

# Histological evaluation of dentin-pulp tissue response after transplantation of stem cells from periosteum and human deciduous teeth and in rats

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## ABSTRACT

Endodontic treatment offers a high success rate; however, the search for therapeutic alternatives that promote regeneration of the dentin-pulp complex has elicited great interest. **Aim:** The aim of this study was to evaluate, in an in vivo model, the potential for dentin-pulp regeneration by transplantation of stem cells from two sources: pulp of human deciduous teeth and periosteum. **Materials and Method:** Eighteen mesiobuccal canals of maxillary first molars of immunosuppressed rats were distributed into three groups (n=6): group DT (deciduous teeth pulp), received stem cells from deciduous tooth pulp in hydrogel vehicle; group P (periosteal), received periosteal stem cells in hydrogel vehicle; and group NC (negative control) received only hydrogel. After pulpectomy and canal preparation, the teeth received the respective materials, and the cavities were sealed. After 12 weeks, the animals were euthanized and the specimens underwent histological processing and qualitative evaluation of intracanal fibrous connective tissue, odontoblast-like cells, intracanal mineralized tissue, and inflammatory cell infiltrate. **Results:** In the NC group, abundant presence of inflammatory cells was observed throughout the canal, in addition to dentin chips and remnants of amorphous substance from pulp tissue. In groups DT and P, in the apical region, new tissue formation suggestive of repair was observed, with deposition of bone-like mineralized tissue and periodontal ligament-like connective tissue containing blood vessels and osteocyte- and fibroblast-like cells. **Conclusions:** Transplanted stem cells from pulp of human deciduous teeth and periosteum did not show potential for regeneration, but for tissue repair, with formation of periodontal ligament-like and bone-like tissue.

**Keywords:** regeneration - dental pulp - periosteum - rats - histology

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## Avaliação histológica da resposta do tecido dentino-pulpar após transplante de células-tronco do periosteio e de dentes decíduos humanos e em ratos

### RESUMO

O tratamento endodôntico oferece alta taxa de sucesso; contudo, a busca por alternativas terapêuticas que promovam regeneração do complexo dentino-pulpar tem despertado grande interesse. **Objetivo:** O objetivo deste estudo foi avaliar, em modelo in vivo, o potencial de regeneração dentino-pulpar mediante transplante de células-tronco provenientes de duas fontes: polpa dentária de dentes decíduos e periosteio humanos. **Materiais e Método:** Dezoito canais mesiovestibulares de primeiros molares superiores de ratos imunossuprimidos foram distribuídos em três grupos (n=6): grupo DT (polpa de dentes decíduos), recebeu células-tronco da polpa de dentes decíduos veiculadas em hidrogel; grupo P (periosteio), recebeu células-tronco do periosteio veiculadas em hidrogel; e grupo NC (controle negativo), recebeu apenas hidrogel. Após pulpectomia e preparo dos canais, os dentes receberam os respectivos materiais e as cavidades foram seladas. Após 12 semanas, os animais foram eutanasiados, e os espécimes submetidos a processamento histológico e avaliação qualitativa de tecido conjuntivo fibroso intracanal, células semelhantes a odontoblastos, tecido mineralizado intracanal e infiltrado celular inflamatório. **Resultados:** No grupo NC, observou-se presença abundante de células inflamatórias ao longo de todo o canal, além de raspas de dentina e restos de substância amorfa do tecido pulpar. Nos grupos DT e P, na região apical, observou-se neoformação tecidual sugestiva de reparo, com deposição de tecido mineralizado semelhante a osso e de tecido conjuntivo semelhante ao ligamento periodontal, contendo vasos sanguíneos e células semelhantes a osteócitos e fibroblastos. **Conclusões:** Os transplantes de células-tronco da polpa de dentes decíduos e do periosteio não apresentaram potencial de regeneração, mas de reparo tecidual, com formação de tecido semelhante ao ligamento periodontal e osso.

**Palavras-chave:** regeneração - polpa dentária - periosteio - ratos - histologia

## INTRODUCTION

Dentoalveolar trauma and carious lesions often lead to the development of pulpitis and pulp necrosis, which are commonly treated by pulp removal and replacement with inorganic materials (gutta-percha and endodontic cement). Although current endodontic treatment modalities offer high success rates, altered or necrotic pulp tissue should optimally be removed and replaced with new healthy, vital pulp tissue<sup>1-2</sup>.

Tissue engineering is the field of science that seeks to restore function, structure, and physiology in tissues compromised by disease or trauma. It is based on the triad of stem cells, signaling molecules (e.g., growth factors), and a scaffold, the medium which will serve as the structural framework for cells during tissue formation<sup>3-4</sup>.

Regeneration has been defined as “the restoration of damaged tissue by tissue similar to the original tissue with concomitant return of biological function”<sup>5</sup>. Therefore, regeneration of the dentin-pulp complex translates into formation of a tissue identical to the damaged native pulp, with the exact same architecture and cellular distribution, composed of fibroblasts, odontoblasts, stem cells, and host defense cells, as well as vascularized and reinnervated<sup>6</sup>. The regenerated tissue must be able to provide sensation, supply nutrition, form new dentin, and mount a response to infection just as the original dentin-pulp complex<sup>4</sup>.

Essentially, two pathways to pulp regeneration have been researched: cell recruitment or stem-cell transplantation. In the first model, a scaffold doped with chemotactic signaling molecules is inserted into a previously prepared root canal so that endogenous progenitor cells are recruited into the canal to form new tissue<sup>4</sup>. In the second model, stem cells are isolated, cultured, and added to a scaffold, which is then transplanted directly into the previously prepared root canal<sup>7-8</sup>.

Gronthos et al.<sup>9</sup> were the first to isolate dental pulp stem cells (DPSCs): mesenchymal cells with multipotential capacity to differentiate into cells similar to those which make up pulp tissue and therefore act as precursors for tissue regeneration in endodontics. In addition to DPSCs, other mesenchymal stem cells have been identified and characterized, such as stem cells of apical papilla (SCAPs), pulp stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem

cells (PDLSCs), bone marrow-derived mesenchymal stem cells (BM-MSCs), and cultured autogenous periosteal cells (CAPCs), among others<sup>10-12</sup>.

To make stem-cell transplantation for pulp regeneration a viable procedure in everyday dental practice, research has been carried out in animal models, with ectopic, semi-orthotopic, and orthotopic transplants<sup>13</sup>. Clinical research in this line has also advanced, with several reports of outcomes in human patients<sup>8,14-17</sup>. However, these studies have only been able to provide subjective data from imaging modalities and pulp sensitivity testing<sup>8</sup>. Rats approximately 3 months of age were used in this study which have complete root formation and, therefore, minimal relationship between the dentin-pulp complex and the periapical tissue. This fact guided this study with the purpose of evaluating the possible regenerative potential of the dentin-pulp complex in immunosuppressed rats in pulp conditions similar to those of an adult human being<sup>18</sup>. There are already reports in the literature on the regenerative potential of stem cells from human deciduous teeth<sup>19-20</sup>; however, to the best of our knowledge, to date there have been no published reports of research evaluating the potential of periosteal stem cells to regenerate the dentin-pulp complex. As it can be readily accessed throughout the life span via a single superficial tissue layer in the oral cavity, the periosteum is an extremely advantageous source of stem cells<sup>21</sup>.

In view of the foregoing and given the vast range of possible combinations of the elements of the tissue engineering triad, regenerative endodontic therapy is a broad field for research seeking to consolidate the much-desired goal of regeneration of the dentin-pulp complex and integrate it into the clinical armamentarium of endodontics practice. The objective of the present study was to evaluate, in an *in vivo* model, the potential for dentin-pulp complex regeneration after transplantation of stem cells derived from human exfoliated deciduous teeth and periosteum into rat molars with complete root formation.

## MATERIALS AND METHOD

Cryopreserved SHEDs and CAPCs, obtained through primary culture, were donated by R-Crio (Campinas, SP, Brazil), as approved by the Brazilian National Research Ethics Committee

(Certificate of Submission for Ethical Approval: 52269321.5.0000.5374; 52217321.7.0000.5375).

Sample size calculation was based on prior work by Pelegrine et al.<sup>22</sup>. One-way ANOVA was performed (G Power 3.1.9.4, Franz Faul, University of Kiel, Germany) with  $\alpha = 0.05$ ,  $\beta = 0.80$ , and effect size  $f = 3.40$ . The minimum sample size was calculated as 6 per group.

### Animal Selection and Preparation

Once the study protocol had been approved by the Institutional Animal Care and Use Committee (protocol nos. 2021/31 and 2021/32), male Wistar rats (*Rattus norvegicus albinus*; age 12 weeks, weight ~300 g) were selected. All experiments were performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and the National Research Council *Guide for the Care and Use of Laboratory Animals*.

Considering the possibility of rejection of human cells, an immunosuppression protocol was started 10 days before the experiment, as described by Lekhoo et al.<sup>23</sup>, and continued until euthanasia. The animals were anesthetized by intraperitoneal injection of 10% ketamine hydrochloride 50 mg/mL (Quetamina®; Vetnil, Louveira, SP, Brazil) and 2% xylazine hydrochloride 10 mg/mL (Sedanil®; Vetnil). The animals were placed in dorsal decubency on a wooden platform. To serve as a mouth gag, orthodontic elastics were wrapped around the incisors of both arches and the other end attached to the platform on which the animal was placed. To keep the cheeks apart, small retractors were made from #08 orthodontic wire (Morelli, Sorocaba, SP, Brazil) folded into a convenient shape.

### Study Groups

Eighteen mesiobuccal canals of maxillary first molars from 12 rats were distributed into three groups (n=6 each): in Group DT (deciduous teeth pulp), canals received stem cells from human exfoliated deciduous teeth in hydrogel vehicle; in Group P (periosteal), canals received periosteal stem cells in hydrogel vehicle; and in Group NC (negative control), canals received only hydrogel. For the DT and P groups, the same six animals underwent the transplant procedure, with the maxillary first molars on the right side receiving SHEDs and the maxillary first molars on the left side receiving CAPCs. For the NC group, another six animals were used.

### Endodontic Procedures

Access to the pulp chamber was obtained with a No. 1/2 long-shank high-speed round carbide bur (Microdont, São Paulo, Brazil) under refrigeration. After locating the mesiobuccal canal and irrigating the pulp chamber with 2.5% NaOCl (Fórmula e Ação, São Paulo, Brazil), the canal was explored with a #10 K-file (Dentsply Maillefer, Ballaigues, Switzerland) and the working length established 1 mm short of the apical foramen, with the aid of an electronic apex locator (Propex pixi; Dentsply Sirona, Ballaigues, Switzerland). Instrumentation was performed with Medium (35.06) WaveOne Gold system files (Dentsply Sirona, Ballaigues, Switzerland), driven by an X-Smart Plus engine (Dentsply, Ballaigues, Switzerland) set to the WAVEONE GOLD program. The tooth was instrumented in thirds with small-amplitude in-and-out movements (“pecking motion”) until the working length was reached. After every three pecking motions, the canal was irrigated with 1 mL 2.5% NaOCl via a NaviTip 30-gauge needle (Ultradent, South Jordan, UT, EUA) and hypodermic syringe (Injex, São Paulo, Brazil), and apical patency was confirmed with a #10 K-file. After chemomechanical preparation, the canal was irrigated with 1 mL of ethylenediaminetetraacetic acid (EDTA) 17% (Fórmula e Ação, São Paulo, Brazil) for 1 minute and 1 mL of 0.9% sodium chloride (Fórmula e Ação, São Paulo, Brazil). Final aspiration was performed with a Capillary Tip (Ultradent, South Jordan, UT, USA), and the canal was then dried with absorbent paper points (Dentsply Maillefer).

### Hydrogel Compounding and Stem Cell Transplantation

The vehicle for stem cell delivery was a 1:1 chitosan/xanthan gum-based hydrogel ( $1 \times 10^6$ /mL). The method used to obtain the hydrogel was based on a 1:1 mass ratio of chitosan to xanthan gum<sup>24</sup>. To do so, a 1% w/v chitosan solution (Sigma-Aldrich, Cat. No. 448877, 83% deacetylation) was prepared in a solution composed of 2% v/v lactic acid (Merck, Cat. No. W261106) dissolved in ultrapure water (Milli-Q Direct Q 8/16 System) and homogenized in a mechanical overhead stirrer with a pitched-blade (turbine-type) impeller (Tecnal), at a rotation speed of 1000 rpm. Then, xanthan gum (Sigma, Cat. No. G1253) was dissolved at 1% w/v in ultrapure water (Milli-Q Direct Q 8/16 System) and homogenized in the overhead stirrer at 1000 rpm.

Finally, 100 mL of the chitosan solution was added to 100 mL of the xanthan gum solution with the aid of a peristaltic pump (Tecnal) at a rate of 5 mL/min, temperature of 25°C, under constant stirring at 2000 rpm. After homogenization was complete, stirring at 2000 rpm continued for an additional 5 minutes.

For the *in vivo* experiment, the chitosan/xanthan gum hydrogel was combined with the mesenchymal stem cells. The advanced therapy product was manufactured by mixing, in a 1:1 ratio, sterilized chitosan/xanthan hydrogel with a final concentration of  $1.0 \times 10^6$  cells/mL resuspended in culture medium (Human Mesenchymal-XF Expansion Medium, Sigma–Aldrich) supplemented with 10% v/v human serum (Sigma–Aldrich, H4522), 1% v/v L-glutamine (Sigma–Aldrich), and 1.1% v/v penicillin/streptomycin (Sigma–Aldrich).

In groups DT and P, approximately 25  $\mu$ L of the stem cell/hydrogel solution was instilled into the prepared root canal using a 30-gauge NaviTip needle attached to a 1 mL syringe. A Teflon barrier (Isotape®; TDV, Pomerode, SC, Brazil) was then placed and the cavity was sealed with a layer of mineral trioxide aggregate (MTA; Angelus, Londrina, PR, Brazil), followed by a layer of light-curing glass ionomer cement (Ionofast®; Biodynamics, PR, Ibioporã, Brazil). In the NC group, the procedure was similar, but the root canal was filled with hydrogel alone. After 12 weeks, the animals were euthanized by saturation with 2.5% isoflurane (Isoforine®; Cristália, São Paulo, SP, Brazil).

### Histological Processing

Specimens obtained from the maxilla were fixed in 10% buffered formalin for 24 hours, decalcified in

a 20% formic acid solution for 10 days, and rinsed under running water for 24 hours. They were then dehydrated in a graded ethanol series, cleared in xylene, and embedded in histological paraffin. For histological evaluation, serial longitudinal sections 4  $\mu$ m thick were obtained, stained with hematoxylin-eosin, and subsequently mounted on glass slides with Permount Mounting Medium (Fisher Scientific, Fair Lawn, NJ, USA).

Images of the slides were captured with a computerized acquisition system (AxioVision Rel. 4.8; Carl Zeiss, Oberkochen, Germany) coupled to a microscope (Axioskop 2 Plus; Carl Zeiss) and evaluated by a single examiner blinded to group allocation.

The following data were collected and qualitatively analyzed: intracanal fibrous connective tissue; morphologically odontoblast-like cells; cementum-like and/or bone-like and/or periodontal ligament-like intracanal mineralized tissue; inflammatory cell infiltrate; and presence of dentin chips.

### RESULTS

In samples from the NC group, histological analysis revealed an abundant, predominantly polymorphonuclear inflammatory infiltrate throughout the entire canal, in addition to dentin chips resulting from the instrumentation procedure and remnants of amorphous substance from pulp tissue (Fig. 1).

On histological examination of specimens from groups DT and P, dentin chips, remains of amorphous substance from pulp tissue, and mononuclear inflammatory infiltrate were also observed; however, the latter was less evident. Furthermore,

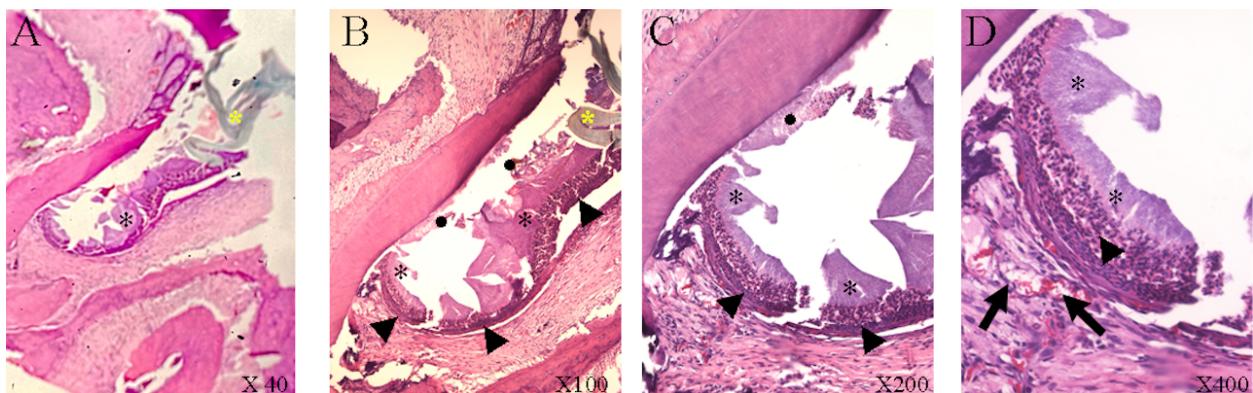


Fig. 1: Representative photomicrograph of longitudinal sections of a mesiobuccal root from a maxillary first molar of a rat from the NC group (H&E; X40, X100, X200, X400 original magnification). Note representative images of dentin chips (\*), amorphous substance (•), polymorphonuclear inflammatory cells (▶), and blood vessels (†). The yellow asterisk shows the Teflon barrier.

in the apical region of the root canal (intracanal), new tissue formation suggestive of repair was observed, with deposition of a bone-like basophilic material consistent with mineralized tissue and of a loose, periodontal ligament-like connective tissue, which included the presence of blood vessels and osteocyte- and fibroblast-like cells (Figs. 2, 3).

## DISCUSSION

Modern endodontics practice requires harmony between good technique and the best science. Once this balance is achieved, the objective is to ensure health, maintain and/or restore function, and guarantee the longevity of the treated teeth. Regenerative endodontic therapy is becoming tantalizingly closer to daily clinical practice. Advances in research, as well as in our understanding

of the mechanisms that guide tissue engineering, have cleared the way towards achieving the much-desired goal of regeneration of the dentin-pulp complex. The present study evaluated the potential of stem cell transplantation to regenerate the dentin-pulp complex, since the cell recruitment strategy commonly results in tissue repair, not regeneration<sup>5</sup>. Stem cells are indispensable in the tissue engineering triad. As noted above, there are several sources of stem cells with potential for regeneration of the dentin-pulp complex: DPSCs<sup>7-9, 25-26</sup>, SCAPs<sup>12,27</sup>, BM-MSCs<sup>16</sup>, umbilical cord mesenchymal stem cells (UCMSCs)<sup>14</sup>, and SHEDs<sup>19</sup>, among others. In a clinical trial, Xuan et al.<sup>19</sup> evaluated the regenerative potential of DPSCs and observed, in the histological analysis of a single case, complete regeneration of the dentin-pulp complex, concluding that this cell type

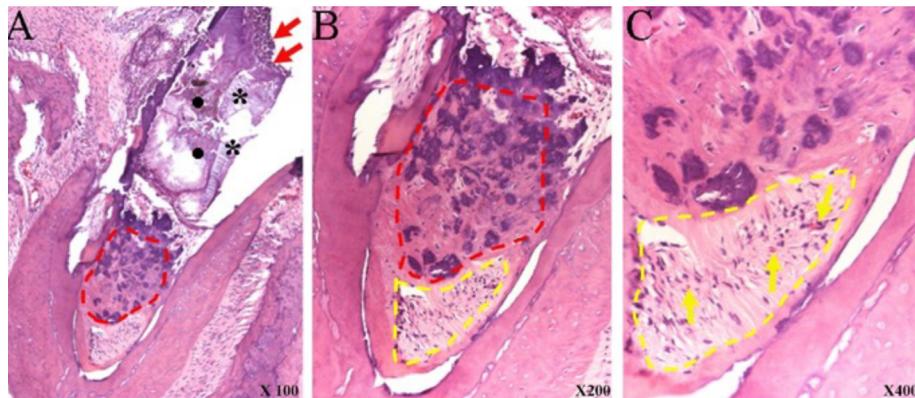


Fig. 2: Representative photomicrograph of longitudinal sections of a mesiobuccal root from a maxillary first molar of a rat from the DT group (H&E; X100, X200, X400 Original magnification). Note the presence of dentin chips (\*), amorphous substance (•), polymorphonuclear inflammatory cells (red arrows), blood vessels (yellow arrows). The dotted lines represent bone-like mineralized tissue (red) and periodontal ligament-like loose connective tissue (yellow).

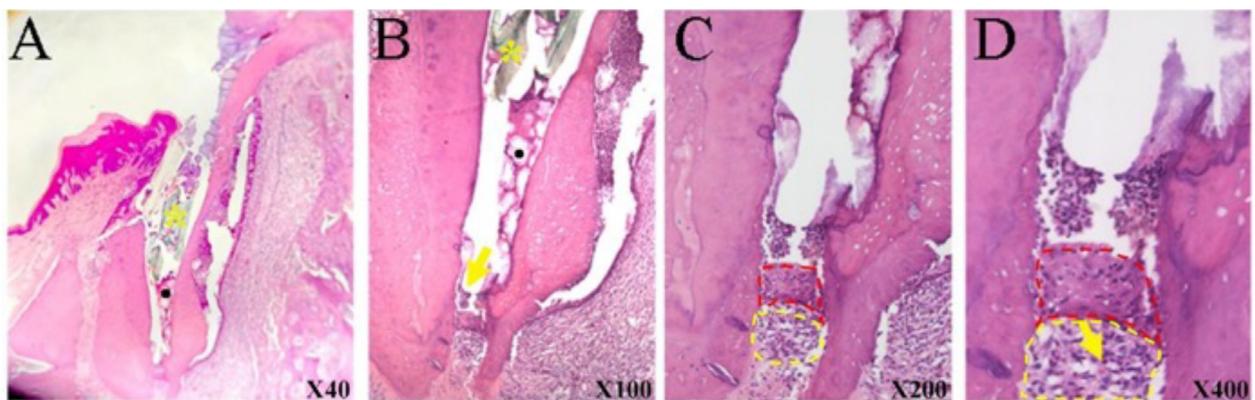


Fig. 3: Representative photomicrograph of longitudinal sections of a mesiobuccal root from a maxillary first molar of a rat from the P group (H&E; X40, X100, X200, X400 original magnification). Note representative images of amorphous substance (•), polymorphonuclear inflammatory cells (red arrow), and blood vessels (yellow arrow). The dotted lines represent bone-like mineralized tissue (red) and periodontal ligament-like loose connective tissue (yellow). (\*) Teflon barrier.

was safe and efficacious for this purpose. Conversely, research recently published by our group<sup>20</sup>, despite confirming the presence of odontoblast-like cells, found that the transplantation of SHEDs to rat molars resulted in the formation of a cementum-like reparative tissue rather than regeneration. Regarding stem cells of periosteal origin, to the best of our knowledge, there is no research reporting their use in regenerative endodontic therapy, despite their ease of collection<sup>28</sup>.

Several groups have advanced research into regenerative therapies by conducting clinical trials with promising results<sup>8,14-16,19</sup>. However, the vast range of possible combinations of the elements of the tissue engineering triad and the lack of standardization in regenerative endodontic therapy protocols mean both *in vitro* and *in vivo* research pursuits are still warranted. Animal studies allow histological examination of newly formed tissues, which is a major limitation of clinical trials. In this context, the use of animal models, especially rat models, is well established in the literature<sup>9,12,27,29</sup>. Nevertheless, the use of orthotopic and heterotopic models is uncommon. A systematic review found only 10 studies using orthotopic models between 2010 and 2017, only one of which was in rats<sup>25</sup>.

Rats were selected for the present study because this species combines several desirable characteristics of an animal model, namely: affordable cost, ease of ethical approval, similarity to the human genome, and speed in achieving endpoints of interest<sup>30</sup>. One month of life in a rat is comparable to 3 years of human life; therefore, significant results can be obtained in relatively little time<sup>31</sup>. In this context, we chose to use 12-week-old rats, an age at which complete root formation is already present but degenerative changes are absent<sup>18</sup>. Furthermore, the 12-week period between the experiment and euthanasia is commonly reported in the literature, justifying its adoption in the present study<sup>32-33</sup>.

Due to their anatomical, physiological, histological, and biological characteristics, the first molars of rats can be considered miniatures of human molars and, although their small dimensions make cell transplantation difficult, it is not impossible<sup>34</sup>. The mesiobuccal root was chosen to carry out the experiment for its high buccal slope, ease of access, clearer distinction from other canals, and larger volume<sup>35</sup>.

No histological similarity was observed between the

newly formed tissue seen in the DT and P groups and normal pulp tissue. Contradicting the results of Xuan et al.<sup>19</sup> and corroborating those of Zhu et al.<sup>7</sup> and Santos et al.<sup>20</sup>, the present study did not observe any regenerative potential of dental pulp stem cell transplantation but did observe tissue repair potential. Likewise, periosteal stem cell transplantation – to the best of our knowledge, not assessed for this purpose in any previous study – also demonstrated only tissue repair potential rather than any regenerative potential.

Among several factors that may explain these findings, a particularly relevant one is the blood supply of the intracanal region<sup>13</sup>. Adequate blood supply is of great importance to promote tissue regeneration, considering its ability to promote angiogenesis, and ensure the nutrition of stem cells in a timely fashion. The small size of the apical foramen can render this supply difficult, especially in molars<sup>36</sup>, hence impairing cell nutrition throughout the entire length of the root canal and potentially explaining a recurring observation in samples from groups DT and P with new tissue formation restricted to the apical region. Huang et al.<sup>37</sup> reiterated the importance of establishing a protocol that would allow satisfactory vascularization, thus enabling cell development up to the cervical third of the tooth. According to the authors, combined transplantation of endothelial cells, administration of growth factors, or apical enlargement – approaches not attempted in the present experiment – could meet this need. Taking together, these factors can explain the pattern of newly formed tissue observed in this trial. In a recent study, Buss et al.<sup>38</sup> evaluated the potential for bone regeneration after filling of critical calvarial defects in rats with xenografts combined with conditioned medium obtained from a stem-cell culture of human dental pulp lineage. Immunohistochemical analysis for markers of angiogenesis (VEGF and anti-CD34) showed that vascularization was stimulated by the conditioned medium. In future research, this could potentially be tested in the intracanal region to evaluate the potential for dentin-pulp complex regeneration.

A key finding in our histological analyses was the abundant presence of acute inflammatory cells throughout the length of the canal in samples from the NC group, whereas this was much less evident in the DT and P groups. This may have been due to the immunomodulatory characteristics of dental

pulp stem cells and periosteal stem cells, which can release anti-inflammatory cytokines such as IL-6 and IL-8 as well as growth factors implicated in angiogenesis, such as VEGF, FGF, and PDGF<sup>39</sup>. This would explain the development of a more favorable microenvironment in the specimens which received stem cell transplants.

Another important finding was the presence of debris (dentin chips and pulp tissue remnants) in all groups. The persistence of debris within the root canal system after chemomechanical preparation is well established in the endodontic literature, since current endodontic instruments are incapable of touching all dentin walls and irrigation/agitation is unable to remove all mineral debris<sup>40-41</sup>. Our results corroborate these well-established findings, indicating that instrumentation was unable to cover all dentin walls and that irrigation, in turn, was unable to remove all the debris generated by instrumentation.

The conception of this study was to outline a protocol for animal model research to transplant

stem cells from two different sources into the minute space of the rat root canal system. Future studies could evaluate the regenerative potential of cell transplantation in conjunction with other elements that might enhance its outcomes, e.g., growth factors, other cell types that stimulate angiogenesis/neurogenesis, and apical enlargement.

In an animal model, transplantation of stem cells derived from dental pulp and periosteum resulted in partial new tissue formation in the dentin-pulp complex. Further studies are needed to consolidate a predictable protocol for regeneration of the dentin-pulp complex in mature human teeth.

## CONCLUSIONS

Within the limitations of the present study, we conclude that transplanted stem cells obtained from the pulp of deciduous teeth and periosteal stem cells, delivered in a hydrogel scaffold, did not show potential for pulp regeneration, but rather for root canal tissue repair, with formation of intracanal vascularized fibrous tissue and bone-like tissue.

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## CONFLICT OF INTERESTS

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

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