presence (+) or absence (empty crown chamber or necrotic tissue). When the dental pulp was preserved their status was classified based on the Inflammation signs: presence (+) or absence (–). These signs evaluated were presence of dilated and congested blood vessels, red blood cells out of the vessels, infiltration of inflammatory cells (such as polymorphonuclear leukocytes and neutrophils) or microabscesses.

3. Results

3.1. In vitro assessment

3.1.1. Immunophenotypic profile of cells

The characterization of hDPSCs through the detection of mesenchymal stem cells surface markers using flow cytometry showed that the cells expressed the typical levels of these markers (Fig. 3). The cultures expressed the MSC-associated markers [CD44 (93.4 %), CD105 (64.68 %) and CD146 (53.2 %)]. The non-associated markers, such as hematopoietic [CD45 (0.06 %) and CD34 (0.046 %)] and endothelial [CD14 (0.41 %)] markers showed absent or minimal expression.

3.2. In vivo assessment

In some histological sections (5 of 24 for dentin bridge formation analysis and 4 of 24 for pulp tissue analysis) it was not possible to precisely locate the pulp exposure site. Therefore these samples were not considered for histological analysis.

3.2.1. Dentin bridge formation

The percentages of samples showing dentin bridge formation for each group and experimental time are presented in Table 1. Dentin bridge formation failed in 40 % and 20 % of the samples of the MTA.
Dentin bridge formation occurred in all samples of the MTA-CM group at the both experimental times and almost all of them were complete. When the conditioned medium was associated with MTA, there was a high percentage of samples presenting formation of dentin bridges in both experimental periods. Representative histological aspects of dentin bridges formation at 4 and 8 weeks are illustrated in Fig. 4.

In MTA samples the dentin bridges were mostly non-homogeneous dentin bridge, with pulp remains retained inside the hard tissue (Fig. 5a). When the conditioned medium was associated with MTA, there was a high percentage of samples presenting formation of complete dentin bridges in both experimental periods. Moreover, roof and floor of pulp chambers in this group frequently appeared with extensive calcification, bringing these two walls closer together (Fig. 5b).

### Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>MTA</th>
<th>MTA+</th>
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<tbody>
<tr>
<td>Experimental Time</td>
<td>4W 8W</td>
<td>4W 8W</td>
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<tr>
<td>Dentin Bridge formation analysis</td>
<td></td>
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<tr>
<td>Samples (n=n)</td>
<td>5 5</td>
<td>4 5</td>
</tr>
<tr>
<td>Dentin Bridge Formation</td>
<td>60% 80%</td>
<td>100 100%</td>
</tr>
<tr>
<td>Complete Dentin Bridges</td>
<td>66.6% 100%</td>
<td>100% 80%</td>
</tr>
<tr>
<td>Samples (n=n)</td>
<td>5 5</td>
<td>5 5</td>
</tr>
</tbody>
</table>

Pulp Tissue analysis

| Presence of Dental Pulp tissue | 100 100% |
| Presence of Inflammation Signs | 83.4% 75% |

#### 3.2.2. Pulp tissue condition

The vast majority of samples preserved the dental pulp tissue for both groups in both experimental times. Percentage of specimens of MTA group presenting inflammatory signs was high (Table 1). Fig. 6 illustrates pulp tissue conditions.

### 4. Discussion

The pulp tissue has important functions, participating in the dental root formation, acting as biosensor to perceive external stimuli, providing nutrition and sustaining teeth (da Rosa et al., 2018). In order to maintain the vitality of the pulp tissue, direct pulp capping is considered a minimally invasive therapy for exposed vital pulp. In an in vitro and in vivo study, Kim et al. (2018) evaluated the anti-inflammatory and mineralization effects of ProRoot MTA and Endocem MTA and concluded that successful healing of dental pulp tissue depends on the control of inflammation levels and, consequently, that successful direct pulp capping procedure for the treatment of inflamed pulps requires more effective pulp capping materials.

The experimental hypothesis tested in the present pilot study was that an alternative pulp capping material composed by MTA manipulated with medium conditioned by hDPSC when applied to exposed dental pulp would induce an improved response when compared to that to MTA alone, a well-known pulp capping material. This hypothesis was based on the presence of growth factors in the hDPSCs’ conditioned medium that would control the inflammatory and regenerative process in the dental pulp tissue. Indeed the results showed that the hDPSC-CM was able to improve the performance of MTA in forming dentin bridges, in the organization of this newly formed hard tissue and, mainly, in the modulation of the inflammatory response.

Based on the revised literature, this is the first study that evaluated the influence of a stem cell conditioned medium on direct pulp capping. Prior to that, hDPSC-CM had already been used in in vivo models for pulp...
regeneration therapies (de Cara et al., 2019; Murakami et al., 2015) and for dental replantation (Al-Sharabi et al., 2017), demonstrating favorable results that indicated its promising potential on regeneration of dental tissues. All of these studies, like the present one, used human stem cells for application in rats. Even using human stem cells conditioned medium in rats, with the possibility that these animals might not have receptors for all the human growth factors putatively present in this medium, it was possible to verify positive effects of this alternative pulp capping material over the results of the MTA alone. Possibly, even better results could be reached if future studies with medium conditioned by stem cells isolate from of rats.

To minimize the bias inherent in qualitative analyses, the histological slides were evaluated by an experienced pathologist and all samples were randomized and blinded to the evaluator. This analysis showed that the specimens of the MTA-CM group presented dentin bridge formation in all samples at both experimental times, whereas the MTA group presented 40 % and 20 % of failure in forming bridges at 4 weeks and 8 weeks, respectively. Thus, the association of hDPSC-CM with MTA in fact positively influenced the repair response.

The MTA group presented disorganized dentin bridges’ structure, especially due to the presence of pulp tissue trapped inside these bridges and a tendency to calcification in the roof and floor regions. Those findings are in accordance to those of Simon et al. (2008), that described dentin bridges with globular surface morphology in MTA-treated pulp exposures, and of Dammaschke et al. (2019), which stated that the new calcified hard tissue formed after pulp capping with MTA was not regular dentine and did not seem to be the product of genuine odontoblast differentiation. Instead, the newly formed tissue was more characteristic of a cicatricial process than a genuine regeneration response. This result is worry because if in only 8 weeks this amount of calcification was formed inside the dental pulp, one can infer that in a longer period of time maybe the whole coronary dental pulp will be calcified. In this case, if endodontic therapy would be indicated in the future, the localization of the root canal entrances will be a way difficulted. In addition, calcification of the pulp chamber may lead to a lack of sensitive response to aggressive stimuli, such as dental caries, impairing the defense mechanisms of the dentin-pulp complex. These results pointed out to the need of new materials with formulations that could control this dental pulp calcification process.

MTA and MTA-CM mostly preserved the coronal dental pulp tissue. However, the inflammatory signs in these dental pulps differed in function of time and experimental groups.

At 4 weeks, the inflammatory response was more pronounced than in 8 weeks, especially in the MTA group. Although inflammation is an important process towards regeneration, it needs to be controlled so that the pulp tissue can restore its physiological condition. If the inflammation is too severe, or if it persists for longer than desired, the pulp may be compromised. The association of hDPSC-CM with MTA demonstrated a higher percentage of samples with pulp tissue with absence of inflammatory signs (75 % at 8 weeks), when compared to MTA used alone (25

Fig. 5. Representative photomicrographs of MTA samples at 8 weeks of (a) extensive calcification inside the dental pulp and dental bridges englobing soft tissue and, (b) calcification at the roof and floor of pulp chamber (arrows), bringing these walls closer together (H&E staining; bars indicate 200 μm, original magnification 200X).

Fig. 6. Photomicrographs showing pulp tissue conditions in the corners far from the dental pulp exposure after pulp capping. (a) Dental pulp tissue demonstrating presence (+) in inflammation signs, with hyperemia and inflammatory infiltrate in the MTA group and (b) Pulp tissue demonstrating absence (-) of inflammatory signs in the MTA-CM group (H&E staining; bars indicate 200 μm, original magnification 200X).
The association of hDPSC-CM and MTA showed beneficial effects on direct pulp capping in rats, superior to those of MTA when applied solo. There was an improvement in the percentage of dentin bridges formation, in the morphological quality of these newly formed hard tissue and, mainly, in controlling pulp tissue inflammation. Thus, hDPSC-CM has the potential to improve the performance of pulp capping materials and deserves to be considered for future studies in regenerative dentistry.

Funding
This work was supported by National Council for Scientific and Technological Development (CNPq) [grant numbers 306423/2018-9 and 434750/2018-2] and Foundation for Research Support of the State of São Paulo (FAPESP) [grant number 2017/16777-5]. Giovanna Sarra was supported by CAPES. The authors thank Dentsply Sirona for donating the MTA ProRoot.

Author’s contributions
Giovanna Sarra contributed to conceptualization, in vivo and in vitro experimental procedures and manuscript writing. Manoel Eduardo L. Machado contributed to conceptualization and manuscript writing and revisions. Marcia M. Marques contributed to conceptualization, histological analysis and manuscript writing and revisions. Héctor V. Caballero-Flores contributed to in vivo experimental procedures and manuscript revision. Maria S. Moreira contributed to literature review, in vivo experimental procedures and manuscript revision. Ana Clara F. Pedroni contributed to literature review and in vitro experimental procedures. All authors have read and accepted the final version of this manuscript.

Declaration of Competing Interest
The authors deny any conflicts of interest related to this study.

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