



**THE EFFECT OF AUTOLOGOUS BONE MARROW CONCENTRATE  
AND DEMINERALIZED FREEZE- DRIED BONE ALLOGRAFT IN  
MANAGEMENT OF EXPERIMENTALLY INDUCED INTRABONY  
PERIODONTAL DEFECTS IN RATS  
(IMMUNOHISTOCHEMICAL AND RADIOGRAPHIC STUDY)**

Abeer S. Gawish\*, Hany Shalby\*\*, Mohammed Ghoniem\*\*\* and Manar A.A.Selim\*\*\*\*

**ABSTRACT**

**Objectives:** The aim of the present study was to evaluate periodontal regeneration/ alveolar bone fill with the use of autologous bone marrow concentrate alone and with a combination of demineralized freeze dried allograft in the treatment of intrabony defects (IBDs). The evaluation was based on histological, immunohistochemical and radiographic analysis.

**Materials and Methods:** Forty adult male albino rats with experimentally induced periodontitis were included in the present study and divided as follows, **group I** involved 10 rats and served as positive controls, **group II** involved 10 rats were treated with demineralized freeze dried allograft (DFDBA) only, **group III** involved 10 rats were treated with autologous bone marrow concentrate (BMSCs) only and **group IV** involved 10 rats were treated with combined demineralized freeze dried allograft and autologous bone marrow concentrate. Five rats of each group were sacrificed two weeks after the beginning of the experiment and the other 5 rats were sacrificed six weeks after the beginning of the experiment. Harvested jaw specimens were fixed in formalin, then embedded in paraffin wax, and serially sectioned at 4 $\mu$ m for histological, immunohistochemical assessment using of TGF- $\beta$ 1. Furthermore radiographic assessment was done at the end of study period.

**Results:** The histological examination of control positive rats revealed degeneration of the collagen fibers associated with alveolar bone resorption. In group II DFDB allograft treated rats showed signs of PDL regeneration associated with increase in the thickness of bone trabeculae. BMSCs treated rats (group III) showed formation of new bundles of collagen fibers of PDL with increase in alveolar bone thickness. In group IV rats treated with combined DFDBA and BMSCs showed the best regenerative features in the PDL fibers and cells with more increase in the thickness of alveolar bone.

\*Vice dean and professor of Oral Medicine, Faculty of Dentistry, Sinai University & Alazhar University.

\*\*Associate professor of Oral Medicine, Faculty of Dentistry, Suez Canal University, Ismailia, Egypt.

\*\*\*Lecturer of Oral surgery, Faculty of Dentistry, Sinai University, Arish, Egypt.

\*\*\*\*Lecturer of Oral biology, Faculty of Dentistry, Suez Canal University, Ismailia, Egypt.

**Immunohistochemical results of TGF- $\beta$ 1** of group I showed negatively to weakly positive TGF- $\beta$ 1 reaction. While, PDL of group II presented moderately positive TGF- $\beta$ 1 staining. PDL of group III revealed weakly to moderately positive TGF- $\beta$ 1 stain. However, group IV presented strongly positive TGF- $\beta$ 1 staining.

**Radiographic analysis:** showed a statistical significant difference between the three treated groups compared to the positive control group at 2 and 6 weeks respectively indicating that the bone density increased gradually in the three treated groups with the best radiographic evidence of increased bone density in group IV (treated with combined DFDBA and BMSCs).

**Conclusion:** The present investigation revealed that all the treatment modalities showed favorable regeneration of the intrabony defect. The combination of DFDBA with BMSCs may positively influence the treatment of a periodontal attachment gain with enhancing resolution of osseous defects compared with other treatment modalities. Regenerative combination (DFDBA with BMSCs) emerging as a promising option in the treatment of periodontal osseous defects.

**KEY WORDS:** Periodontitis, bone marrow stem cells, bone graft, TGF- $\beta$ 1.

## INTRODUCTION

Periodontitis is a chronic inflammatory disease that causes pathological alterations in tooth-supporting tissues, which can lead to tooth loss if left untreated<sup>1</sup>. The significant burden of periodontal disease and its impact on general health and patient quality of life suggest a clinical need for the effective management of this condition<sup>2</sup>. The ultimate goal of periodontal therapy is the predictable regeneration of the functional attachment apparatus that is destroyed by periodontitis, which involves at least three unique tissues, including the cementum, periodontal ligament (PDL) and alveolar bone.

Regeneration is defined as the reproduction or reconstitution of lost or injured a part of the body in such a way that the architecture and function of the lost or injured tissues are completely restored. Therefore, the key to periodontal regeneration is to stimulate the progenitor cells to re-occupy the defects. Growth factors are vital modulators during this process which can induce the migration, attachment, proliferation, and differentiation of periodontal progenitor cells<sup>3</sup>.

To date, several regenerative procedures have been developed in an attempt to treat periodontitis,

including guided tissue regeneration (GTR), bone graft placement and the use of bioactive agents, such as growth factors<sup>(2,4)</sup>. However, the current therapeutic techniques used either alone or in combination have limitations in producing complete and predictable regeneration, especially in advanced periodontal defects. In these cases, remaining deep intraosseous defects following periodontal therapy are high-risk sites for the further progression of periodontitis<sup>5</sup>.

Recent advances in stem cell biology and regenerative medicine have enabled the use of cell-based therapy in periodontal diseases<sup>6</sup>. A large number of studies have indicated that ex vivo-manipulated stem cells derived from either bone marrow or the PDL can be used in conjunction with different physical matrices (autografts, xenografts, allografts, and alloplastic materials) to regenerate periodontal tissues in vivo<sup>(7-9)</sup>.

Allograft material has been used in periodontal therapy for last three decades. It is generally used in one of two forms: freeze-dried bone allograft (FDBA) and demineralized freeze-dried bone allograft (DFDBA). DFDBA provides an osteoconductive surface. Moreover, it provides a source of osteoinductive factors<sup>10</sup>.

The chemical inductive agent exposed after bone demineralization was termed bone morphogenetic protein (BMP). BMP is composed of a group of acidic polypeptides that have been cloned and sequenced and stimulate the formation of new bone by osteoinduction<sup>11</sup>

The available BMPs in DFDBA stimulate host osteoprogenitor cells to differentiate into osteoblasts and begin new bone formation<sup>(12,13)</sup>.

Bone marrow concentrate contains mesenchymal stem cells (MSCs), hematopoietic stem cells, platelets (containing growth factors), and cytokines. The anti-inflammatory and immunomodulatory properties of bone marrow stem cells (BMSCs) can facilitate regeneration of tissue. Additionally, BMSCs enhance the quality of cartilage repair by increasing aggrecan content and tissue firmness. Following bone marrow aspiration (BMA), BMC is easily prepared using centrifugation, and is available for a same-day procedure with minimal manipulation of cells<sup>14</sup>.

The basic concept underlying conventional periodontal regenerative therapy is first to remove the source of infection and then to provide a space into which neighboring cells can grow. Regenerative periodontal/bone therapy was originally based on the use of scaffolds. Tissue regeneration requires three key elements: cells, scaffolds, and signaling molecules. Cells (e.g., bone marrow stromal cells, osteoblasts, and periodontal ligament cells), scaffolds (e.g., type I collagen [Col-I], hydroxyapatite [HA], and b-tricalcium phosphate [b-TCP]), and signal molecules (e.g., platelet-derived growth factor, basic fibroblast growth factor [bFGF], and bone morphogenetic protein [BMP]) have been used in regenerative therapy as single factors and in combination<sup>15</sup>.

The hypothesis of the present study is that autologous bone concentrate combined with DFDBA

graft material can providing an ideal conditions for regeneration of alveolar bone and periodontal ligament tissues for the treatment of induced periodontal intraosseous defect using histological, immunohistochemical and radiographic assessment methods.

## MATERIALS AND METHODS

Forty adult male albino rats with induced periodontitis, their body weight range from 200-220 g, were used as an experimental animal model in this study. Five rats were housed in each cage and maintained under 12-hour light/dark cycle at a temperature of 23°C and relative humidity of 50% with access to standard rat chow pellets and water ad libitum throughout the whole experimental period, in the animal house of the faculty of Dentistry, Suez Canal University.

### Induction of Periodontitis:

All procedures of periodontal disease induction for all of the experimental rats were performed under general anesthesia by intramuscular injection with a solution of Ketamine 10% and Xylazine 2% (2:1), 0.12 ml /100 g body weight. Anesthesia was installed in 4-5 min after administration. After anesthesia, ligatures in “8” with sterile 4/0 silk (Ethicon, Johnson e Johnson, São Paulo, SP, Brazil) were placed around the mandibular anterior teeth. This ligature acted as gingival irritants for 14 days and promoted the accumulation of plaque and subsequently development of periodontal disease<sup>16</sup>.

### Treatment:

Following the induction of periodontitis, the animals were divided into four groups as follows ten on each and subjected to different treatment modalities:

**Group I** : receiving open flap debridement only and served as positive controls.

**Group II** : treated with DFDBA \*

**Group III**: treated with autologous bone marrow concentrate (BMSCs).

**Group IV**: treated with combination of autologous bone marrow concentrate and DFDBA.

#### Harvesting procedure for bone marrow aspiration

##### *In groups III & IV autologous bone marrow had been harvested as following*

After anesthetizing of rats of with an intraperitoneal injection of 60 mg/kg ketamine with 6 mg/kg xylazine, and then placing the animal in dorsal recumbency. The anterior face of the thigh was shaved and disinfected the area with ethanol. The rat's leg held firmly in place. The needle is used to pierce perpendicularly through the thigh skin and musculature on the anterior face, above the knee joint until it reaches the solid surface of the femur. With the needle perpendicular to the bone, a hole is made in the femur at the junction between epiphysis and diaphysis by a firm, rotating, and grinding movement. Next, the needle is moved so that it is almost parallel to the bone and the needle is inserted into the diaphysis channel. After attaching a syringe to the needle the investigator advanced it gradually up its cylinder by pushing and rotating the needle as it moves up, periodically aspirating the marrow. To prevent the coagulation of the diaphysis content one preloads the syringe with 0.1 ml heparin (5,000 µg/ml). Then, the wound is cleaned and disinfected, and processes the bone marrow accordingly.<sup>17</sup>

#### Animal sacrifice

Five rats of each group were sacrificed under general anesthesia by cervical dislocation two weeks after the beginning of the experiment and

the other 5 rats were sacrificed six weeks after the beginning of the experiment. The jaws of the lower incisors regions of the sacrificed animals were dissected out for histological examination and immunohistochemical analysis.

The jaw specimens were decalcified in 10% ethylene diamine tetra acetic acid (EDTA) PH 7-7.4. After complete decalcification, jaw specimens were prepared for light microscopic examination. They were dehydrated in alcohol, cleared in xylene, embedded in paraffin, sectioned and subjected to:

- 1- **Hematoxylin and eosin stain**: for histological examination of the periodontal ligament and alveolar bone of teeth.
- 2- **Masson's trichrome stain**: for collagen fibers demonstration<sup>18</sup>.
- 3- **Immunohistochemical localization** of TGF-β1 in PDL & alveolar bone using a kit of antibody against TGF-β1 antigen.

#### Immunohistochemistry

Polyclonal rabbit anti-rat TGF-β1\*\*; Igs were used. Sections were deparaffinized in xylene and rehydrated in a series of 100, 95, 85 and 70% alcohol, then in distilled water and phosphate-buffered saline (PBS). Enzymatic pretreatment with 0.1% (wt/vol) trypsin (Zhongshan, Beijing, China), for 15 min at 37°C, was performed to increase accessibility of antibody to the epitopes, after which the sections were washed in PBS and soaked in 3% hydrogen peroxidase solution (Zhongshan) for 15 min to block endogenous peroxidase activity. The sections were then blocked with 5% normal goat serum for the staining of TGF-β1, for 20 min at room temperature, followed by incubation of the primary antibody (TGF-β1, 1 : 250 dilution) overnight at 4°C. Negative controls were obtained by replacing

\* Straumann allograft ,LLC,60 Minuteman Road. Andover, MA01810 USA.

\*\* (Santa Cruz Biotechnology, TX, USA)

primary antibody with PBS. After three washes in PBS, the sections were incubated with a biotinylated secondary antibody followed by incubation with conjugated streptavidin–peroxidase (Zhongshan) for 20 min. Specific immunostaining was visualized by incubation with 3,3'-diaminobenzidine tetrachloride solution (Zhongshan) for 3 min and the sections were washed twice with distilled water. Then the sections were counterstained with hematoxylin solution, rinsed in running tap water, dehydrated in a series of ethanol (70, 85, 95 and 100%, 3 min each) and cleared with xylene. All sections were viewed and photographically recorded using an Olympus Microscope\*

### Radiographic assessment

Standardized digital periapical X-ray films\*\* were taken at, baseline, 2, and 6 weeks. A standardized rectangle was drawn at the interdental areas between the two central incisors of the rats to calculate the bone density changes.

### Statistical Analysis

All the data regarding the expression of TGF- $\beta$ 1 as well as the bone density were tabulated and statistically analyzed using Unpaired t test was used for comparison between groups. ANOVA test was used to compare all groups, used for comparison between groups.

## RESULTS

### a) Hematoxylin and eosin stain:

The jaw specimens of **control positive** rats revealed dissociation of the collagen fibers associated with widening of the blood vessels after 2 weeks. Alveolar bone resorption with appearance of osteoclasts in their Howship's lacunae (arrows) and widening of the bone marrow cavities is apparent.

While the specimens taken after 6 weeks showed severe degeneration of PDL fibers with loss of their attachment to the bone and cementum surfaces (Fig 1A,B). **Group II (DFDBA)** treated rats for two weeks revealed signs of PDL regeneration in the form of well-defined but unorganized fibers found close to alveolar bone associated with mild increase in the thickness of bone trabeculae associated with disappearance of osteoclasts in their Howship's lacunae (arrows, Fig 1C). While specimens taken after six weeks were characterized by the presence of well vascularized connective tissue having apparent number of viable and undamaged periodontal cells and oriented fibers with apparent increase in the thickness of bone trabeculae. Alveolar bone also showed the presence of reversal lines (arrow) which indicated the past osteoclastic activity (Fig 1D). **Group III (BMSCs)** treated rats showed formation of new bundles of collagen fibers with mild degree of periodontal degeneration after two weeks. Alveolar bone presented increase in its thickness with appearance of resting lines which indicates the formation of new layers of bone. While specimens taken after six weeks revealed more regeneration of PDL fibers & alveolar bone (Fig 1E,F). **Group IV** rats treated with **combined DFDBA and BMSCs** for two weeks showed the least degenerative changes in the periodontal ligament fibers and cells with more increase in the thickness of bone trabeculae when compared with groups II&III. However, specimens treated for six weeks showed the best regenerative features when compared to groups II and III (Fig 1G,H).

### b) Masson's trichrome stain:

Jaw specimens of **control positive** rats revealed localized degeneration of the collagen fibers of PDL with weakly positive reaction to Masson's trichrome stain after two weeks. Moreover, the

\*(Olympus, Tokyo, Japan).

\*\* Digora computer image analysis program version 2.7.

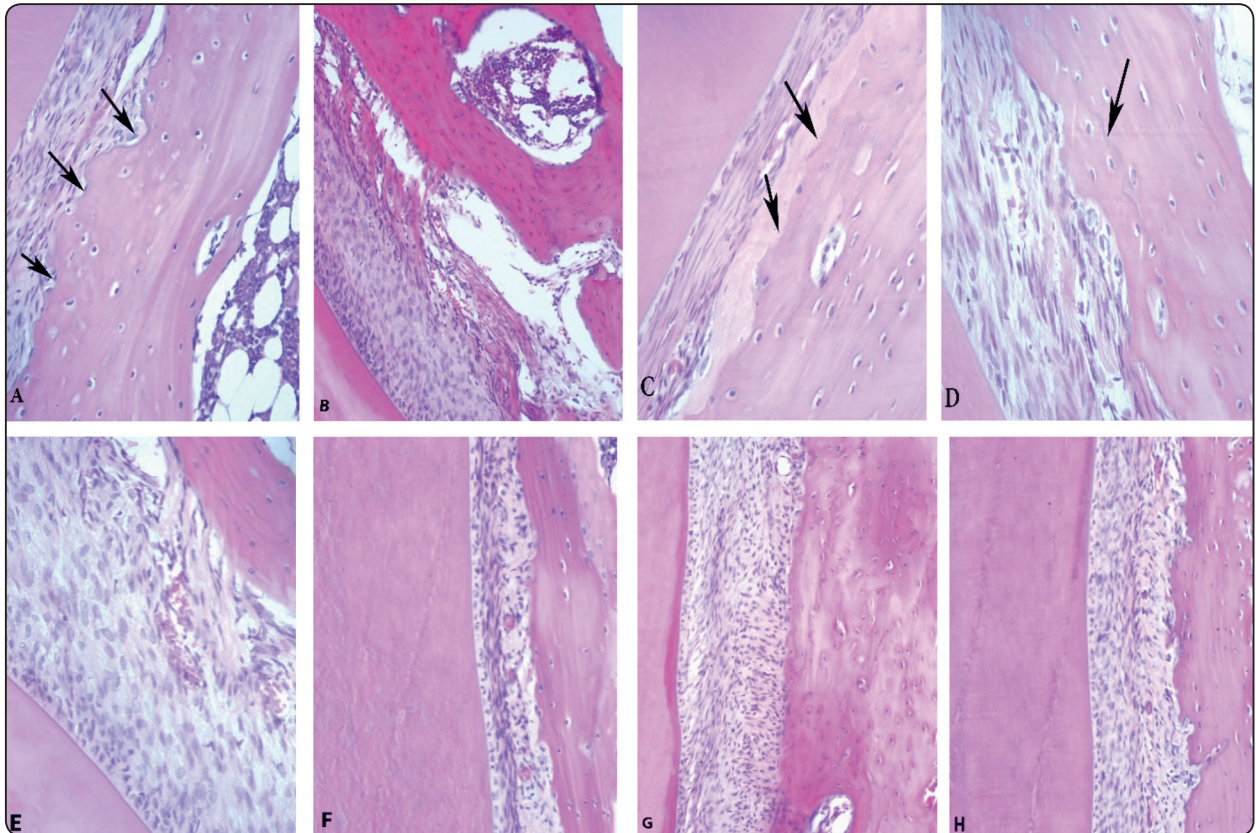


Fig. (1) Showing histological examination of the periodontal ligament and alveolar bone of **Control positive rats (A,B)**, **DFDBA treated rats (C,D)**, **BMSCs treated rats (E,F)** , **Combined DFDBA and BMSCs treated rats (G,H)** . (H&E.orig.mag.400).

specimens taken after six weeks showed generalized massive degeneration of the collagen fibers which stained negatively to weakly positive reaction to Masson's trichrome stain (Fig 2A,B). While, PDL fibers of **DFDBA** treated rats presented the number of fibers was increased and denser collagen fibers formed with weak tomoderate positive stain after two weeks and moderate positive stain after six weeks (Fig 2C,D). **BMSCs** treated rats revealed

weak tomoderate positive stain after two weeks and moderate positive stain after six weeks (Fig 2 E,F). However, Jaw specimens of rats treated with **combined DFDBA and BMSCs** presented further increase of collagen fibers with moderately to strongly positive stain after two weeks and well organized bundles of collagen fibers with strongly positive stain after six weeks (Fig 2 G&H).

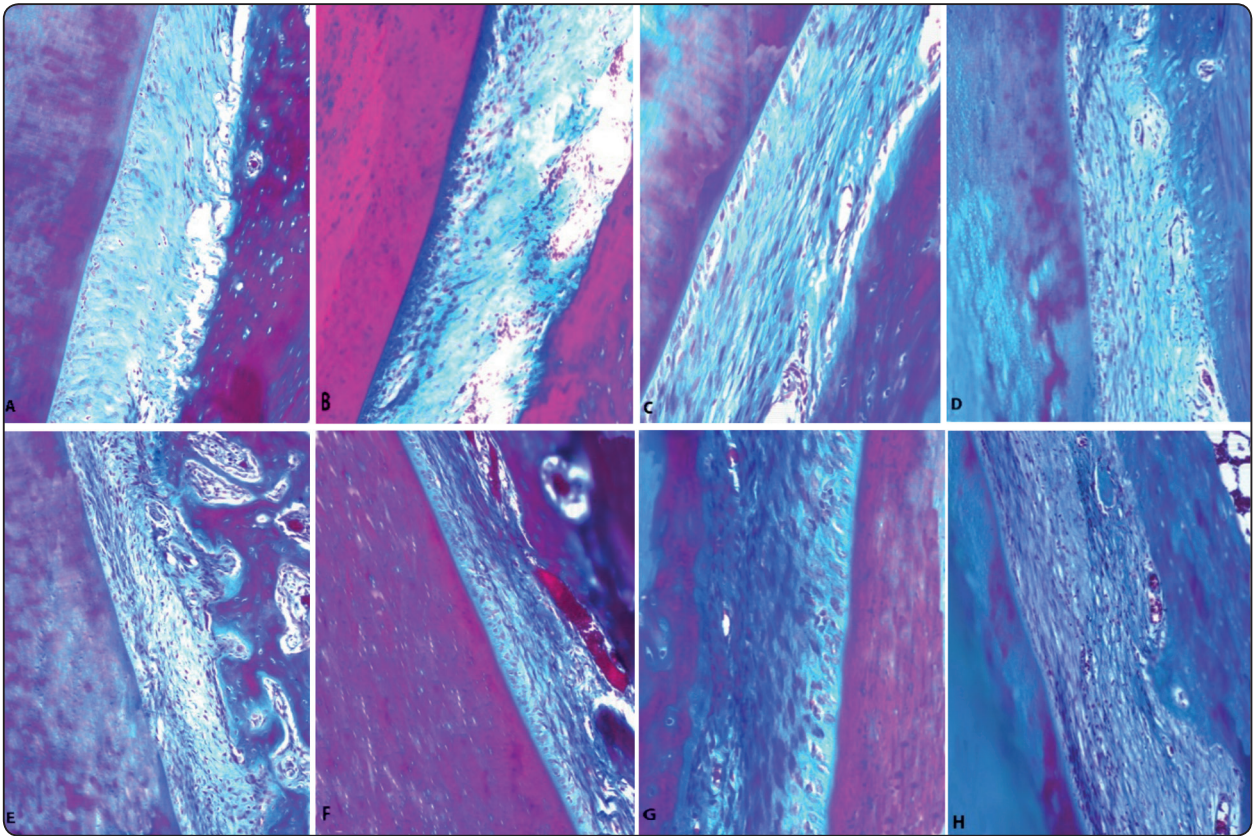


Fig. (2) Showing collagen fibers of the periodontal ligament of Control positive rats (A,B), DFDBA treated rats (C,D), BMSCs treated rats (E,F), Combined treated rats (G,H). (Masson's trichrome, Origin. Mag.400).

**c) Immunohistochemical detection of TGF $\beta$ 1:**

Jaw specimens of **control positive** rats showed weakly positive TGF- $\beta$ 1 reaction in the PDL fibroblasts, extracellular matrix & bone marrow cavities after two weeks and negatively to weakly positive TGF- $\beta$ 1 reaction after six weeks (Fig 3 A,B). While, PDL fibers of **DFDBA** treated rats presented mild to moderate positive TGF- $\beta$ 1 stain

after two weeks and moderate positive stain after six weeks (Fig 3 C,D). **BMSCs** treated rats revealed weak to moderate positive TGF- $\beta$ 1 stain after two weeks and moderate positive stain after six weeks (Fig 3 E,F). However, Jaw specimens of rats treated with **combined DFDBA and BMSCs** presented moderately to strongly positive stain after two weeks and strongly positive stain after six weeks (Fig 3 G,H).

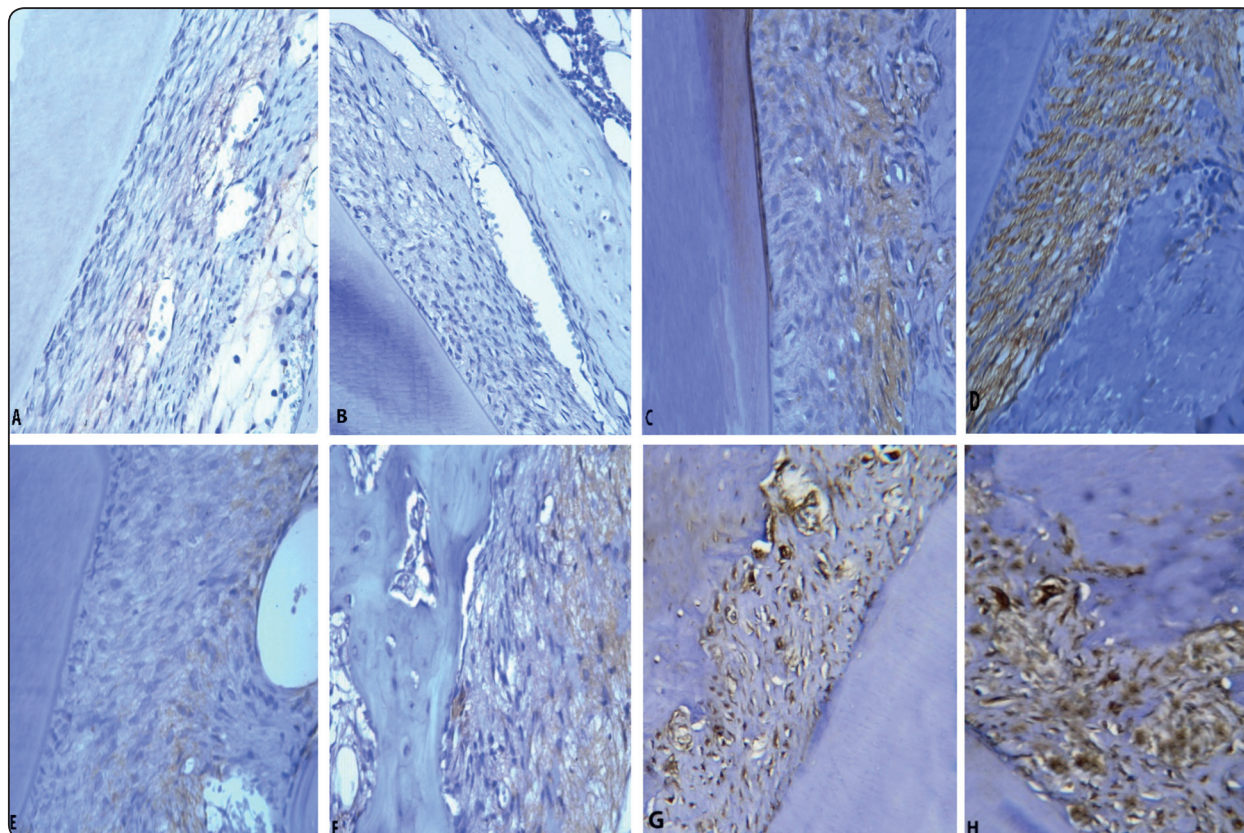


Fig. (3) Showing immunohistochemical localization of TGF-β1 in PDL & alveolar bone of **Control positive** rats (A, B), **DFDBA** treated rats (C,D), **BMSCs** treated rats (E,F), **Combined DFDBA and BMSCs** treated rats (G,H). (TGF-β1, Origin. Mag.400).

**Evaluation of TGFβ immunostaining by image analysis:**

TABLE (I) Illustrates the mean labeling index of TGF-β1 in PDL at the different groups:

	<b>Positive control group I</b>	<b>DFDBA group II</b>	<b>BMSCs group III</b>	<b>DFDBA and BMSCs group IV</b>	<b>ANOVA test</b>
<b>2 week</b>	102.617 ± 5.716	133.451 ± 4.605	123.537 ± 3.291	152.781 ± 3.121	P ≤ 0,5 *
<b>6 week</b>	85.531 ± 2,53	155.621 ± 4.551	142.642 ± 3.908	175.426 ± 4.523	P ≤ 0,5 *
<b>t-test</b>	P ≤ 0,5 *	P ≤ 0,5 *	P ≤ 0,5 *	P ≤ 0,5 *	
	Group II versus group III P ≤ 0,5 * Group II versus group IV P ≤ 0,5 * Group III versus group IV P ≤ 0,5 *				

*Data represented as mean ± standard deviation.*  
*\*P<0.001 versus positive control.*



There is a significant statistical difference between the rats of treated groups II, III, and IV when compared with those of group I (control positive) using one-way ANOVA test. Also, the analysis of variance clarified a high significant differences ( $p < 0.01$ ) between the groups IV when compared with those of groups II & III. As the mean values are considered significant when the P value  $< \text{or} = 0.05$ . This means that the immunostaining of TGF- $\beta$ 1 in PDL of groups II, III and IV rats was gradually increased during the treatment periods when compared with group I rats. While marked increase TGF- $\beta$ 1 immunostaining in groups IV when compared with those of groups II & III.

**Radiographic Results**

The mean bone density in the region of interest was  $(26.13 \pm 3.2)$ ,  $(41.87 \pm 2.1)$ ,  $(32.39 \pm 1.9)$ , and  $(50.32 \pm 2.8)$  for the positive control, allograft treated, bone marrow treated, and allograft + bone marrow treated groups at 2 week respectively, while at 6 week the results was  $(30.23 \pm 2.3)$ ,

$(52.82 \pm 2.8)$ ,  $(38.24 \pm 2.4)$ , and  $(64.36 \pm 2.9)$  for the positive control, allograft treated, bone marrow treated, and allograft + bone marrow treated groups respectively.

The one way ANOVA test showed a statistical significant difference between the three treated groups compared to the positive control group at 2 and 6 week respectively. Moreover, Using t-test the result showed a statistical significant difference within the group at 2 week compared to 6 week in all studied groups.

The comparison between the three treated groups by using t-sample t-test (bone marrow, allograft, and bone marrow + allograft treated groups showed better statistical significant difference between the bone marrow + allograft treated group compared to the bone marrow treated group or allograft treated group ( $p \leq 0.5$ ). additionally, the allograft treated group showed better statistical significant difference results compared to bone marrow treated group (Fig 4-7).

TABLE (II) Showed Mean and standard deviation of bone density of the four groups in 2 and 6 week post evaluation period.

	Positive control group I	DFDBA group II	BMSCs group III	DFDBA and BMSCs treated group IV	ANOVA test
<b>2 week</b>	$26.13 \pm 3.2$	$41.87 \pm 2.1$	$32.39 \pm 1.9$	$50.32 \pm 2.8$	$P \leq 0,5^*$
<b>6 week</b>	$30.23 \pm 2,3$	$52.82 \pm 2,8$	$38, 24 \pm 2,4$	$64,36 \pm 2,9$	$P \leq 0,5^*$
<b>t-test</b>	$P \leq 0,5^*$	$P \leq 0,5^*$	$P \leq 0,5^*$	$P \leq 0,5^*$	
	Group II versus group III $P \leq 0,5^*$ Group II versus group IV $P \leq 0,5^*$ Group III versus group IV $P \leq 0,5^*$				

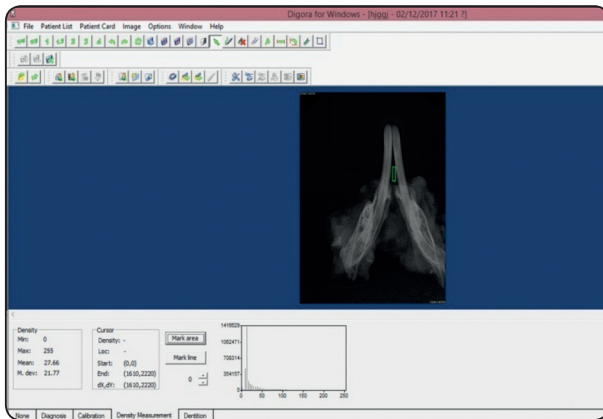


Fig. (4) Showed the mean bone density in the interdental bone between the two central incisors in a rat from positive control group

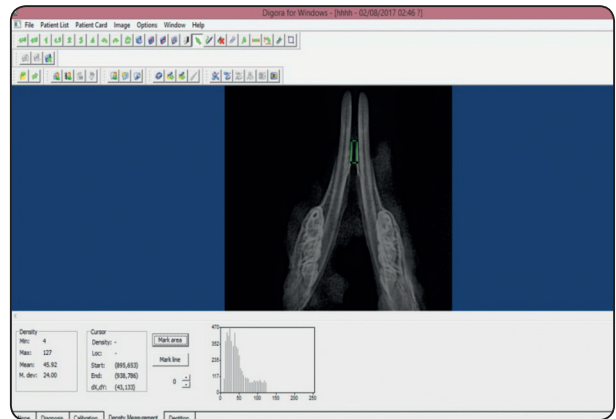


Fig. (5) Showed the mean bone density in the interdental bone between the two central incisors in a rat from allograft treated group at 6 week

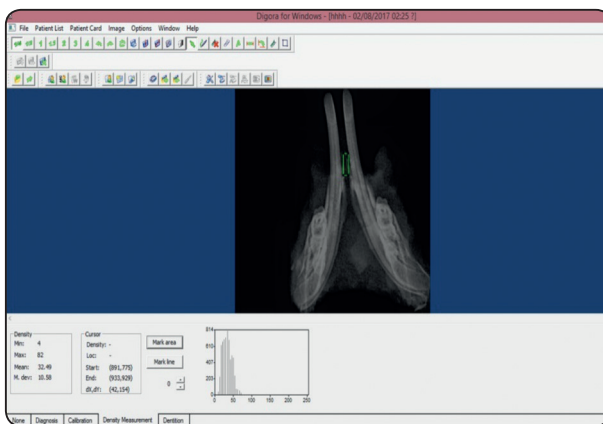


Fig. (6) Showed the mean bone density in the interdental bone between the two central incisors in a rat from bone marrow treated group at 6 week

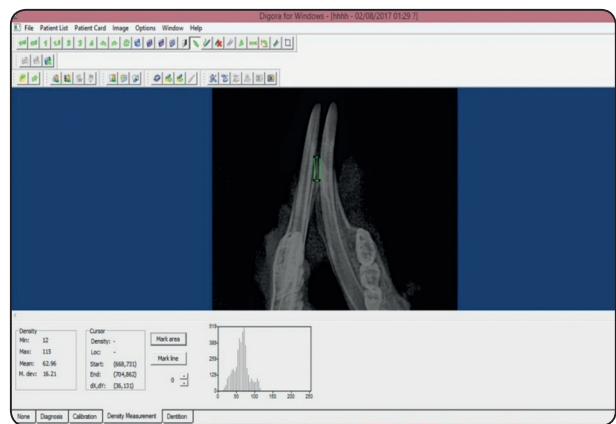


Fig. (7) Showed the mean bone density in the interdental bone between the two central incisors in a rat from allograft + bone marrow treated group at 6 week

### DISCUSSION

The primary objective of the present study was to evaluate the regenerative power of autologous bone marrow concentrate alone or in combination with DFDBA in treatment of induced intrabony defects.

Tissue engineering involves three strategies adapted for the creation of new tissue, isolated stem cells, signal molecules and biocompatible scaffold.<sup>19</sup>

In the present study, we have shown that placement of a silk thread around the cervical region of the lower incisors induced gingival inflammation

and the first symptoms of periodontitis from the third day of experiment. Significant alveolar bone loss was proven by histopathological analysis after 14 days the data obtained being in accordance with results of previous study. Many animal studies provided evidence that MSCs can be safe and effectively used to support periodontal regeneration.<sup>20</sup>

Bone marrow stromal cells used in the present study have the multilineage potential to become osteoblasts, chondroblasts, fibroblasts, and more, and the differentiation into each type of cell depends on the culture environment <sup>21</sup> . These properties

in conjunction with bone formative capacity of DFDBA can be effectively used in the treatment of intrabony defects. To best of our knowledge this is the first histological, immunohistochemical and radiographic study reports this combination therapy in the scientific literature.

Autologous bone grafts are currently considered the gold standard for regeneration of the hard tissue in oral surgery because of their osteoconductive, osteoinductive and osteogenic properties. In recent years, bone grafting techniques have taken great advantage of the establishment of Tissue Banks, which have allowed an increasing diffusion of demineralized freeze-dried bone allografts (DFDBA). The present study demonstrated that rats with induced periodontitis treated with demineralized freeze-dried bone allograft (DFDBA) for two weeks revealed signs of PDL regeneration in the form of well-defined but unorganized fibers found close to alveolar bone associated with mild increase in the thickness of bone trabeculae associated with disappearance of osteoclasts in their Howship's lacunae. Moreover, further improvement in the jaw specimens which taken after six weeks and characterized by the presence of well vascularized connective tissue having apparent number of viable and undamaged periodontal cells and oriented fibers with apparent increase in the thickness of bone trabeculae. Alveolar bone also showed the presence of reversal lines which indicated the past osteoclastic activity. These results are in agreement with Acocella et al (2012) who concluded that DFDBA alone can have an osteoinductive effect, elicit the bone resorption when implanted in mesenchymal tissues and suitable for reducing heterologous bone tissue antigenicity without inducing biomechanical changes in the material <sup>22</sup>.

Bone marrow-derived MSCs, also named bone marrow stromal cells, are multipotent and have the ability to differentiate into osteoblasts, chondrocytes, adipocytes, and smooth muscle cells <sup>23</sup>.

In the current study the experimental rats with induced periodontitis treated with autologous bone marrow concentrate (BMSCs) for two weeks showed formation of new bundles of collagen fibers with mild degree of periodontal degeneration. In addition to, increase in thickness of alveolar bone with appearance of resting lines which indicates the formation of new layers of bone. Interestingly, further regeneration of PDL fibers & alveolar bone of experimental rats after six weeks of their surgical treatment. This is supported by Nemeth et al (2009) who reported that the therapeutic effect of MSCs through the stimulation of host macrophages to produce IL-10, which has a potent inhibitory effect on osteoclastogenesis <sup>24</sup>. Moreover, maximum bone repair and almost complete anatomical restoration with no permanent loss of the operated alveolar bone is achieved if the preoperative bone is the thick cancellous type with many marrow spaces which contain mesenchymal stem cells (MSCs) <sup>25</sup>.

In the last years, a tissue engineering procedure has been set up in which autologous mesenchymal stem cells (MSCs) are used in combination with an osteoconductive scaffold as a graft material. In the present investigation the experimental rats with induced periodontitis which treated with combination of autologous bone marrow concentrate and DFDBA for six weeks showed the best regenerative features in the periodontal ligament fibers and cells with further increase in the thickness of bone trabeculae when compared to the other experimental groups which treated with DFDBA only or autologous bone marrow concentrate only. Our results are highlighted with the recent studies which demonstrated that MSCs are capable of regenerating large bony defects when used in combination with bone substitutes and increasing allograft osteointegration <sup>26</sup>.

These results are supported by Masson's trichrome staining of the Jaw specimens of the different experimental groups which revealed that

localized degeneration of the collagen fibers of PDL of positive control rats with weakly positive reaction to Masson's trichrome stain after two weeks. Further massive degeneration of the collagen fibers of positive control rats after six weeks which stained negatively to weakly positive reaction. While, PDL fibers of DFDBA treated rats presented the number of fibers was increased and denser collagen fibers formed with weak to moderate positive stain after two weeks and moderate positive stain after six weeks. BMSCs treated rats revealed weak to moderate positive stain after two weeks and moderate positive stain after six weeks. However, Jaw specimens of rats treated with combined DFDBA and BMSCs presented further increase of collagen fibers with moderately to strongly positive stain after two weeks and well organized bundles of collagen fibers with strongly positive stain after six weeks.

Bone is a rich source of growth factors, of which TGF- $\beta$  has been extensively characterized and has a profound effect on bone formation. TGF- $\beta$  has been proven to be inducer of osteoblast proliferation and inhibitor of alkaline phosphatase (ALP) activity and osteoblast mineralization indicating potential application for bone growth induction in bone tissue engineering<sup>27</sup>.

The results of the current study are confirmed by the immunohistochemical detection of TGF- $\beta$ 1 where Jaw specimens of control positive rats showed weakly positive TGF- $\beta$ 1 reaction in the PDL fibroblasts, extracellular matrix & bone marrow cavities after two weeks and negatively to weakly positive TGF- $\beta$ 1 reaction after six weeks. While, PDL fibers of DFDBA treated rats presented mild to moderate positive TGF- $\beta$ 1 stain after two weeks and moderate positive stain after six weeks. BMSCs treated rats revealed weak to moderate positive TGF- $\beta$ 1 stain after two weeks and moderate positive stain after six weeks. However, Jaw specimens of rats treated with combined DFDBA and BMSCs

presented moderately to strongly positive stain after two weeks and strongly positive stain after six weeks.

This is in agreement with Rodrigues et al (2007) who demonstrated that TGF- $\beta$ 1 is implicated in periodontal wound healing and regeneration<sup>28</sup> as TGF- $\beta$  plays a role in osteoblast and cementoblast differentiation<sup>29</sup>. In the present study, the healing of the intrabony defects and the new bone area ratio gradually increased with the healing time for regeneration of alveolar bone and periodontal ligament.

The radiographic results of the present study showed a statistical significant difference between the three treated groups compared to the positive control group at 2 and 6 week respectively indicating that the bone density increased gradually in the three treated groups compared to the positive control with the best radiographic parameters rats treated with combined DFDBA and BMSCs followed by DFDBA treated group and group treated with BMSCs alone.

These radiographic changes are in accordance with Chandrashekar et al (2009) who reported that biograft bone regenerative materials are both bio-compatible and bioactive and hydroxyapatite phase is the major constituent. These materials of porous crystalline structure provide osteoconductivity. Hydroxyapatite has a stoichiometry similar to natural bone structure and provides an osteoconductive scaffold in the bone regenerative process<sup>30</sup>. Combination of DFDBA and BMSCs demonstrated more favorable results compared to DFDBA alone. The faster bone formation occurred in the groups treated with autologous bone and DFDBA as seen in study done by Arenaz-Bua et al (2010)<sup>31</sup>.

Furthermore, the results of the present investigation are in accordance with Gothi et al (2015) who reported an increase in the radiodensity in intrabony defect treated with DFDBA<sup>32</sup>.

## CONCLUSION

The present study provides further support to the notion that DFDBA bio-engineered with BMSCs can significantly improve bone formation when compared with DFDBA or BMSCs alone and can represent a promising treatment of periodontal osseous defects.

## REFERENCES

- Burt B. Position paper: epidemiology of periodontal diseases. *J Periodontol.*;76:1406–19. doi: 10.1902/jop.2005.76.8.1406,2005.
- Lu H, Xie C, Zhao YM, Chen FM. Translational research and therapeutic applications of stem cell transplantation in periodontal regenerative medicine. *Cell Transplant*;22:205–29. doi: 10.3727/096368912X656171,2013.
- PolimeniGXiropaidis AV, Wikesjö UM. Biology and principles of periodontal wound healing/regeneration. *Periodontol 2000*;41:30-47,2006.
- Villar CC, Cochran DL. Regeneration of periodontal tissues: guided tissue regeneration. *Dent Clin North Am.*;54:73–92. doi:10.1016/j.cden.2009.08.011,2010.
- Bosshardt DD, Sculean A. Does periodontal tissue regeneration really work? *Periodontol 2000*.51:208–19. doi: 10.1111/j.1600-0757.2009.00317.2009.
- Chen FM, Sun HH, Lu H, Yu Q. Stem cell-delivery therapeutics for periodontal tissue regeneration. *Biomaterials*. 2012;33:6320–44. doi: 10.1016/j.biomaterials.05.048,2012.
- Lin NH, Gronthos S, Mark BP. Stem cells and future periodontal regeneration. *Periodontol 2000*. 51:239–51. doi: 10.1111/j.1600-0757.2009.00303.2009.
- Bartold PM, McCulloch CA, Narayanan AS, Pitaru S. Tissue engineering: a new paradigm for periodontal regeneration based on molecular and cell biology. *Periodontol 2000*. 24:253–69. doi: 10.1034/j.1600-0757.2000.2240113,2000.
- Catón J, Bostanci N, Remboutsika E, De Bari C, Mitsiadis TA. Future dentistry: cell therapy meets tooth and periodontal repair and regeneration. *J Cell Mol Med*. 15:1054–65. doi: 10.1111/j.1582-4934.2010.01251,2011.
- Committee on Research, Science and Therapy of the American Academy of Periodontology. Tissue banking of bone allografts used in periodontal regeneration. *J Periodontol*;72:834-8 , 2001.
- Urist MR, Sato K, Brownell AG, Malinin TI, Lietze A, Huo YK, et al. Human bone morphogenetic protein (hBMP). *Proc Soc Exp Biol Med*;173:194-9, 1983.
- Wood R, Mealey B. Histologic comparison of healing after tooth extraction with ridge preservation using mineralized versus demineralized freeze-dried bone allograft. *J Periodontol*; 83:329-336, 2012.
- Borg T, Mealey B. Histologic healing following tooth extraction with ridge preservation using mineralized versus combined mineralized-demineralized freeze-dried bone allograft: a randomized controlled clinical trial. *JPeriodontol*; 86:348-355, 2015.
- Sampson S1, Botto-van Bemden A, Aufiero D. Autologous bone marrow concentrate: review and application of a novel intra-articular orthobiologic for cartilage disease. 2013 Sep;41(3):7-18. doi: 10.3810/psm.09.2022,2013.
- Lee JS, Wikesjö UM, Jung UW, et al. Periodontal wound healing/regeneration following implantation of recombinant human growth/differentiation factor-5 in a beta-tricalcium phosphate carrier into one-wall intrabony defects in dogs. *J Clin Periodontol*;37: 382-389, 2010.
- Ionel A, Lucaciu O, Moga M, Buhatel D, Ilea A, Tabaran F, Catoi C, Berce C, Toader S, Campian RS. Periodontal disease induced in Wistar rats - experimental study. *HVM Bioflux*;7(2):90-95, 2015.
- Valentin L. Ordodi, MD, Felix A. Mic, MD, PhD , Ani A. Mic, PharmD, Gabriela Tanasie. Bone marrow aspiration from rats: a minimally invasive procedure. Volume 35, No.5. May 2006.
- Bancroft B. and Steven, N. : Theory and practice of histological techniques. 4th ed. P.129,1996.
- R. Langer and J. P. Vacanti, "Tissue engineering," Science, vol. 260, no. 5110, pp. 920–926, 1993.
- Chumakova Y, Vishnevskaya A, Kakabadze A, Karalashvili L, Kakabadze Z. Clinical and biochemical analysis of ligature-induced periodontitis in rats. *Georgian Med News*;(235):63-69,2014.
- Ozono S, Fujita T, Matsuo M, et al. Co-treatment with basic fibroblast growth factor and 17 $\beta$ -estradiol in the presence of dexamethasone accelerates bone formation by rat bone marrow stromal cell culture. *Nihon Hotetsu Shika Gakkai Zasshi*;52:366-374, 2008.

22. Acocella A, Bertolai R, Nissan J, Ellis E, 3rd, Sacco R. Maxillary sinus lift using fresh frozen bone chips in presence of sinus cyst: clinical and histological report. *Cell Tissue Bank*. 2012 Jun;13(2):327–32. doi: 10.1007/s10561-011-9259,2012.
23. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells*. 25:2739–2749,2007.
24. Nemeth K, Leelahavanichkul A, Yuen PS, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med*.15:42–49,2009.
25. Carnevale G, Kaldahl WB. Osseous resective surgery. *Periodontol* 2000.22:59–87,2000.
26. Dallari D, Fini M, Stagni C, Torricelli P, NicoliAldini N, Giavaresi G, Cenni E, Baldini N, Cenacchi A, Bassi A, Giardino R, Fornasari PM, Giunti A. In vivo study on the healing of bone defects treated with bone marrow stromal cells, platelet-rich plasma, and freeze-dried bone allografts, alone and in combination. *Journal of orthopaedic research: official publication of the Orthopaedic Research Society*.24:877–88,2006.
27. Bosetti M, Boccafoschi F, Leigh M, Cannas MF. Effect of different growth factors on human osteoblasts activities: a possible application in bone regeneration for tissue engineering. *Biomol Eng*. 24:613–8,2007.
28. Rodrigues TLS, Marchesan JT, Coletta RD *et al*. Effects of enamel matrix derivative and transforming growth factor-beta1 on human periodontal ligament fibroblasts. *J Clin Periodontol*. 34:514–522,2007.
29. Strayhorn CL, Garrett JS, Dunn RL, Benedict JJ and Sommerman MJ. Growth factors regulate expression of osteoblast-associated genes. *J Periodontol.*; 70:1345-54, 1999.
30. Chandrashekar KT and Saxena C. Biograft-HT as a bone graft material in the treatment of periodontal vertical defects and its clinical and radiological evaluation: Clinical study. *J Indian SocPeriodontol*. 13:138-44, 2009.
31. Arenaz-Búa J, Luaces-Rey R, Soliva S, Seijas B, Rozado Á, Sastre R, Granados J, -Franco F, Vázquez I. A comparative study of platelet-rich plasma, hydroxyapatite, demineralized bone matrix and autologous bone to promote bone regeneration after mandibular impacted third molar extraction. *Med Oral Patol Oral Cir Bucal*. 1;15 (3):e483-9. 2010
32. Gothi R, Bansal M, Kaushik M, Khattak B, Nikhil Sood N, Vishal Taneja V. A comparative evaluation of freeze dried bone allograft and decalcified freeze dried bone allograft in the treatment of intrabony defects: A clinical and radiographic study . *Journal of Indian Society of Periodontology* – 19; 411-415, 2015.