BIOLOGICAL EVALUATION OF ADIPOSE TISSUE-DERIVED STEM CELLS ON ALVEOLAR BONE HEALING IN RATS WITH LIGATURE INDUCED PERIODONTITIS

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ABSTRACT

INTRODUCTION: Periodontal diseases are the most prevalent infectious illnesses affecting tissues supporting the teeth, as well as the most common type of bone pathology. In the field of periodontology, stem cells, as well as tissue engineering, have been introduced and have shown promising results in periodontitis treatment. Researchers have been particularly interested in non-scaffold tissue engineering strategies such as cell injection and cell sheet. The fundamental advantage of cell injection is that it is a non-invasive procedure. Local injection of Adipose stem cells (ASCs) may be a helpful cells source that is used in tissue-engineering techniques since adipose tissue is relatively inexpensive to procure and is available in enormous amounts.

OBJECTIVES: To evaluate the biological effect of the therapeutic role of adipose-derived stem cells on healing of alveolar bone with ligature induced periodontitis in rats using histological assessment, Scanning Electron Microscope analysis, and Energy Dispersive X-ray analysis (EDXA).

MATERIALS AND METHODS: In this research, 36 adult, male, albino, six months rats (200-250 grams) were used. The rats were split equally into three groups: group A; (control group), group B; (induced periodontitis), and group C: (induced periodontitis treated with Adipose Stem Cells). In each group, 12 rats were euthanized after four weeks from the beginning of the study respectively.

RESULTS: The histological, SEM and EDX results revealed restoration of the alveolar bone level around mandibular first molar.

CONCLUSION: Adipose-derived Stem Cells accelerated healing of alveolar bone in induced periodontitis rats and enhanced the osteoblastic activity.

KEY WORDS: Adipose tissue derived-Stem Cells, Periodontitis, Alveolar bone, Rats

INTRODUCTION

The periodontium is a collection of tissues that support and invest the teeth. Cementum, periodontal ligament, bone lining the alveolus and the area of the gingiva that faces the tooth make up the periodontal pocket. Only structural integrity and interaction between these distinct tissues allow the periodontium to function properly. The alveolar process is the bone tissue that surrounds a fully erupted tooth and develops in harmony with the teeth’ development and eruption (1). The morphology of the teeth, their axis during eruption, and their eventual inclination all influence the structure of the alveolar process (2).

Periodontitis is a multifactorial inflammatory illness characterized by progressive tooth-supporting apparatus loss and is associated with dysbiotic plaque biofilms. The most common symptoms include a decline in periodontal tissue support, as evidenced by clinical attachment loss (CAL) and radiographically determined alveolar bone destruction, periodontal pocketing development and gingival bleeding (3).

Periodontitis is a serious issue of public health because of its great prevalence, as well as the fact...
that it can induce tooth impairment, affect chewing function and aesthetics, generate social disparity, and reduce quality of life (3). It is characterized by microbiologically-associated, host-mediated inflammation (4). Failure to treat periodontitis can result in tooth loss. The fundamental purpose of currently used periodontitis treatments is to reduce the subgingival bacterial burden using surgical or non-surgical instrumental debridement (5,6). As a result, some cases of periodontitis may be resistant to standard treatment, necessitating the creation of innovative periodontal therapeutic regimens (7).

Tissue engineering advancements have opened up new possibilities in nascent areas of regenerative medicine in recent years. The discovery of the unique abilities of the stem cells to self-renew and differentiate along various lineage paths sparked an exciting new field of biological research. The most versatile cells are embryonic and induced pluripotent stem cells; yet, they have been accused of tumor growth and are hampered by serious ethical concerns (8). Post-natal tissues also include stem cell reserves that aid in tissue maintenance and regeneration.

Recent data reveals that adult stem cells can develop into various cell types in vitro and in vivo, contrary to the traditional belief that they can only produce identical types of cells (9). Adult-tissue stem cells are of specific importance in this regard since they are unrelated to the prior concerns. From various sources, adult stem cells have been extracted and cultivated. Among these are the bone marrow, umbilical cord blood, skeletal muscles, nerves, periosteum, dental pulp, pancreas, liver and adipose tissue (10).

Although mesenchymal stem cells, which are collected from the bone marrow stroma, are emblematic of this type of cell, attracting a lot of attention from researchers. Bone marrow harvesting is not easy and generates a small amount of cells (11).

On the other hand, adipose tissue is found throughout the body and may be extracted with minimal morbidity using minimally invasive methods such as liposuction aspirations. Bone, cartilage, muscle, endovascular, neuronal, and other cells can be differentiated from multipotent Adipose-tissue derived Stem Cells (12). They are abundant, proliferate rapidly and are multipotent cells that can develop into bone, cartilage, muscle, endovascular, neuronal and other cell types. In both experimental and clinical trials, Adipose tissue-derived Stem Cells emerged as one of the most appealing cell subsets. Many key scientific and medical problems remain unresolved despite the fact that ADSCs give unique opportunities for researching prospective therapeutic treatments for a wide range of illnesses (10). Prior to clinical use in humans, animal models are studied to see if they can meet these expectations. Their importance in biological research arises from the fact that their physiology is very similar to humans' (13). Only a few research have investigated the therapeutic effects of ADSCs injected locally on nonsurgical periodontal therapy. This research aimed to examine therapeutic potential of local injection of Adipose tissue-derived Stem Cells on healing of alveolar bone in rats with ligature induced periodontitis.

The null hypothesis for this study proposed that, no effect of the Adipose tissue-derived Stem Cells will be obtained in healing of alveolar bone in rats with ligature induced periodontitis.

**MATERIALS AND METHODS**

The current study followed the ethical guidelines for conduct of research on experimental animals by the Faculty of Dentistry, Alexandria University. In this study, the researcher used 36 adults, male, Sprague–Dawley, six months rats, weighing 200-250 grams. Animals were collected from the Faculty of Dentistry’s animal house at Alexandria University. In the experimental animal house, animals were held under similar environmental and nutritional conditions.

The rats were split equally into three groups, each with 12 rats:

- **Group A:** Control group
- **Group B:** Ligature induced periodontitis (Periodontitis group)
- **Group C:** Ligature induced periodontitis treated with Adipose tissue-derived Stem Cells (ADSCs group)

**Induced periodontitis preparation**

Under general anaesthesia with a mix of ketamine and xylazine anaesthesia (Nikon Instruments Inc., NY, USA), a 4-0 silk ligature (Roboz Surgical Instrument Co., MD, USA) was applied at the gingival sulcus level of the mandibular right first molar (M1) of all animals. After two weeks, the silk ligature was removed and periodontal symptoms such as bleeding and gingival inflammation were examined clinically. The gingival tissues were swollen, resulting in the creation of a pocket, debris collection and ulceration (14).

**Adipose-derived Stem Cells Isolation (15)**

**Collecting adipose tissue sample**

The operation room was disinfected thoroughly, and instruments were sterilized to avoid contamination. Adipose tissue was harvested from six months old adult male albino rats (weighing 330-380 grams) in a safety cabinet class II. After anesthesia, abdominal skin was disinfected with 70% ethyl alcohol. Sagittal cuts were made along the linea alba at the ventral surface of the abdomen and the subcutaneous adipose layer was exposed (Figure 1A). A sufficient quantity of subcutaneous adipose tissue was extracted and placed in a sterilized container before being transferred to the stem cell lab at...
Alexandria University’s Faculty of Medicine in the Center of Excellence for Research and Regenerative Medicine and Applications (CERRMA).

**Isolation Procedures**

1. The sample was washed many times with sterile phosphate buffer saline (PBS), minced finely with a scalpel, then added to 0.1 mg/ml collagenase type I solution (Sigma Aldrich, St. Louis, MO, USA) and placed in a 37°C shaking water bath for 30-60 mins (Figure 1 B-D)

2. After digestion, enzymatic activity was neutralized by the addition of an equal volume of complete cell culture medium (Dulbecco's modified Eagle's medium-F12 (DMEM-F12) composed of DMEM-F12, 2 M M L-glutamine, 10% fetal bovine serum (FBS) and 0.5% gentamycin) (Figure 1 A).

3. Sample was centrifugated, the supernatant was removed, and the cell pellet was cultured in a flask of cell culture and incubated at 37°C in a humidified atmosphere of 5% CO₂ (Figure 2 B-C).

4. The medium was altered after 72 hours and every 2-3 days till reaching 80% confluency.

5. Cells were split at 80% confluence using 0.025% (w/v) trypsin/EDTA (Thermo Fisher Scientific) in a ratio of 1:3.

ADSCs from passage three, P3, were described and employed in this study. An inverted phase contrast microscope (Olympus CKX41SF, Japan) with a digital camera was used to monitor cultured cells.

**Immunophenotype Characterization of ADSCs (16)**

Cells were characterized using fluorescently labeled monoclonal antibodies (mAb) for CD90 (Abcam, UK) and CD45 (Abcam, UK) markers with monoclonal phycoerythrin (PE)-conjugated antibody for CD45 and monoclonal Allophycocyanin-conjugated antibody for CD90 incubated (Anti-Thy1.1). A Becton Dickinson FACS caliber flow cytometer with Cell Quest software was used to assess immunofluorescence on cells.

The ligatures were removed and ADSCs (2.0 × 10⁶) were suspended in 200 µl phosphate-buffered saline (PBS; Invitrogen) using a 26 gauge syringe and injected once in interdental papilla on both buccal and lingual mandibular first molar sides (16) (Figure 2D). After four weeks, 12 rats were euthanized.

**Euthanasia**

- To put animals to sleep, 20 mg/kg thiopental (0.5 g Thiopental, Crista 'Lia, Sao Paulo) was given (17).

- Animals were sacrificed four weeks after the ligature was removed. Each rat's mandible was divided and removed from muscle and soft tissue. For histological and SEM investigation, just the mandibular molar teeth segment along with the nearby alveolar bone was produced.

**Histological Examination (18)**

A light microscope was used to examine the specimens. The biopsies were fixed in 10% neutral buffered formalin in molar area segments. Following the fixation of the specimens, they were washed, decalcified, washed, dehydrated, cleared, infiltrated, embedded in paraffin wax, cut into sections with 5 µm thickness, mounted, and stained. Specimens were stained with Eosin and Hematoxylin stain.

**Scanning Electron Microscopic (SEM) Examination (19)**

Specimens were fixed in 2.5 percent glutaraldehyde in phosphate buffer (pH 7.3) for 48 hours before being washed three times in the same buffer. Afterwards, specimens were dehydrated for an hour in a graded series of aqueous ethanol solutions containing 50, 70, 90, and 100 percent ethanol. After air drying, they were silver-painted on aluminum SEM stubs and sputter-coated with gold using an ion coater (sputter coater). Specimens...
were studied buccally at magnifications of X30 using a scanning electron microscope (SEM) with a 20-kV accelerating voltage to verify alveolar bone surface integrity. Bone resorption was indicated by irregularities, pits and depressions caused by osteoclasts forming Howship's lacunae.

**Statistical analysis of the data**

The obtained data from EDXA was collected and analyzed using Analysis of Variance (ANOVA) test to compare the overall difference between the 3 groups. Pairwise comparison between each 2 groups was done using **Post Hoc Test (Tukey)**.

**RESULTS**

**Light Microscopic Results**

**Control group (Group A)**

After four weeks, an examination of the control group's alveolar bone revealed normal and smooth construction. The bone surface in the middle area of the alveolar bone was smooth and consistent. The osteoblasts were plump and organized in a continuous layer lining the surface of the bone. Osteocytes were shown to be normal in size and quantity in their lacunae in the bone. There were also darkly stained incremental lines that looked to be regular and parallel resting lines were also observed. Normal-sized, cellular and well-vascularized bone marrow compartments were found to be surrounded by dense bony trabeculae. Periodontal ligament thickness was normal with well-organized periodontal fibers linking bone and cementum, **Figure (3A)**.

**Periodontitis Group (Group B)**

Examining the alveolar bone of this group four weeks after the ligature was removed revealed a punched out uneven outline. The middle area of the alveolar bone was exceptionally rough with a scalloped appearance and extensive alveolar bone loss. In their Howship's lacunae, many multinucleated osteoclasts were seen. Osteocytes were big in size and had pyknotic nuclei. Bone remodeling was also indicated by darkly pigmented reversal lines. Thin and uneven bony trabeculae with significant expanding bone marrow spaces were found in the alveolar bone. In addition, a marked inflammatory cell infiltration was observed. The periodontal ligament gaps were found to be widened, **Figure (3B)**.

**ASCs Group (Group C)**

At the same time frame, this group showed less alveolar bone deterioration than the periodontitis group B. In comparison to group B, the middle portion of the alveolar bone had a reasonably regular and smooth bone surface. Howship's lacunae were found to have multinucleated osteoclasts. While some osteocytes were big and had pyknotic nuclei, the majority looked to be normal in size. There were also darkly stained incremental lines, as well as homogeneous parallel resting lines, indicating new bone development. There were numerous deeply stained reversal lines indicating new bone growth. Bony trabeculae surrounding moderately large, vascularized bone marrow spaces were much denser in the alveolar bone than in group B. In comparison to the periodontitis group, there was a slight expansion of the periodontal ligament gaps along with a relative restoration of the periodontal fibers, **Figure (4)**.

**Figure (3) :** (A) Light micrograph (LM) of of the control group (A) revealing typical alveolar bone architecture showing regular bone surface facing the oblique fibers of periodontal ligament disrupted by Volkmann canal (Arrow). The osteoblasts were plump and organized in a continuous layer lining the surface of the bone as well as normal size and distribution of osteocytes. Parallel homogenous resting lines were noticed (arrowheads). AB: alveolar bone. D: dentin. PDL: periodontal ligament. (H&E) x 100.

(B) Light micrograph (LM) of the periodontitis group (B), four weeks interval revealing irregular resorbed alveolar bone with large bone marrow spaces. Osteocytes with pyknotic nuclei and osteoclasts within Howship's lacunae were observed. Bone remodeling was indicated by incremental lines (arrows). PDL fiber separation was observed (arrowheads). AB: alveolar bone. D: dentin. PDL: periodontal ligament. (H&E) x 100.

**Figure (4):** Light micrograph (LM) of the periodontitis treated with adipose tissue-derived stem cells group (C), four weeks interval showing alveolar bone with regular boundary. Osteocytes were normal in size and Howship's lacunae contained multinucleated osteoclasts (arrow). Incremental lines, reversal lines as well as homogeneous parallel resting lines indicating new bone formation were observed (arrowheads). Expansion of the periodontal ligament fibers was noticed. AB: alveolar bone. D: dentin. PDL: Periodontal ligament. (H&E) x 100.
Results of the scanning electron microscope

Control Group
Alveolar bone’s buccal cortical plate had a consistent, smooth and homogeneous surface topology. Around the mandibular first molar, the alveolar bone surface was intact with a normal level, Figure (5A).

Periodontitis Group (Group B)
The buccal cortical plate's surface topography at the first molar revealed overall roughness with irregular resorptive craters and porosities. With extensive resorption in the level of the alveolar bone surrounding the mandibular first molar, severe discontinuance of alveolar bone surface architecture was noted, Figure (5B).

ASCs group (group C)
In comparison to group B, the buccal cortical plate surface topography showed moderate surface roughening and porosity. A smooth and homogeneous bone surface alternated with roughened uneven patches that had shallow depressions was observed. Around the mandibular first molar, partial alveolar bone repair was observed, Figure (6).

Figure (5): (A) Scanning electron micrograph (SEM) image of control group (A) demonstrating intact buccal cortical plate and alveolar crest between first and second molars (black arrow). The buccal cortical plate of alveolar bone has a homogenous, smooth, and regular surface texture (white arrow). (X30) (B) Scanning electron micrograph (SEM) of periodontitis group (B), revealing a decrease in the alveolar bone crest level between the mandibular 1st and 2nd molars. The surface topography revealed a widespread pattern of surface porosity, roughening, and abnormalities, as well as alveolar bone loss (yellow arrows). (X30)

Figure (6): Scanning electron micrograph (SEM) of ADSCs group (C), showing the restoration of the buccal cortical plate and the alveolar crest between the first and second molars. The buccal cortical plate of alveolar bone has a homogenous, smooth, and regular surface topography (yellow arrows). (X30)

Results of Energy Dispersive X-Ray Analysis (EDXA)
Results of EDX regarding the percentage of calcium after four weeks showed the control group with the highest values, then came group C (ASCs group). However, periodontitis group B showed the lowest value (Table 1). At four weeks interval, there was a statistically significant difference between group A and B as well as in comparison to group C. Moreover, groups B and C had a statistically significant difference.

A comparison between the three studied groups concerning the percentage of phosphorus at four weeks revealed that the control group A had the highest value, then came group C, while the periodontitis group B showed the lowest value (Table 1). The difference between the control group A and group B was statistically significant, and it was also significant in comparison to group C. In addition, groups B and C had a statistically significant difference.

Table (1): Comparison between the three studied groups according to Ca and P (4 weeks)

<table>
<thead>
<tr>
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<th>control (n = 4)</th>
<th>+ve Control (n = 4)</th>
<th>Treatment (n = 4)</th>
<th>F</th>
<th>p</th>
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<tbody>
<tr>
<td>Ca</td>
<td>12.55 ± 0.16</td>
<td>5.18 ± 0.12</td>
<td>6.19 ± 0.58</td>
<td>513.44*</td>
<td>&lt;0.001*</td>
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<tr>
<td>Sig. bet. grps.</td>
<td>p&lt;0.001*, p&lt;0.001*, p&lt;0.001*</td>
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<tr>
<td>P</td>
<td>8.01 ± 0.65</td>
<td>3.75 ± 0.47</td>
<td>5.59 ± 0.36</td>
<td>70.455*</td>
<td>&lt;0.001*</td>
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<tr>
<td>Sig. bet. grps.</td>
<td>p&lt;0.001*, p&lt;0.001*, p&lt;0.001*</td>
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Data was expressed by mean ± SD.
F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the three studied groups
p1: p value for comparing between Control and +ve control
p2: p value for comparing between Control and Treatment
p3: p value for comparing between +ve control and Treatment
*: Statistically significant at p ≤ 0.05

DISCUSSION
Healing of periodontitis is a major process that is studied experimentally and clinically with the goal of positively facilitating and documenting it using various approaches, including biomechanical measurements, surgical procedures, as well as
effects of various aspects and medications on periodontal pocket healing (20-22). Traditional therapies, including tissue engineering materials implantation, mechanical and chemical root conditioning, growth factors, guided tissue regeneration, besides several combinations of the previous methods failed to achieve the required therapeutic effects. These approaches' outcomes are frequently linked to a lack of clinical predictability (23). Recent research has concentrated on stem cell-based regenerative techniques, they have tremendous therapeutic potential in regenerative medicine, providing a biological supply for the regeneration and restoration of various missing periodontal tissues (PDL, cement, and bone) (24). The goal of the current research was to assess the therapeutic effect of locally injected adipose tissue derived stem cells on healing of alveolar bone in ligature induced periodontitis rats on a histological and ultrastructural level. The local injection of cells suspension in medium into the site of injury without being carried on a scaffold is simple and minimally invasive, thus might be in privilege to better healing (25). Tobita et al. (26) used locally injected adipose stem cells in healing periodontitis. He observed a periodontal ligament like structure along with alveolar bone, suggesting that adipose stem cells can promote healing of alveolar bone and periodontal tissue regeneration in vivo. Also, Zhang et al. (27) suggested that local application of BMMSCs in periodontal lesion sites can improve the inflammatory microenvironment as well as promote alveolar bone healing and restore periodontal homeostasis. In addition to, Du J et al. (28) who showed that locally injected Bone Marrow-derived Mesenchymal Stem Cells (BMMSCs) into mice periodontal abnormalities exerted anti-inflammatory and immunomodulatory effects at the target location as well as contributed to new tissue regeneration.

The four weeks interval as a follow-up from time of ligature removal was estimated in this study according to Zambrano et al (29). He found that this period is enough to examine periodontitis complications on the alveolar bone. The control group's results revealed typical alveolar bone development and architecture, which was lined by plump active osteoblasts. Furthermore, the size and distribution of osteocytes, which were osteoblasts and got encased in bone matrix, were found to be normal. Bony trabeculae with dense lamellar trabeculae were noted and typical cellular and vascularized bone marrow was also observed. The periodontal ligament was found to be normal in thickness with well-organized periodontal fibers. These findings matched those of Sanz et al. (30) who studied the normal structure of alveolar bone in rats.

The periodontitis group B showed an uneven scalloped contour of the middle part of the alveolar bone after four weeks of ligation removal, as well as numerous multinucleated osteoclasts, indicating substantial bone resorption and discontinuity of surface osteoblasts. The major resorptive cells, according to Hienz et al. (31), are osteoclasts. Alveolar bone injury necessitates local activation of osteoclast activity. Furthermore, thin bony trabeculae appeared around severely enlarged bone marrow spaces with inflammatory cell infiltration. These results were in harmony with Menezes et al. (32), who demonstrated the inflammatory changes and damage in whole regions of the alveolar bone in rats exposed to experimental periodontitis. At four weeks interval, the periodontal ligament in group B showed enlargement of the PDL space, as well as severe damage and degeneration of the periodontal fibers toward the alveolar bone. Compared to the periodontitis group B at the same interval, the alveolar bone surface in the adipose stem cells group in the current study revealed alternating areas of smooth and rough surfaces with less damage, showing the anti-inflammatory effects of adipose stem cells. These results were in accordance with Shaafiean et al. (33), who demonstrated that application of PRP-assisted hADSCs could induce regeneration of bone tissue in canine alveolar bone defects and therefore, presented a helpful option in bone tissue healing. Furthermore, a layer of osteoblasts laid over the surface of the alveolar bone, as well as deeply pigmented reversal lines representing bone remodeling and new bone creation were observed. At the same interval, periodontal ligament gaps widened slightly in contrast to the periodontitis group, with relative restoration of periodontal fibers. These results were in agreement with Mohammed Khalil et al. (16), who noticed that ADSCs are promising regenerative approaches that can be used in conjunction with other traditional treatments to improve the result of nonsurgical periodontal therapy.

In the current experiment, the SEM results of the control group revealed broadly smooth and uniform surface topography of the buccal cortical plate of the alveolar bone. These findings backed up Maia's et al. (34) findings, which showed that the control group had uniformly intact alveolar bone. At four weeks interval, the SEM topography of periodontitis group B demonstrated generalized roughness of the buccal cortical plate surface, significant porosity, and profound erosion. These results were in agreement with Li et al. (35), who ensured that there is a correlation between alveolar bone damage and periodontitis. He observed that...
the increase in alveolar bone loss in animals with periodontal disease was due to stimulation of the release of inflammatory cytokines. In comparison to group B, the SEM results for group C (ASCs) at four weeks revealed a decrease in surface roughness of the buccal cortical plate with moderate porosities. These results were in accordance with Wofford et al. (36) who showed that Adipose-derived MSCs are a prospective bioactive addition for bone tissue engineering materials due to their reported osteogenic potential when applied and sustained within a bone defect. Concerning the Energy Dispersive X-Ray used in this study, this efficient method confirmed the SEM observations. In the periodontitis group, the calcium level showed the lowest value in comparison to control and ASCs treated groups, whereas, phosphorus showed a decreased value. These results were in agreement with Kourkoumelis et al. (37) who stated that "There is a strong relationship between lowered Ca/P ratio and induced bone loss, as Ca/P ratios of cortical bone are significantly reduced in all osteoporotic cases. The EDX results of the ASCs group showed the highest level in calcium percentage while phosphorus showed the lowest value. Pieri F. et al (38) mentioned that adipose stem cells differentiated into osteoblast-like cells and regenerated bone in calvarial defects. With a production of 5000 ASCs/g of adipose tissue, fat tissue possesses the greatest percentage of adult stem cells of any tissue in the body. ASCs can develop into a variety of cell types while retaining the ability to differentiate into many cell lineages. As a result, the use of ASCs for the clinical restoration of oral/maxillofacial bone defects is a realistic and successful option (39). CONCLUSION

Results of this study show that Adipose tissue-derived Stem Cells (ADSCs) speed up alveolar bone repair in induced periodontitis rats. They promote osteoblastic activity and the production of new bone. As a result, it could be used as a supplement to traditional periodontitis treatment.

CONFLICT OF INTEREST

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REFERENCES


Galal et al. Adipose Stem Cells in Rats With Induced Periodontitis


