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Immunohistochemical expression of PCNA, STRO-1 and CD 44 in the healing of experimentally induced periapical lesions in rats

S. ZEB KHAN[1], S. MIRZA[2], M.S.K. SADIQ[3], S. KARIM[4], M.F. ALKAHTANY[5], K.H. ALMADI[5], N. ALDAHIAN[6], A. ABDULWAHED[7], B. ALMUTAIRI[6], M. MUSTAFA[8], F. VOHRA[9], T. ABDULJABBAR[9]

[1]Department of Clinical Pathophysiology, Oral Health Science Center, Graduate School of Tokyo Dental College, Tokyo, Japan
[2]Department of Oral Pathology, Faculty of Dentistry, Ziauddin Medical University, Karachi, Pakistan
[3]Department of Oral Pathology, Bahria University Medical and Dental College, Bahria University, Karachi, Pakistan
[4]Department of Ophthalmology, Hayatabad Medical Complex, Khyber Girls Medical College, Peshawar, Pakistan
[5]Department of Restorative Dental Science, Division of Endodontics, College of Dentistry, King Saud University, Riyadh, Saudi Arabia
[6]Department of Restorative Dental Science – Operative Division, College of Dentistry, King Saud University, Riyadh, Saudi Arabia
[7]Department of Conservative Dental Sciences, Endodontic division, College of Dentistry, Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia
[8]Department of Conservative Dental Sciences, College of Dentistry, Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia
[9]Department of Prosthetic Dental Science, College of Dentistry; Research Chair for Biological Research in Dental Health, College of Dentistry, King Saud University, Riyadh, Saudi Arabia

Abstract. – OBJECTIVE: The aim of the study was to determine the expression of cell proliferating marker, anti-proliferating cell nuclear antigen (anti-PCNA) and mesenchymal stem cell (MSC) markers (anti-STRO-1 and anti-CD44) in periapical periodontitis and their role in the healing of periapical lesion in periapical periodontitis.

MATERIALS AND METHODS: Ninety Sprague-Dawley male rats (100 g) were divided into 3 groups: Experimental group I (EG I: n = 30), experimental group II (EG II: n=30) and control group (CG: n = 30). Periapical lesions were experimentally developed by leaving the dental pulp of maxillary first molars mesial root open to oral environment for 4 weeks. Conventional root canal treatment was performed in EG II. Maxillary first molars along with alveolar bone were resected and fixed. The processed samples were stained with routine hematoxylin and eosin (H&E), and evaluated immunohistochemically using antibodies against anti-PCNA, anti-STRO-1, and anti-CD44 polyclonal antibodies. Data were analyzed using Chi-square test and a *p*-value of <0.05 was considered significant.

RESULTS: Immunostaining of anti-PCNA showed 30%, 70% and 53.3% positive staining in CG, EG I, and EG II, respectively ($p<0.001$). Moreover, the CD44 staining was 20% in CG in contrast to 63.6% in EG I and 43.3 in EG II. STRO-1 staining in CG was 10%, 50% in the EG I and 36.6% in the EG II ($p<0.001$).

CONCLUSIONS: Periapical inflammatory tissues expressed significant proliferative cell marker PCNA and mesenchymal stem cell markers STRO-1, and CD44. These findings further reaffirm the promising role of mesenchymal stem cells in the healing of periapical periodontitis.

Key Words:
Mesenchymal stem cells, Periapical periodontitis, Proliferating cell nuclear antigen, Tooth root, Rats, Sprague-Dawley.

Introduction

Dental pulp generates local inflammatory responses upon exposure to microorganisms, dental caries, fracture of tooth and operative procedures[1]. As a consequence of these inflammatory respons-

Corresponding Author: Tariq Abduljabbar, MD; e-mail: tajabbar@ksu.edu.sa

es, inflammation of periapical tissue occurs resulting in pulp necrosis, eventually generating a periapical lesion[2]. Apical periodontitis is defined as an inflammatory disease in the peri-radicular area in response to the infected pulp. When left untreated, the possible sequelae of apical periodontitis may include periapical abscess (caused due to the progressive odontogenic infection) or periapical cyst (formation of cavity lined by epithelium).

The destructive-proliferative inflammation in apical periodontitis has different clinical characteristics, depending upon the etiology, individual reactivity, and diverse structure of the apical periodontium, leading to the lysis and metaplasia of the periodontal components[3,4]. Peri-radicular pathologies are worldwide, involving the apical periodontium effecting the population with no predominance of age, gender, or race[5,6]. Inflammation of tooth-supporting structures without sufficient repair process, causes severe damage to the tissues with subsequent bone and tooth loss[7]. Conservative endodontics is the usual treatment modality for treating the periapical pathologies, resulting in peri-radicular health and tooth function without surgical intervention[8]. In addition, dental pulp and periodontal ligaments are considered as rich source of progenitor stem cells. Like the bone marrow derived mesenchymal stem cells, the cells from the sources of dental pulp and periodontal ligaments have shown the ability of enhanced multipotent differentiation, colony-forming activity as well as self-renewal capacity. Due to their multifaceted potential, such as increased proliferation rates, the ability to differentiate into different cells and tissues has made them desirable candidates for their potential utilization in oral and maxillofacial tissue regeneration[9].

The recognition of extent of proliferative activity especially in periapical lesions can be beneficial in predicting the biological behavior of numerous cell types. Proliferating cell nuclear antigen (PCNA) is a nuclear protein with the mass of 36 kDa, that takes significant part in synthesis of DNA and its repair, cell proliferation and progression of cell cycle[10]. It is suggested that the evaluation of proliferative ability of PCNA has a critical role in hepatic, gastric, colorectal, oral premalignant and malignant lesions[11,12]. On the other hand, mesenchymal stem cells (MSCs) are multipotent progenitor cells that are frequently found in many tissues and organs, though limited in bone. MSCs have ability to differentiate into mesenchymal and non-mesenchymal lineages and are key determinants of healing[13]. Inflammation causes activation of MSCs in the periapical region. Patel et al[13] reported the existence of multipotent MSCs in granulation tissues of rats that were formed in response to foreign bodies, while Alongi et al[14] and Park et al[15] found the presence of MSCs in inflamed PDL and dental pulp concluding that pathological dental tissues possess many similarities with MSCs from healthy tissues. It is also suggested that STRO-1 and CD44 proteins are markers of MSCs and have been used in many studies for the identification and isolation of MSCs[16,17]. Therefore, it becomes imperative to determine additional sources of MSCs in other dental tissues, such as periapical region, in order to establish potential alternative source of MSCs and assess their subsequent role in tissue healing during inflammation.

It is hypothesized, that the MSCs present around the apex of the root in the presence of acute inflammation play a role in the healing process of periapical lesion following root canal treatment and can be assessed using the MSC markers, such as STRO-1 and CD 44. Therefore, this study aimed at determining the immunohistochemical expression of STRO-1 and CD 44 MSC markers and the proliferating cell marker PCNA in the healing of experimentally induced periapical lesions in rats.

Materials and Methods

Rat models are frequently used for histopathological analysis due to small size and resemblance with human teeth. In the present study, SD rats were used for histopathological analysis of periapical lesion to observe the expression of PCNA and STRO-1 and CD 44 in resolution and healing process of the periapical lesion. The research protocol was reviewed and approved by the Research Ethical Committee of the Graduate School of Tokyo Dental College. Ninety Sprague-Dawley (SD) male rats with mean body weight of 100 ± 20 g were included. They were housed in plastic cages with wood shavings under 12 hours/12 hours light/dark cycles at 55% humidity in an air temperature of 22°C. A standard laboratory diet was fed to the rats during the experimental period with proper *ad libitum*.

Ethical Statement

The ethical approval of the present experimental protocol was obtained from Ethical Committee of Tokyo Dental College (Code: 223, 206) Japan.

Groups

The animal model studies were conducted according to the standard ARRIVE guidelines (Animal Research: Reporting *In Vivo* Experiments). All rats were anesthetized with Sodium thiopental (Ravonal, Tanabe, Osaka, Japan) with the dose of 0.3 ml/100 g body weight and were equally divided into 3 groups:

1. Control group (CG) (n=30), without inducing any lesion or intervention.
2. Experimental group (EG I) (n=30), inducing periapical lesions using tungsten carbide bur #06 produced from stainless steel (Meisinger Germany) for preparing access cavity in the first maxillary molar's mesial root. After four weeks of exposure, periapical periodontitis was developed by following the protocol of Khan et al[18]. Histopathological analyses and immune expression of the lesions were performed.
3. Experimental group (EG II) (n=30), after the development of periapical lesion, conventional root canal treatment was performed. The dental pulp was extirpated utilizing conventional Nickel Titanium (NiTi) reamers (25 mm) and H-files (MANI INC., Japan) and the cavity exposed to oral flora. After cleaning and shaping, root canal was obturated with conventional gutta-percha points (GC corporation, Tokyo, Japan) and caviton (GC corporation, Tokyo, Japan) and kept aside for the period of 4 weeks. Both histological and immune expression of markers were performed.

Histological Analysis

After 4 weeks, all the animals in three groups were dissected by cervical dislocation and the mesial root of maxillary first molars together with alveolar bone and muscular tissue were harvested. All the tissue samples were fixed for the duration of 5 days in 10% neutral buffered formalin solution. Maxillary molars were kept for 4 weeks in 10% ethylenediamine tetra-acetic acid (EDTA) solution in order to decalcify the tissue samples for subsequent sectioning. After processing, the tissue samples were stained with hematoxylin and eosin (H&E) for morphological observation.

Immunohistochemistry

Serial paraffin sections of 4 μm thickness were de-parafinized with xylene, washed with 100% alcohol, and then, washed with distilled water. Tissue specimens were kept in 30% H_2O_2 and methanol solution for 30 min to block activity of endogenous peroxidase. Following de-parafinization, tissue slides were placed inside microwave for 30 min in 0.01 M citrate buffer (pH 6.0) solution at the temperature of 65°C. Following microwave, specimens were cooled down at room temperature and washed thrice in PBS for 5 minutes each. The sections were incubated for 10 min with 10% serum to prevent nonspecific reactions. The primary antibody anti-PCNA (code # M0879, DAKO DK-2600 Glostrup, Denmark) at dilution of 1:200 was applied for the expression of newly proliferating cells, and the primary antibodies STRO-1 (code # sc-47733, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and CD 44 (code # sc-7297, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted at 1:200 were used to confirm MSCs. These antibodies were kept at 4°C overnight. The sections were rinsed in PBS for 5 min each three times, after the primary antibody reaction.

Subsequent to overnight reaction of primary antibodies, tissue specimens were incubated with a secondary antibody (biotinylated): NichireiHistofine simple-stain MAX-PO (Nichirei, Tokyo, Japan) for 45 min at 25°C. After incubation, secondary antibody specimens were washed with PBS thrice for briefly 3 min each. To visualize, samples were stained with Nichirei-Histofine simple stain DAB (Nichirei) and later counterstaining was performed using hematoxylin. Finally, paraffin sections were observed, under 40× magnification, employing UPM Axiophoto microscope (Carl Zeiss, Germany). For each slide, the levels of staining intensity, stained epithelial cell percentage and H-score grading were noted. The grading system for staining was scored as follows: 0, complete absence of staining; 1, weak cytoplasmic staining; 2, <50% positive cells with strong cytoplasmic staining; 3, >50% positive cells with intense level of cytoplasmic staining.

PCNA-positive cells, STRO-1, and CD-44 in the periapical areas of the control and experimental groups were counted as:

PCNA/ STRO-1/ CD44 = number of positive cells per 100 μm^2.

Results were shown as the percentage of PCNA positive cells.

Statistical Analysis

Data was analyzed by using statistical program for social sciences (SPSS-Version 22, Armonk, NY, USA) and a p-value < 0.05 was considered

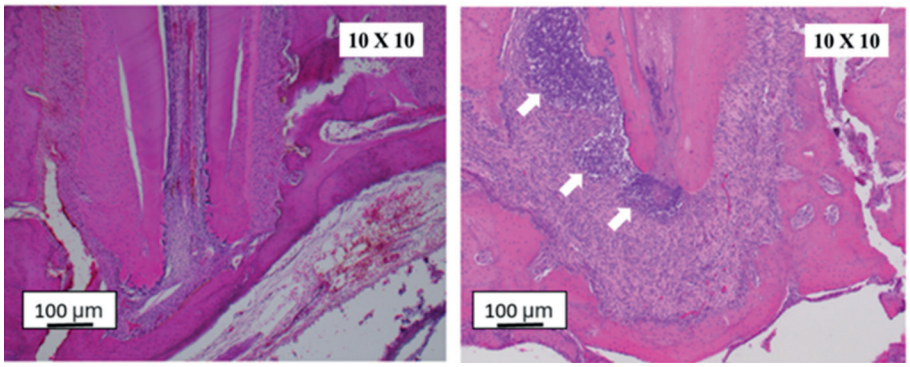

Figure 1. A, H&E images depicting mesial root apex of rat's maxillary molar with no periapical lesion and **(B)** with periapical lesion showing densely mixed primarily inflammatory mononuclear infiltrate in cells (white arrows).

significant. Chi square test was tested to see the distribution of PCNA within experimental and control groups; and to determine the distribution of Anti STRO-1 and CD44 among PCNA positive cells both in the control and experimental groups.

Results

Histological Findings

In the CG, the histological section of rat's maxillary first molar's mesial root showed no pathological changes. Dental pulp containing blood vessels and odontoblast cell layer was observed. On outer side of the pulp, dentin and periodontal ligaments were observed with open root apex (Figure 1a).

In EG I, the periapical lesion was developed after four weeks, comprised of granulation tissue, acute and chronic inflammatory cells, PMN leukocytes, lymphocytes, and monocytes. Periapical area expansion with resorption of alveolar bone, surrounding the apex of the mesial root was observed (Figure 1b).

In EG II, after four weeks following root canal treatment, reduction in the chronic inflammatory cell were observed, mainly of lymphocytes. Cellular regeneration in the form of fibroblast cells in the periodontal ligament were also observed as shown in (Figure 2).

Immunostaining

Control group-PCNA: PCNA was employed for inspecting proliferation of cells in both experimental groups as it indicates complete phases of cell cycle. As compared to EG, fewer number of PCNA +ve cells were seen around mesial root's apex in the CG n=9(30%) (Figure 3a) (Table I).

Experimental Groups-PCNA: Expression of PCNA in periapical lesion at 4 weeks (EG I) was n=21 (70%) as compared to EG II n = 16 (53.3%) after root canal treatment (Figure 3b).

Control group STRO-1: 3 (10%) samples of STRO-1 +ve cells with random distribution were reportedly seen in the periodontal ligament of the CG at the apex of mesial root. The shape of majority of STRO-1-positive cells was observed as round, that comprised of limited cytoplasm and intensely stained nuclei and (Figure 4a).

Experimental group STRO-1: Positive staining for STRO-1 was higher n = 15 (50%) in the EG I as compared to EG II n = 11 (36.6%) (Figure 4A). Majority of cells were found around the middle and apical portion of the mesial root in experi-

Table I. Expression of markers in control and experiment groups.

Expression markers N= 90	CG n (%)	EG I n (%)	EG II n (%)	*p*-value
PCNA	9 (30%)	21 (70%)	16 (53.3%)	0.001
STRO-1	3 (10%)	15 (50%)	11 (36.6%)	0.001
CD44	6 (20%)	19 (63.3%)	13 (43.3%)	0.001

Abbreviations: N, Population size; n, sample size. * Total sample size is 90 with 30 in control and each experimental group.

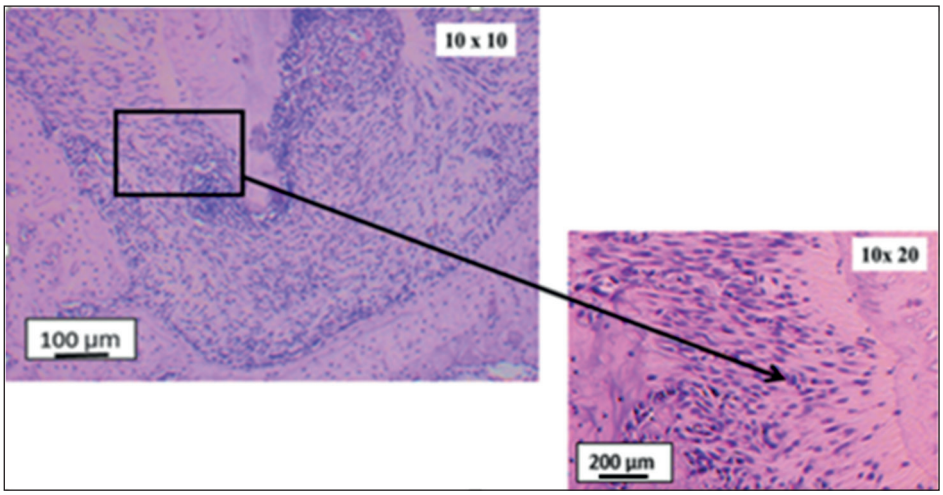

Figure 2. H&E sections depicting mesial root apex of rat's maxillary molar after obturation where spurting newly formed fibroblasts can be seen (High Magnification- black rectangle).

mental groups. The STRO-1+ve cells showed two distinct morphologies; group with extended cytoplasm and nuclei, or ovoid nuclei like endothelial cells, and others having large oval-shaped or rounded nuclei with intense staining of hematoxylin and little cytoplasm; these cells were sporadically seen in the experimental as compared to the control group (Figure 4b).

Control group CD44: Immunostaining intensity of paraffin sections for CD44 was of greater degree in both control and experiment groups than immune staining of STRO-1. The difference was because of relative specificity of progenitor cells for each marker. The distribution of staining for CD44 was not confined to any particular area of periodontal ligament, although generalized background staining was the difference between the STRO-1 and CD 44 immunostaining.

CD 44 positive cells expression was n = 6 (20%) in CG. The cells were mainly distributed near apex and alveolar bone of mesial rot, perivascular area of the periodontal ligament as well as in extravascular spaces of the periapical area (Figure 5a).

Experimental groups CD44: The distribution of positively stained CD 44 cells in EG I & II were different than CG. Immune staining for CD44 biomarker were found in n = 19. Moreover, in the EG I and EG II compared to CG, more intense nonspecific background staining was noticed. Nonspecific background staining observed in the perivascular and extravascular areas in both experimental groups (EG I and EG II), and in the CG was the similar. Positively stained cells in the extravascular region consisted of nucleus resembling endothelial cells located within an elongated cytoplasm (Figure 5b).

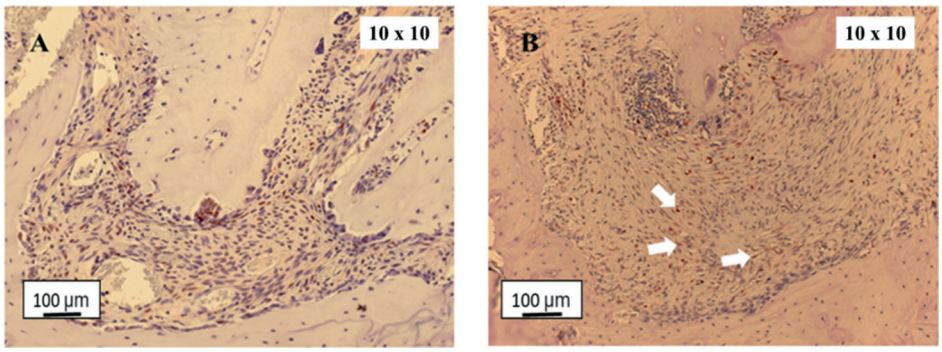

Figure 3. **(A)** Immunohistochemically staining for PCNA on the apical third of control mesial root. PCNA-positive cells showed intense positive brown staining around the apex of normal root **(B)** PCNA-positive cells post obturation with gutta percha indicated staining for PCNA-positive cells in positive brown. It is note-worthy the increased PCNA-cells showing advanced proliferative activity (white arrowheads).

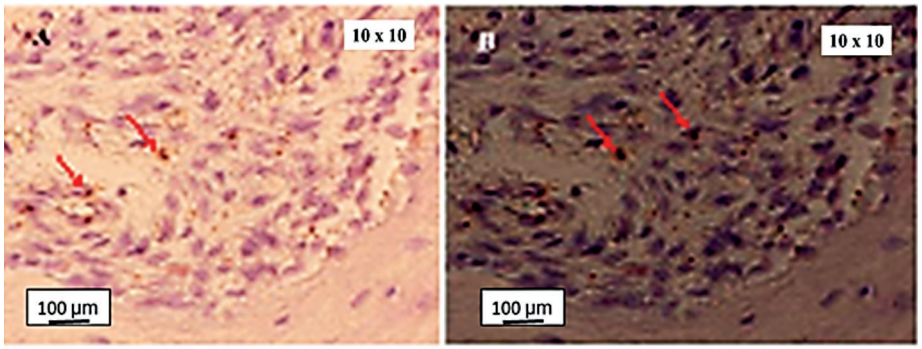

Figure 4. (A) Anti-STRO-1 antibody +ve staining in PDL in control group showing cells with rounded nuclei and small amount of cytoplasm (red arrows) and **(B)** in test group after obturation depicted elongation of nuclei and endothelial cells resembling cytoplasm and spherical nuclei within scarce cytoplasm (red arrows).

Discussion

In the present study, Sprague Dawley rats were used for histopathological analysis of periapical lesion to determine the expression of PCNA and MSC biomarkers, such as, STRO-1 and CD 44, during the healing process of the periapical lesion. Periapical inflammatory lesions occur when dental pulp is infected, resulting in microbes and products of inflammation leaching into the periapical area through the apical foramen. This change in turn results in the loss of bone tissues, which can be diagnosed during routine radiographic examination[19]. Histologically this tissue contains various chronic inflammatory cells with collagen and neovascularization[20]. When a source of infection is removed by extraction or endodontic treatment, the resolving periapical lesion undergoes healing process in the presence of stem cells with the potential of differentiation and regeneration of bone[21,22]. Dammaschke et al[23] has reported that the healing process in other mammals is almost similar to rat molar teeth as far as different stages of wound healing and biological reactions of the pulp were concerned. It was observed in the present study from the histopathological assessments after four weeks, that periapical lesion composed of chronic inflammatory cell infiltrate was similar to that observed in humans. The EG I reported the highest immunohistochemical expression of PCNA, STRO-1 and CD44 biomarkers among the three groups therefore, reestablishing the prominent role of mesenchymal stem cells in the healing of the periapical periodontitis.

In the present study, PCNA was used for evaluating cell proliferation during periapical healing. The detection of this specific synthesized protein is strictly related to the quantitative amount of proliferant cells. In a periapical lesion this protein biomarker was highly positive (70%). This positive reaction to PCNA has reported to differ depending mainly upon the type of periapi-

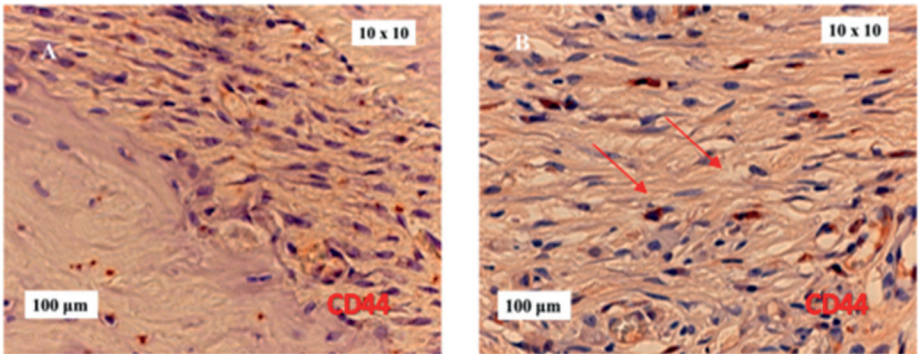

Figure 5. A, CD44 antigen expressed in periodontal ligament showing strong CD44 staining in peri- and extravascular regions in control group. Two types of morphology observed. **B,** Positively stained CD44 cells in experimental group showing nonspecific staining of background and with both forms of morphology reported. CD44 cell that were stained positively were distributed irregularly in apical area.

cal lesion, as seen in periapical granulomas and periapical cysts[24]. In addition, Chepra et al[25] reported that mesenchymal cells are still active in the capsule of periapical cysts and are found in a great quantity inside acute periapical lesion. Our study revealed statistically significant differences between expression of PCNA within CG and EG I showing presence of proliferative cells in response to inflammation. Numerous researchers employed PCNA for assessing of epithelial cell proliferation as they considered PCNA as a definitive indicator of proliferation. In one such study, Bologna-Molina et al[26] assessed PCNA expression in ameloblastic tumors and found significant differences between the ameloblastic carcinomas and the desmoplastic ameloblastomas.

The viability and activity of the MSCs resulting in the healing of the periapical lesion was expressed immunohistochemically by PCNA and the presence of MSCs biomarkers (STRO-1 and CD 44) in the present study. It is reported in literature, that when cells are lost due to the exfoliation process in human tissues, MSCs promote growth and wound healing which is in accordance with our study. Similar outcomes have been observed using multiple preclinical and clinical models[27]. Estrela et al[28] in their study concluded that CD44 marker expression was significantly higher in the periapical abscesses therefore, indicating a strong association between MSCs and the histopathological diagnosis of an abscess. Therefore, the authors suggest that MSCs could be the potential tool for tissue regeneration and repair[29,30]. Stem cells extracted from the human periapical cyst have shown MSC-like properties of high proliferation, ability of self-renewal, and immune expression[31,32].

The immunohistochemical expression of STRO-1 and CD44 in the healing of periapical lesion in the present study were previously observed in revascularization treatment for the immature permanent tooth with periapical periodontitis by Estrela et al[28]. They observed a positive CD44 immunostaining in MSCs found in the periapical cyst specimens[28]. In addition, the expression of positive immunostaining in the present study was in line with previous studies reporting strong positive immune expression of mesenchymal stem cell markers like STRO-1, CD90 and CD146, suggesting the presence of MSCs in periapical lesions and reduction in count of these cells after healing suggesting strong role in inflammation and healing[33,34].

Our immunostaining data reported the expression of MSC markers in the tissues, subsequently indicating to the presence of stem cells, although their properties might have possibly been altered. The isolated cells showed characteristic mesenchymal cell immune phenotype with a potential to form mineralized matrix *in vitro* and *in vivo*. The quantification of positive cells remains one of the substantial strengths of our study. However, the limitation of our present study is that we could not perform the immunohistochemistry in the same rats for both experiments at one time. Therefore, in future, development of the periapical lesion can be confirmed with the MICRO-CT, after 4 weeks and then after another 4 weeks of the root canal treatment. We can compare the resolution of the periapical lesion. Similarly, we can apply other MSCs markers together with STRO-1 and CD44 and use these cells in tissue regeneration procedures *in vitro.*

Conclusions

Periapical inflammatory tissues expressed proliferative cell marker PCNA and mesenchymal Stem Cell markers such as STRO-1, and CD44. These findings further re-affirm the promising role of Mesenchymal Stem Cells in the healing of the periapical periodontitis.

Conflicts of Interest

The authors declare no conflicts of interest.

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