Stem Cells from Human Exfoliated Deciduous Teeth in Delayed Tooth Replantation: Histologic and Histomorfometric Analyses in Rats

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Abstract: *Aim:* The complication in delayed replantation is root resorption that leads to loss of reimplanted teeth. This study was designed to evaluate the influence of stem cells from human exfoliated deciduous teeth (SHEDs) on the healing process of periodontal tissues after delayed tooth replantation in rats.

Material and Methods: Forty-five rats had their right upper incisor extracted and maintained in dry storage for 60 minutes. Then dental papilla was removed; root canal was prepared and filled with calcium hydroxide paste and root surface treatment was processed. Rats were randomly assigned into three groups: Control group: the incisor was replanted without any substance in the dental socket. SHEDs group: the tooth was replanted after infusion of SHED, re-suspended in platelet-rich-fibrin in the dental socket; platelet-rich-fibrin group: the incisor was replanted with platelet-rich-fibrin in the dental socket. Animals were euthanized on days 15, 30 and 60 after replantation; histological and histomorphometric analyses were performed.

Results: Root resorption was present in all experimental groups. No statistically significant differences were observed in the final period of study. The control group showed more replacement resorption but SHEDs group, showed dental tissues were more preserved. Inflammatory resorption nearly did not affect any group, as expected in this protocol ($p<0.005$).

Conclusions: The findings of this study suggest that in the SHEDs group, teeth were more preserved with ankyloses occurrence, witch holds teeth in the socket for a longer period of time, when compared to replacement resorption or inflammatory resorption in the healing process of delayed replantation.

Keywords: Periodontal regeneration, platelet-rich plasma, SHEDs, stem cells.

1. INTRODUCTION

Traumatic dental injuries are complex because various tissues and multiple structures are involved. The goal of the healing process is to restore continuity between margins and reestablish the function of tissues. Even when the damage results in small lesions, periodontal ligament repair after dental replantation is difficult to achieve [1]. These injuries carry one of the poorest outcomes for dentoalveolar trauma and the majority of replanted teeth are lost prematurely.

Clinical experience has shown that storage of replanted teeth in dry or in low humidity media cause periodontal ligament cells necrosis, invariably leading to development of external root resorption [2]. External root resorption is a serious complication after dental replantation and both inflammatory resorption and replacement resorption are major causes of teeth lost. Reimplanted teeth resorption can be classified as superficial resorption, replacement resorption, ankyloses and inflammatory resorption [1, 3].

Considering current knowledge of the reimplanted teeth healing process and biological events in the absence of periodontal ligament, a therapeutic approach should be based on inflammatory resorption prevention with neutralization of contamination, performing endodontic treatment, systemic antibiotic therapy and teeth surface treatment [4].

Adult stem cells are undifferentiated and responsible for producing specialized cells during tissue differentiation, cell renewal and the tissue repair process. Just like embryonic, adult stem cells have the capacity of self-renewal and differentiation into specialized cell types during cell division. These

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characteristics guarantee maintenance of undifferentiated cells that are capable of perpetuating repair capacity and tissue renewal [5, 6].

Besides, plasticity, another advantage of adult stem cells is a possibility of autologous use, assuring no risk for immune rejection. Currently, stem cells have been identified in almost all tissues and this hopefully enlightens future possibilities for treatment of degenerative and traumatic diseases that still do not have an available therapy [6].

Classically, mesenchymal stem cells are extracted from bone marrow but there are other sources of stem cells available. In the clinical therapeutic context, it is important to consider that bone marrow aspiration is very invasive and painful procedure [7].

Dental tissues are considered a rich mesenchymal stem cells source for application in tissue engineering. Several studies have shown the presence of stem cells in the dental pulp of deciduous teeth, in permanent teeth and also periodontal ligament. Human exfoliated deciduous teeth (SHED) are affordable and a relatively easy source of adult stem cells [8]. It is known that human primary teeth are promising source of mesenchymal stem cells for use in bone and dental tissue engineering since these cells have potential to differentiate in many cell types, including osteoblasts, neurons, odontoblasts, chondrocytes and adipocytes [9-14].

Stem cells from human exfoliated deciduous teeth (SHED) transplantation have shown to provide similar, and even better, therapeutical results than bone marrow stem cells in mouse models, suggesting that stem cells derived from human pulp deciduous teeth may be a possible source of stem cells for medicine therapy [13].

Researchers compared characteristic and properties of stem cells derived from dental tissues (SHEDs), with mesenchymal stem cells from bone marrow and concluded that SHEDs proliferate faster and larger amounts. *In vivo* and *in vitro* studies have shown mesenchymal stem cells derived from pulp dental tissues have the ability of cell proliferation and differentiation [15].

The aim of this study was to evaluate through histological and histomorphometrical analyses the influence of stem cells from human exfoliated deciduous teeth (SHED) transplantation in healing process of periodontal tissues after delayed tooth replantation in rats.

2. MATERIALS AND METHODS

2.1. Animals

Fifty-four Wistar male rats (Rattus norvegicus, albinus) weighing 250-350 g were used. Animals were fed with ground solid ration (Anderson and Clayton S.A.) and water *ad libitum*. Rats were housed under controlled conditions (12 h light/12 h dark).

Research protocol was approved by the Animal Research Ethics Committee of Sagrado Coração University, Bauru, São Paulo, Brazil (protocol number 015/14).

2.2. Stem Cells from Decíduos Teeth (Sheds)

2.2.1. Cultivation, Immunophenotype and Characterization and of Sheds

Dental pulp tissue was extracted from tooth previously selected for exodontia, with endodontic files, under a biosafety hood, and plated in culture flasks with DMEM - 10% fetal bovine serum and 50mg/ml gentamicin, at 37°C and 5% CO_2. Growth medium was exchanged every three days. After SHEDs confluence cells were dissociated with trypsin (Gibco Life Technologies Coorporation) and some cells were separated for cryopreservation.

SHEDs phenotypic characterization was performed in the sixth passage by flow cytometry and immunofluorescence analysis to further confirm mesenchymal stem cells.

2.2.2. Flow Cytometry and Immunofluorescence Analysis of Sheds

SHEDs, in the sixth passage (P6), were selected to detection of surface and intracellular antigens. The following antibodies were used: FITC-conjugated anti-CD90, PE-conjugated anti-CD38, PE-Cy5-conjugated anti-CD54, APC-conjugated anti-CD45, FITC-conjugated anti-CD105, PE-conjugated anti-CD166, PE-Cy5-conjugated anti-CD117, FITC-conjugated anti-CD44, PE-conjugated anti-CD73, PE-conjugated anti-CD31, FITC-conjugated anti-CD133 (BD Biosciences). PE-conjugated anti-OCT-4, and anti-7-STRO-1 (RandD Systems®, Inc.). Data acquisition and analysis were performed in flow cytometer (FACSCalibur flow cytometr, BD Biosciences) with specific software (BD CellQuest Pro software). At least 50,000 events were collected and analyzed. Unlabeled antibodies cells were used as controls.

For immunofluorescence analysis, cells were fixed with 4% paraformaldehyde for 15 minutes, washed in PBS, blocked with PBS 5% BSA for 30 minutes and incubated with primary antibodies overnight at 4°C. The following primary antibodies and dilutions were used: Anti-CD44, diluted 1:100; Anti-CD34, diluted 1:100 (Invitrogen); Anti-CD45, diluted 1:100 (BioSource, Life Technologies); Anti-CD90, diluted 1:100; Anti-CD105, diluted 1:100; Anti-CD73, diluted 1:100 (BD Bioscences); Anti-CD117, diluted 1:100; Anti-CD133, diluted 1:25 (Abcam); Anti-myosin, diluted 1:200 (Sigma-Aldrich); Anti-Cytokeratin 18, diluted 1:50 (Santa Cruz Biotechnology); Anti-SSEA-1, diluted 1:50; Anti-SSEA-4, diluted 1:50; Anti-TRA1-60, diluted 1:50, Anti-TRA1-81, diluted 1:50 (Chemicon); Anti-Collagen type I diluted 1:50 (Santa Cruz Biotechnology); Anti-GFAP, diluted 1:200 (Dako); Anti-Myelin, diluted 1:100 (Invitrogen); and Biotin-conjugated Anti-NeuN, diluted 1:50 (Chemicon).

On the following day, cells were washed and incubated with secondary antibodies or streptavidin, combined with Phalloidin for 1 h at room temperature. After this step, the following secondary antibodies diluted 1:200 were used: Anti-mouse IgG Texas Red-conjugated, Anti-mouse IgG Texas Alexa Fluor 568-conjugated, and Anti-rabbit IgG Alexa Fluor 568-conjugatted (Life Technologies). Slides stained with biotin-conjugated primary antibodies were incubated with Streptavidin (Life Technologies). Actin filaments were stained with Phalloidin Alexa Fluor 488-conjugated (Life Technologies). Cell nuclei were counterstained with DAPI (Vector Lab).

2.3. Genetic Control of Cultivated Sheds

2.3.1. Cytogenetic Analysis and Cell Viability Test

Chromosomal stability determination is the key point in stem cell lines establishment. Cytogenetic analysis may be performed in cell culture to monitor genetic instability and chromosomal abnormalities. G-banding or Giemsa banding, a cytogenetics technique, is used to produce visible karyotype by stained condensed chromosomes [16].

Cells were prepared for cytogenetic analysis as previously described and analyzed by G-banding technique [17]. Slides were observed in an optical microscope under 1,000X magnification, and approximately 20 metaphases of each sample were analyzed for numerical and/or structural cytogenetic abnormalities, according to the International System for Chromosome Nomenclature [18]. Images were captured *via* software (Image Pro Band View Plus). All cells obtained from passages 5 and 6 showed normal female karyotypes (karyotype: 46, XX), certifying SHEDs chromosomal stability. After freezing/defrosting and rinsing processes cells were re-suspended in PRF, and cell viability was checked with Trypan blue reagent. The sample was considered suitable for transplantation if at least 80% viable cells were found. Assuring infusion cellularity, approximately 2.0 x 10^6 *ex vivo* expanded SHEDs were added in PRF aliquots.

2.4. Platelet-Rich-Fibrin (PRF) Preparation

Nine mL of rat whole blood was withdrawn from cardiac puncture in a 10mL syringe containing 1mL of 3.2% sodium citrate. PRF fraction was obtained with a double centrifugation method [19]. After collection, whole blood was homogenized and transferred to collection tubes (BD Vacutainer®) and they underwent first centrifugation subjected at 200G for 10minutes at 23°C promoting blood cells separation. Supernatant plasma fog area (buffy coat), consisting of platelets and leukocytes, was transferred to a new sterile tube. Collected plasma was subjected to second centrifugation at 400G for 10 minutes at 23°C concentrating at least 1.000.000/µl platelets. Finally, supernatant was removed to achieve 800µl of PRF final volume. PRF was separated in sterile cryotubes containing 150µl of PRF and stored freezing at -80°C to be used later in experiment. A sample was obtained for platelet counting and control to ensure minimum concentration of 1.000.000/µl platelets.

Before surgical procedure PRF was completely thawed, homogenized and divided in 100µl PRF aliquots and added to 2x10^6 stem cells from human exfoliated deciduous teeth. PRF was activated with 10% Calcium Gluconate solution immediately before use and inserted in gel form in dental sockets.

2.5. Surgical Intervention and Tooth Preparation

Surgical procedures were performed under general anesthesia. Animals received xylazine chlorhydrate 0.03mL/100g body weight, in intramuscular injection for muscular relaxation and were anesthetized with ketamine chlorhydrate 0.07mL/100g body weight. Anterior maxilla asepsis was performed with 10% polyvinylpyrrolidone-iodine with 1% active iodine, followed by non-traumatic extraction of the maxillary right incisor of all animals. Extracted teeth were held by their crowns, fixed on red wax plate and kept dry at room temperature for 60 min.

After that, the periodontal ligament was removed mechanically with saline embedded gauze and teeth were immersed in 20mL of sodium fluoride 2% solution – pH 5.5 for 10 minutes [20]. Dental papilla was removed and pulp tissue was extirpated through retrograde *via* using n. 20° Hedstrom file. Fluid aspiration was performed, and root canals were dried using absorvent paper points. Canals were filled with calcium hydroxide paste composed of calcium hydroxid PA, and propyleneglycol spatulated in a glass plate obtaining pasty consistency. This paste was introduced filling the canal, retrograde *via* using 1mL/cc insulin syringe with 25 x 0.7 needle. The MTA plug was made and placed as a bandage in the root apical region retrograde *via* endodontic file (Sybron Kerr Corporation) and laid up with condensers.

The tooth was replanted in original socket according to random groups. Animals were randomly assigned in 3 groups of 15 specimens each, according to dental socket treatment accomplished before replantation, as follows: In the control group, the incisor was replanted without any substance in the dental socket; In the SHED/PRF group, the tooth was replanted after stem cells from deciduous tooth, resuspended in platelet-rich plasma, infusion in the dental socket; and in the PRF group, the incisor was replanted after platelet-rich plasma infusion in the dental socket.

After replantation, animals received penicillin G benzathine 20,000 IU in a single dose in the posterior left paw.

2.6. Histologic Procedures and Histological Analysis

Five animals for each group were euthanized with anesthetics overdose at 15, 30 and 60 days after replantation. Maxilla´s right side containing replanted teeth was separated with a#15 surgical blade in median line. Specimens were immediately fixed in 10% formalin for 2 days, washed in water for 24 hours and decalcified in 5% ethylenediaminetetraacetic acid (EDTA).

Specimens were embedded in paraffin and longitudinal semi-serial histologic slices (6μm thick) were obtained. Two slices of each specimen were stained with hematoxylin and eosin for histologic and histomorphometric evaluation under optical microscopy.

For histological events descriptive analyses the entire teeth extension were examined through the lingual surface since in rats teeth periodontal ligament fibers connect just in this area.

Histological analysis evaluated along the entire root extension, particularly dentine, cementum, periodontal ligament and bone tissue were also analyzed, using an optical microscopic. The following parameters were considered: connective tissue characteristics in periodontal space and fiber organization, cement presence or absence, replacement resorption, inflammatory resorption and ankyloses occurence.

Replacement resorption was considered when alveolar bone fills reabsorbed area of dentin. Resorbed dentine areas without cement and presence of inflammatory cells in adjacent periodontal ligament was classified as inflammatory resorption. Ankyloses was defined when bone tissue was in direct union with intact root cementum in the absence of periodontal ligament.

A single blinded and experienced examiner assigned histologic analysis.

2.7. Histomorphometric Analysis

For histomorphometric evaluation root surface extension on slice was divided into thirds. The middle third of the tooth was analyzed after microscopic examination attached to a digital camera (MicroPublisher 3.3 Real-Time Viewing; QImaging) connected to a computer. A photograph of each slice was taken at 10x magnification and resulting images were saved. An automated image-analysis system (Image-Pro® Plus 5.1.2 for Windows) was used to perform measurements of resorption areas and total area of each section. Data were exported to excel software, and resorption areas were noted and calculated in relation to total areas.

A single blinded and experienced examiner assigned histomorphometric analysis.

2.8. Statistical Analysis

Results from histomorphometric analysis were subjected to nonparametric tests using Kruskal Wallis analysis. Data analysis was performed in statistical program (SPSS® 20.0, Inc., USA) with significance level at $p<0.05$

3. RESULTS

3.1. Sheds Flow Cytometry and Immuno-fluorescence Results

Cells flow cytometry showed mesenchymal stem cell markers of high expression of CD105, CD73, CD

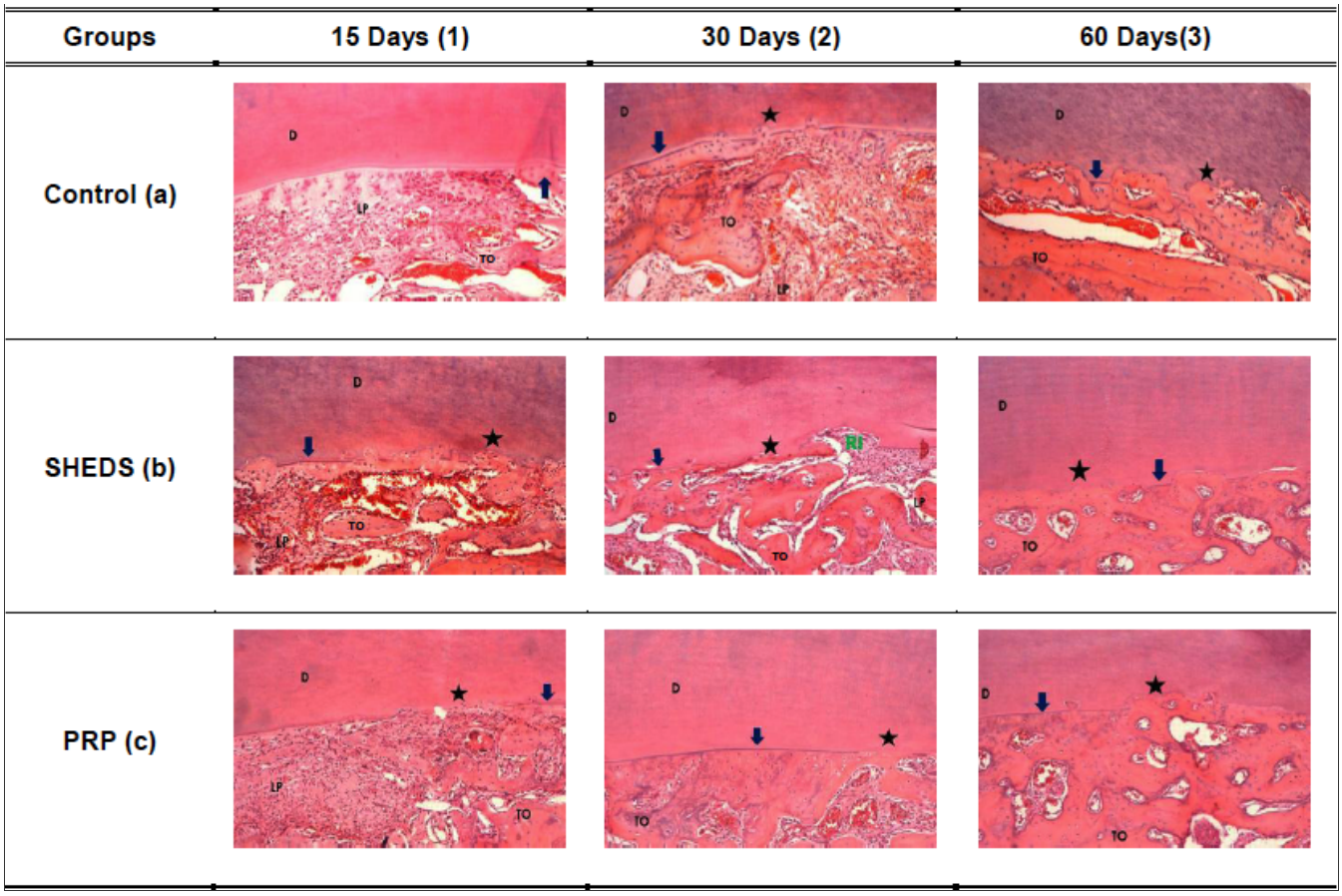

Figure 1: Photomicrographs illustrating histological findings. Hematoxylin and eosin stain, periodontal ligament (LP), bone tissue (TO) and dentine (D), alveolar cement, Replacement Resorption (★), Inflamatory Resorption (RI) and Ankyloses (⬇).

44, CD90, CD166, CD54, OCT-4, and STRO-1. These cell markers, and CK18, α-myosin, myelin, GFAP and collagen I, were also positive by immunofluorescence demonstrating and ensuring mesenchymal stem cell characteristics.

3.2. SHEDs Citogenetics Analyses Results

Cytogenetic analysis performed in cell culture showed genetic stability without chromosomal abnormalities. Cytogenetic analysis showed normal karyotype (46, XY) of cultured SHED at P5 and P6 cells (data not shown).

3.3. Histologic and Histomorfometric Analysis Results

Photomicrographs illustrating histological qualitative evaluation of periodontal tissues findings, stained in HE are presented in Figure **1**. Data of histomorphometric analyses as well as the comparison among experimental groups and the occurrence of root resorption at days 15, 30 and 60 are described in Table **1**.

Table 1: Comparison among Histomorphometric Values Analyzed in the Period of 15, 30 and 60 Days after Tooth Replantation. Reference Values - Total Area: 808.783,6 μm^2 (100%) and Lingual Extension 2.240,3μm (100%)

Parameters	Control			Stem Cells			PRP			*p*		
	15	30	60	15	30	60	15	30	60	15	30	45
Inflamatory Resorption (%)	0.49	0.85	1.39	1.14	1.86	3.22	0.42	1.29	1.56	0.453	0.994	0.177
Replace Resorption (%)	0.04	0.60	6.49	0.69	1.50	5.52	0.28	0.00	3.53	0.259	0.417	0.749
Ankylosis (%)	0.77	10.64	20.30	26.39	33.00	22.96	23.53	6.04	0.00	0.078	0.302	0.281

*Statistic significance at 5% ($p<0.05$).

3.4. 15 Days´ Histological Analysis Results

The control group showed periodontal ligament space preserved in most specimens, however, disorganization of collagen fibers was observed on periodontal ligament. Surface resorption areas were observed in the tooth and at this period, it was possible to observe some ankyloses areas, discrete, but evident.

In the SHEDs group, at the same period, specimens presented loss of dental tissue and extensive resorption areas in some specimens. Replacement resorption and ankyloses areas were the predominant outcome in this period and group.

The PRF group specimens showed periodontal ligament fibers disorganized. Discrete points of surface resorption were also observed. Cementum was preserved throughout almost the entire root surface and ankyloses areas were also present.

3.5. 30 Days' Histological Analysis Results

In the control group, an extensive resorption areas, major dental tissue loss and active root resorption areas with osteoclasts filled were observed. Many specimens had ankyloses areas alternating with replacement resorption areas over the thirds of the analyzed root surface.

The SHEDs group specimens, at this period, showed a predominance of ankyloses and presence of active root resorption areas. Only one specimen presented inflammatory resorption with evident inflammatory response next to intense resorption activity.

In the same period, in the PRF group, the periodontal ligament was deeply disorganized with noted and prevalent ankyloses areas. Active root resorption areas with osteoclasts presence also were observed in specimens.

3.6. 60 Days' Histological Analysis Results

Histological analysis of the control group showed active root resorption areas in almost all specimens and therefore extensive tooth loss. Ankyloses areas were observed on preserved tooth tissue.

At 60 days, in the SHEDs group, ankyloses and severe root resorption were observed in all specimens, however, it is noteworthy that there was greater preservation of tooth tissue in this group when compared to other groups in the same period.

The PRF group specimens showed severe loss of most dental tissue, with rigorous dentin resorption in all specimens. When there was some dental remaining, it was ankylosed. In two specimens, there was vast tooth loss with bone tissue formation in the socket region.

3.7. Histological Analysis Results Observations

All groups presented, at 60 days, vast loss of root structure. The SHEDs group showed, in the last analyzed period (60 days) greater tooth preservation. Even presenting moderate resorption, it can be noted that in this group (SHEDs) teeth were more preserved at the end of study than other groups. PRF specimens showed an acceleration in resorption response as metabolism was activated due to the imposed treatment.

4. DISCUSSION

A delay replantation preclinical model in rats was chosen representing complex situation in the dental clinic, since 73-96% of reimplanted teeth are lost prematurely because of progressive root resorption. Dental replantation success depends on a few factors to increase or delay resorption and ankyloses development and several approaches have been proposed [4, 20-24].

Stem cell infusion in the socket in delayed tooth replantation with endodontic treatment is an inedited approach and so tested in this research.

The stem cells used have origins in one of only two Brazil stem cell centers currently authorized - FIOCRUZ - Hospital St. Raphael in Salvador - Bahia. After transport to the Cytogenetics Laboratory of Blood Center of Marilia Medical School - FAMEMA, under proper care and storage temperature cells were cultured, expanded and underwent cytogenetic and chromosomal analysis confirming no chromosomal and /or genetic changes, securing identity, quality and stem-cell phenotypic characteristics of differentiation [15].

In order to try to repair the complex periodontal ligament and supporting structures damaged in replanted teeth, endodontic treatment and root surface treatment as protocol are already established in delayed tooth replantation [4, 21, 23, 25-27].

Replacement resorption and ankyloses are expected in dental replantation [24, 28, 29]. The occurrence of ankyloses and severe tooth resorption

due to removal of cemental periodontal ligament is a part of the healing process in delayed replantation with endodontic treatment as we also observed in the present research.

These outcomes are endpoints that will keep tooth in position and so are the better outcomes in order to enhance the prognosis when compared to inflammatory resorption [25, 29, 30]. Even though no statistically significant differences were observed, histomorphometric values of replacement resorption and ankyloses were exactly the outcomes we had in our research.

Cementoblasts death seems to define the resorption outcome [30]. Stem cells self-differentiation capacity depends on stimulus, in this preclinical model stem cells seemed incapable to induce new formations of periodontal ligament fibroblasts and collagen fibers, or to induce new cementoblasts, promoting repopulation and protection of the root surface, which could prevent occurrence of resorption.

The results showed that even though no statistical significance was present, stem cell infusion associated with PRF in socket had larger ankyloses and replacement resorption occurrence when compared to control group and PRF group. These findings may also be related to the high osteogenic capacity of stem cells from deciduous teeth and their ability to differentiate in odontoblasts and osteoblasts. In addition to induction of bone and dental tissue formation in tooth replantation healing process, as in the present research [12-14, 31, 32].

Zheng *et al*., [33] suggest that dental pulp cells of the mesenchymal compartment have an innate ability to attenuate osteoclastogenesis and may be responsible for the absence of dentin resorption in homeostasis.

Inflammatory resorption appears in small quantities in histological analysis. This protocol already provides control of inflammatory resorption if we follow endodontic and surface teeth guidelines but it is worth pointing out that stem cells also have immunomodulatory capacity that may have helped to modulate inflammation, reducing inflammatory resorption occurrence [34-36]. Minimum inflammatory resorption can also demonstrate materials placed in the socket in dental replantation - Stem cells and PRF - were not aggressive to periodontal tissues.

Recently, Al-Sharabi *et al*., [37] concluded that pro- and anti-inflammatory cytokines secreted by MSC-conditioned attenuates the initial inflammatory response in the rat dental pulp following tooth replantation.

According to histological analysis at 60 days, the SHEDs group had greater dental element preservation. This shows that stem cells infused in dental socket at the moment of delayed tooth replantation can be alternative for tooth structure greater preservation for longer periods of time when compared to replantation protocolcommonly applied in dental practice [25, 27].

Recently, most research is directed toward regeneration of damaged dentin, pulp, resorbed root and periodontal regeneration. Whole tooth regeneration to replace the traditional dental implants is also in focus. We know that the mesenchymal cells has been a major hope in the dentistry area. Our results cannot be ruled out, although there was no statistical significance of mesenchymal stem cells from deciduous teeth influence in tissues repair after tooth replantation, because it can be a positive outcome with future clinical relevance. Its very important to emphasize then we use extreme experimental condition.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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