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Immunohistochemical Evaluation of Periodontal Regeneration Using Moringa Oleifera Gel and Platelet-Rich Fibrin

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Running title: Osseoinduction by Moringa Oleifera Gel in Rabbits

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ABSTRACT

Purpose: This study evaluated effect of Moringa Oleifera (MO) gel and Platelet-Rich Fibrin (PRF) in treatment of bone defect.

Methods:16 defects were produced in 8 rabbits and divided randomly into 2 groups; group (1) buccal bone defect treated with MO hydrogel and PRF , group (2) buccal bone defect treated with PRF . Immunological and histological examination were assessed at 14 and 28 days. **Results:** After 14 days, PCNA and TGF-β1 showed moderate increased expression of cells in proliferating fibroblast as well as in the organized tissue with ill-defined new bone trabeculae in group 1 while, in group 2 there was mild positive expression in the proliferating fibrous tissue that was filling the defect area and to lesser extent in the proliferating fibroblast. After 28 days, there was increased expression of both PCNA and TGF-β1 of both groups. TGF-β1 was significantly higher in group 2 than group1.Masson's trichrome stained sections revealed that after 14 days, group(1)showed increased amount of collagen than group-2. After 28 days, there was an increased amount of osteoid tissue in group-2 than in group-1. Conclusion: MO gel + PRF has a promising effect in increasing bone regeneration .

Keywords:

- Intrabony Defects; Moringa Oleifera Gel; Periodontal regeneration; Platelet-Rich Fibrin.

Introduction: -

Periodontal disease is a multifaceted inflammatory condition where interactions occur between microbes and host′s inflammatory–immune response. Periodontitis alters the architecture of the bone by causing attachment loss and underlying bone loss, leading to various forms of intra-bony defects [1]. Periodontal therapy's main objective is to restore the periodontal architecture and function as well as to inhibit the development of periodontal disease. Biomaterials that can be utilized to treat intra-bony defects are always needed to be developed [2].

In the search for effective defect filling materials, several techniques utilizing autografts, allografts, xenografts, and other synthetic bone substitutes have been established. [3]. For osseous regeneration, autogenous osseous grafts are frequently regarded as optimum. However, there are drawbacks to using them, including significant donor site morbidity, a lack of availability, and somewhat high and unpredictable resorption. [4] Alloplastic grafts are natural or synthetic materials that function as bone substitutes. Among the alloplastic grafts, few possess osteoconductive properties, whereas many of them act as space fillers. The need for safer alternatives has been driven by the persistent side effects of present conventional therapies. With their capacity to reduce the long-lasting negative effects of synthetic medications and to effectively manage acute metabolic bone diseases or osteoporosis, natural sources and their active components, utilized as therapeutic substances, have sparked a growing interest in community and medicine. In several clinical and preclinical studies, natural substances found in popular culinary spices including curcumin, garlic, ginger, and others have been shown to have osteogenic effects. [5].

Bone healing has been achieved using herbal extracts from a variety of plants. Moringa oleifera leaf is one of the natural components that give various advantages among herbal extracts. It includes high flavonoids (kaempferol and quercetin), saponins, alkaloids, and tannins. Flavonoids inhibits cyclooxygenase enzyme, which reduces the prostaglandin synthesis (PGE_2) as well as the release of histamine and pro-inflammatory cytokines (tumor necrosis factor-α, interleukin [IL]-1, and IL-6), therefore have anti-inflammatory properties. [6] Flavonoids can increase osteoblast proliferation and differentiation, according to research by *Zhang et al*. [7] This is further supported by Patel's research, which found that flavonoids obtained from Moringa leaf extract can enhance osteoblast differentiation, resulting in bone formation. [8]

Also, quercetin can decrease the number of osteoclasts and raise the number of osteoblasts by preventing differentiation and activation as well as inducing osteoclast apoptosis. (9,10) Saponin has an influence on osteogenic activity which encourages the proliferation and differentiation of osteoblasts. [11] Tannin inhibits RANK activation, making it an effective inhibitor of osteoclast differentiation. [12] As a consequence, the number of osteoclasts will decrease, with the result that bone resorption decreases PRF (second platelet concentrates PCs generation), which is easier to produce is simply centrifuged blood with no additives [13]. Many growth factors, including transforming growth factor (TGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), epithelial growth factor (EGF), and fibroblast growth factor (FGF-), are highly concentrated in PRF, and which can influence neo-angiogenesis, cell proliferation, migration, differentiation, and other biological activities to promote wound healing and periodontal regeneration [14].

PDGF, BMPs, IGFs, TGF-, FGF, and VEGF are the most prevalent growth factors associated with bone repair, osteoinduction, and osteoconduction. TGF-, which has several roles in the cell and is released as a protein, regulates cell growth and proliferation as well as differentiation and death. TGF- promotes the extracellular synthesis of proteins such as collagen, proteoglycans, osteopontin, osteonectin, and alkaline phosphatase as well as the proliferation of MSCs, pre-osteoblasts, osteoblasts, and chondrocytes [15,16]. Additionally, it is a strong chemotactic agent for MSCs. It causes osteoprogenitor cells to produce BMP during chondrogenesis and endochondral bone formation, inhibits osteoclast activation, and promotes osteoclast apoptosis. [16].

A marker for cells in the early G1 and S phases of the cell cycle is the proliferating cell nuclear antigen (PCNA). It is a cofactor of DNA polymerase delta that may be found in the nucleus. It functions as a homotrimer and boosts strand synthesis during DNA replication. Because PCNA expression and the cell proliferation cycle are tightly correlated, they provide a realistic representation of cell proliferation. PCNA is an essential protein for the proliferation of osteoblasts [17]. Also, in Masson's trichrome staining, the blue color indicates the regenerated bone, collagen fibers, or osteoid, while the red color indicates the mature old bone[18].

Thus, we postulated that Moringa Oleifera combined with PRF could increase bone healing and, as a result, bone tissue regeneration. So, the goal of this study was to compare the osseous regeneration in rabbit intrabony defects treated with Moringa Oleifera gel and autologous platelet-rich fibrin on a histological and immune-histochemical level.

Materials and methods: -

The present study was carried out after the approval of Research Ethics Committee (REC), **Faculty of Dentistry Suze Canal University** according to ″WHO-2011″ standard Serial number: 384/2021

I. Materials:

Preparation of cold aqueous extracts of fresh and dried leaves [1]:

One hundred grams of Moringa oleifera Lam. fresh leaves were weighed out, crushed using a grinder, and added to 400 ml of cold distilled water in a conical flask. The mixture was then allowed to steep for seven days with intermittent shaking. Filtered through sterile filter paper (Whattman No. 1, Sigma Aldrich, USA) into a clean conical flask, then evaporated in a water bath at the boiling point of the aqueous solvent, which is 100°C [19] .

Moringa Gel preparation:

Polyethylene glycol (PEG) **[22]**, synthesized material that is inert and safe and can be used in medicinal applications, was used to prepare a synthetic hydrogel. After making an aqueous PEG solution, aqueous plant extract was dissolved in it and thoroughly mixed to achieve a homogenous solution. The hydrophilic gel's thixotropic behavior guarantees that it keeps its stability when applied to the specimen.

The vial inversion method was used to assess the gel's thixotropic behavior. Using a vortex genie (Scientific Industries, Inc, USA), the vial's hydrogel was shaken and mechanically compressed for many seconds the gel that had been turned into sol was allowed

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to set at room temperature until it returned to gel condition, which was assessed by visual examination of the bottle inverted. [20]

PRF preparation [3]: -

Before sedation. Five milliliters of blood were drawn from the inner canthus of each rabbit's eye using capillary tubes, placed in syringes without anti-coagulants, and centrifuged for 15 minutes at 30.000 RPM. To make a thin membrane, The PRF was placed between two sterile glass slides after being picked up.

II. Methods:

Animals, Anesthesia, and Surgery:

Eight males New Zealand white rabbits (3.5–4 kg) were utilized. Animals were divided randomly into two groups: group (1) the induced buccal bone defect treated with moringa hydrogel + PRF in the right site, group (2) the induced buccal bone defect treated with PRF in the left site. before the start of the study, all rabbits were given a week to acclimate to the experimental circumstances. Animals will be fed a conventional rodent meal and have unrestricted access to water, as well as be subjected to a 12-hour light/dark cycle at room temperature of 18–22°C and relative humidity of 55–65%. For the duration of the trial, Each animal was housed in its designated cage. [3]

For general anesthesia, 0.15ml/kg of Xylazine hydrochloride and 0.35 ml/kg of Ketamine hydrochloride were injected intramuscularly. On the first day of the study, the surgical field was meticulously shaved in preparation for the surgical intervention, and then sterilized with 70% ethanol. A full-thickness incision was made in the skin and underlying muscles to create an extraoral buccal approach .

In the mandibular alveolus, the distal and buccal roots of the first molar, and the mesial root of the second molar are then exposed via a flap raised without vertical incisions. The intrabony defect was subsequently created using a stopper-premeasured tapered FG drill (Azdent. China) attached to a high-speed motor with sufficient physiological saline irrigation. The surgical defect measured 4 mm deep (buccolingual direction) from the surface of the alveolar bone to the lingual surface of the defect, and 10 mm corono-apical (from the cementoenamel junction to the most apical border of the defect)..

Finally. the flap was repositioned, then sutured. Each rabbit was returned to its designated cage after the procedure and placed on a heating blanket to help it recover from anesthesia without becoming hypothermic. Following surgery, rabbits received ceftriaxone (5 mg/kg) and carprofen (4 mg/kg) for seven days. After seven days, the sutures were removed.

Animals were euthanized at two and four weeks to harvest the lower jaw. All surgery sites were visually evaluated prior to euthanasia to assess wound healing and detect any problems. Ketamine and xylazine were given intramuscularly as a premedication before euthanasia.

Specimens' preparation [4]:

- **Masson Tri-chrome staining:**

The Specimens were washed in tap water over night and then dried out in ascending grades of alcohol, cleared in xylene and then implanted in low melting point (56˚C) paraffin.

3 Modern Veterinary office- Mariotia Rd, El Omraniya, Giza

4 Faculty of Veterinary medicine, Cairo University, Histology lab.

Serial sections of 5 um thickness were cut down with rotary microtome (lyca) and then processed for Masson Tri-chrome staining, then were observed under the light microscope.

- **PCNA and TGF- β1**:

In this experiment, we used Nuclear immune-histochemical marker Proliferating Cell Nuclear Antigen (PCNA). The specimens were fixed overnight in 4% paraformaldehyde and 0.2% picric acid in 0.1 phosphate buffer (PH 7.4). The samples were dehydrated in ascending grades of alcohol, then embedded in paraffin. Serial sections of 5 microns were obtained and were subjected to Immune-histochemical staining for PCNA (according to manufacturers' recommendation) and staining for TGF- β1 (according to manufacturers' recommendation) [21]

Statistical analysis

Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Data was summarized using mean and standard deviation. For comparison of serial measurements within each group repeated measures ANOVA was used [17] 19. P-values less than 0.05 were considered statistically significant.

Result:

A- Immunohistochemistry

1- PCNA

After 14 days: Most of the examined sections from Rt. Side (Moringa+ PRF group 1) (Fig. 1) showed moderate increased expression of PCNA positive cells in proliferating fibroblast mainly as well as in the organized tissue with ill-defined new bone trabeculae while, Lt. side (PRF. Group 2) immune expression of PCNA was detected mild positive in the proliferating fibrous tissue that was filling the defect area and to lesser extent in the proliferating fibroblast (Fig. 2).

After 28 days, PCNA expression was increased in both groups, Rt. Side (Moringa+ PRF group) (Fig. 3) showed severe increased positive expression of PCNA in both fibrous tissue and well-defined large new bone trabeculae and Lt. side (PRF. Group) (Fig. 4) showed increased PCNA expression in the organized tissue with well-defined new bone trabeculae.

As shown in table (1) generally, PCNA expression at the defect area was significantly increased with time. PCNA expression was significantly increased in Rt. Side (Moringa+ PRF group) in comparison to the Lt. side (PRF. Group) on both time points.

Table. (1) Immune expression of PCNA at the defect area

	Rt. Side (Moringa+ PRF group	**Lt. side (PRF. Group)**
14 days	**10.37± 0.58 b**	**6.59±0 .46 a**
28 days	**17.6± 1.07 c**	**12.52± 1.32 b**

Data were presented as mean± SE.
a, b and c indicate statistically significant difference at $P<0.05$.

2- TGF-β1

After 14 days, Rt. Side (Moringa+ PRF group) Figure (5) showed moderate increased TGF-β1 expression in the fibrous tissue and the newly formed bridging bone, while mild expression of TGF-β1 was detected in Lt. side (PRF. Group) in the proliferating fibrous tissue and the few newly formed bone parts. Fig (6)

After 28 days, TGF-β1 expression was increased in both groups but its expression was significantly higher in Rt. Side (Moringa+ PRF group) figure (7) when compared to Lt. side (PRF. Group) figure (8) (Table 2).

Table. (2) Immune expression of TGF-β1 at the defect area

	Rt. Side (Moringa+ PRF group)	**Lt. side (PRF. Group**
15 days	**7.72±1.01 b**	**2.69± 0.58 a**
30 days	**17.9± 0.92 c**	**10.1± 1 b**

Data were presented as mean± SE.
a and b indicate statistically significant difference at P<0.05.

3- Masson's trichrome staining

After 14 days: showed an increased amount of collagen was detected on the Rt. Side (Moringa+ PRF group) (Fig 9) Meanwhile, Lt. side (PRF. Group (Fig. 10) showed moderate amount of blue stained fibrous tissue filling the defect area

After 28 days: Both Rt. Side (Moringa+ PRF group (Fig. 11) and (Lt. side (PRF. Group) (Fig. 12) showed increased collagen bundles and increased MTC stained fibrous tissue.

As illustrated figure (13) after 14 days, significantly higher amount of stained fibrous tissue was detected in Moringa+ PRF group when compared to PRF. Group at the same time point and after 28 days, both groups showed increased fibrous tissue with absence of significant difference in between,with increase amount osteoid tissue in group-2 than in group-1.

Discussion:

Periodontitis is characterized by clinical attachment loss, altering bone architecture and leading to a variety of intrabony defects. It involves complex dynamic interactions among specific bacteria pathogens, destructive host immune response and environmental factors. Different treatment modalities have been described for the regeneration of intrabony defects, as guided tissue regeneration (GTR), the use of bone grafts, and the use of biologically active agents. [22] No biomaterial has been identified as the gold standard for the treatment of intrabony defects. [23]

Periodontal tissue engineering is aimed to regenerate the tooth's supporting tissue through a combination of proper biomaterials, such as growth factors and scaffold materials, which stimulate cells and signaling molecules to produce new healthy tissue. Biomaterials that repair alveolar bone defects require stable biological properties and good biocompatibility.[24]

The introduction of novel biomaterials for human bone repair requires preclinical safety testing using laboratory animals. The present study was conducted on 16 intrabony defects and assessed through immunohistochemically examination at 14 and 28 days. The methodology of the current study agreed with research conducted by Guskuma [25] that showed on day 7 bone defects are still inflamed and enter the early stage of resorption, while on day 30, bone defects begin in the early stage of bone formation. The results were also in line with research conducted by Kresnoadi [26] who reported that the number of osteoblasts on the 30th day increase, while osteoclasts decrease significantly compared to the previous day.

PRF is an autologous, high-density fibrin that includes growth factors (Dohan E. et al,.[27] In addition to its low cost, short preparation period, easy handling and lack of required biochemical modification, it is beneficial for several biological events (Dohan E. et al. [28] Ghanaati et al. [29] Also, the degradability of PRF was evaluated by both in vitro and in vivo studies. An in vivo study using Wistar rat models reported that PRF completely disappeared in 28 days.[30]

Chandradas et al. [31] demonstrated that PRF improves clinical and radiological parameters compared to OFD alone in intrabony defects. Addition of demineralized bone matrix (DBM) enhances the effects of PRF in relative attachment level (RAL) gain and radiographic defect fill. This goes with our study which revealed that new bone tissue is formed within the defect area treated with PRF after 30 days of the beginning of experiment.

Among all platelet concentrates, platelet-rich fibrin (PRFs) has been shown to possess fibrin, leukocytes, and a variety of growth factors that could promote wound healing. Platelet rich fibrin alone in ridge preservation did not provide significant additional benefit when compared to natural healing sockets with regard to bone volume, bone density, and osteoblastic activity [32] Moringa oleifera is essential for activating osteoblastic cells [33],similarly Patel et al. [34] also found that Moringa oleifera may have osteoblastic stimulatory capability. It has a significant quantity of flavonoids (kaempferol and quercetin). Flavonoids have anti-inflammatory properties because they inhibit the cyclooxygenase enzyme and the production of histamine. Quercetin can decrease the number of osteoclasts and increase the number of osteoblast cells by inhibiting differentiation and activity as well as inducing apoptosis in osteoclast cells. [35,36]

Our results showed that after 14 days, mild expression of TGF-β1 was detected in Lt. side (PRF. Group) in the proliferating fibrous tissue and the few newly formed bone parts. Meanwhile, Rt. Side (Moringa+ PRF group) showed increased TGF-β1 moderate expression in the fibrous tissue and the newly formed bridging bone. After 28 days, TGF-β1 expression was severe increased in both groups but its expression was significantly higher in Rt. Side (Moringa+ PRF group) when compared to Lt. side (PRF. Group)

TGF-β1 is an important transcription factor for regulating extracellular matrix (ECM)formation and mineralization. The increased expression of TGF-β1 in the intra fibrillarly-mineralized collagen (IMC)group suggests the involvement of the TGF-β1 signaling pathway in the formation of mineralized ECM as induced by IMC. [37]

Chunmei Xu et al. concluded that TGF-B signaling plays critical roles during early alveolar bone formation via the promotion of PDL mesenchymal progenitor proliferation and differentiation mechanisms. [38] Also, Kuru *et al*. suggested that TGF-β1 could be noticeable in in gingival crevicular fluid and the level of this growth factor rises transiently after regenerative periodontal surgery using non-resorbable membranes. [39]

Our immunohistochemistry results showed that PCNA expression at the defect area was significantly increased with time. PCNA expression was significantly increased in Rt. Side (Moringa+ PRF group) in comparison to the Lt. side (PRF. Group) on both time points. Daniel et al. [40] stated that the concentration of both osteopontin (OPN) and PCNA cells was dramatically higher in areas of intramembranous bone formation.

Our findings are in line with those of Kresnoadi et al., who investigated the effects of Moringa leaf extract and demineralized freeze-dried bovine bone xenograft on the expression of TGF-1 and osteocalcin during the preservation of tooth extraction sockets in Cavia cobaya's alveolar bone. They came to the conclusion that this combination can effectively increase the likelihood of successful post-extraction socket preservation by accelerating alveolar bone regeneration. [41]Regarding our study, the defect area from Rt. side (Moringa + PRF group) after 14 days showed better healing signs, the defect area was filled by organized non-inflammatory fibrous tissue with higher percentage of defect bridging by newly formed bone.

Moreover, after 28 days the defect area on the was almost filled completely by newly formed bone and the surface of bone facing the defect area was filled by increased number of active osteoblasts.

Also, Masson’s trichrome staining results showed significantly higher amount of stained fibrous tissue on the Rt. Side (Moringa+ PRF group) when compared to Lt. side

(PRF. Group which showed moderate amount of blue stained fibrous tissue filling the defect area after 14 days. While, after 28 days both Rt. Side (Moringa+ PRF group and (Lt. side (PRF. Group) showed increased collagen bundles and increased MTC stained fibrous tissue with absence of significant difference in between both groups.

Moringa Oleifera's mechanism of action includes stimulating osteoblastic cells and possessing anti-inflammatory properties such as inhibiting the release of histamine and pro-inflammatory cytokines, whereas PRF's mechanism of action includes rapid proliferation of various cell types, as well as being an essential regulator for the migration, proliferation, and survival of mesenchymal cells. The synergistic impact of Moringa hydrogel and PRF may be superior than their individual usage.

Conclusion:

Strong immunohistochemical expression of PCNA and TGF-β1 in osteoblasts and fibroblasts of fibrous tissue and newly formed bone confirmed the superiority of Moringa+ PRF mix in induced periodontal intrabony defects regeneration in rabbits. Clinical trials should be considered to detect the effectiveness of MO gel in promotion of bone regeneration .

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Rt Side (Moringa+ PRF group 14 days	**Lt. side (PRF. Group) 14 days**

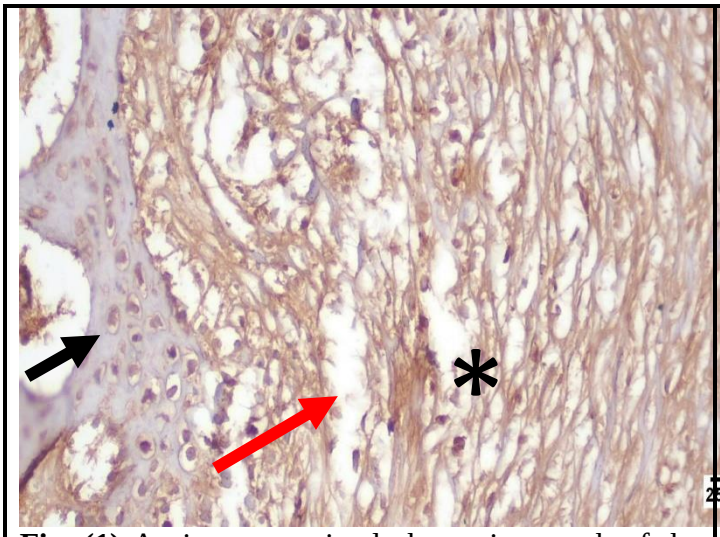

Fig. (1) An immunostained photomicrograph of the defect area, showing moderate positive expression of proliferating fibrous tissue (red arrow) with organized fibrous connective tissue (asterisks) and ill-defined new bone trabeculae (black arrow). (PCNAx200)

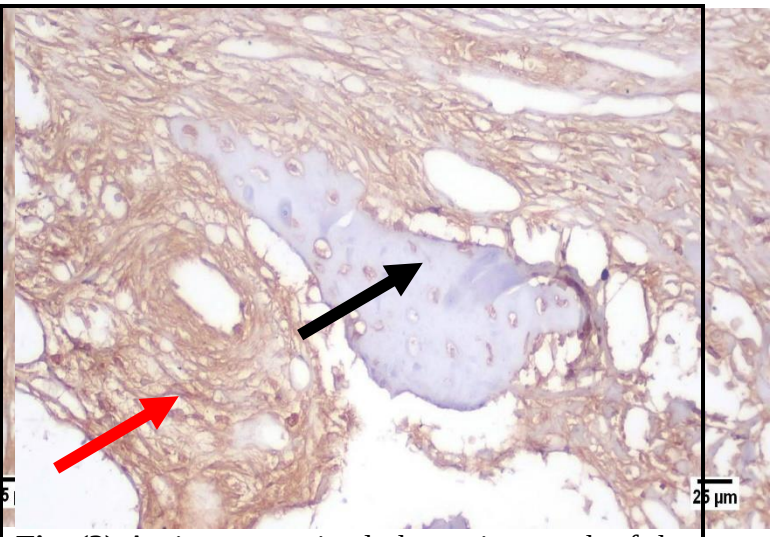

Fig. (2) An immunostained photomicrograph of the defect area, showing mild expression of proliferating fibrous tissue (red arrow) with ill-defend bone trabeculae (black arrow). (PCNAx200)

Rt. Side (Moringa+ PRF group 28 days	Lt. side (PRF. Group 28 days

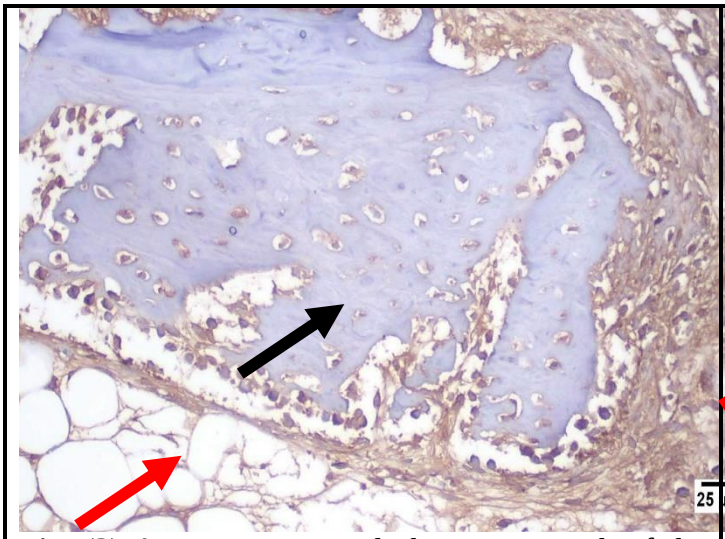

Fig. (3) An immunostained photomicrograph of the defect area, showing severe positive expression of proliferating fibrous tissue (red arrow) and well-defined large new bone trabeculae (black arrow). (PCNAx200)

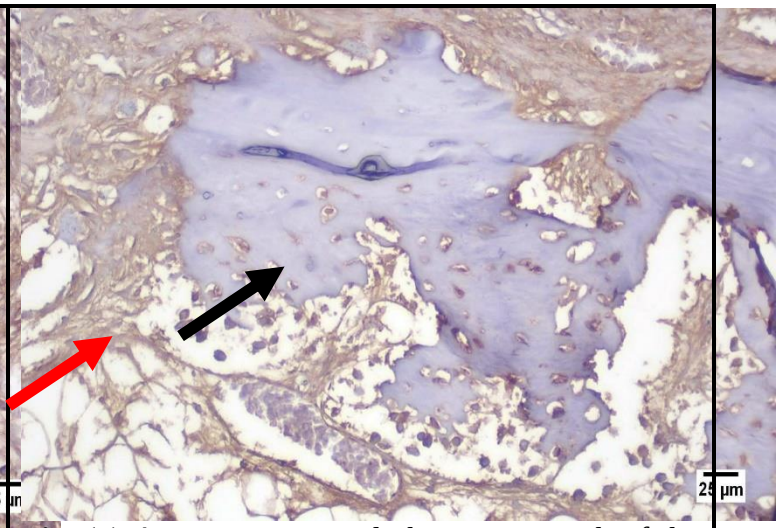

Fig. (4) An immunostained photomicrograph of the defect area, showing severe expression of proliferating fibrous tissue (red arrow) with well-defined new bone trabeculae (black arrow). (PCNAx200)

Rt. Side (Moringa+ PRF group) 14 days

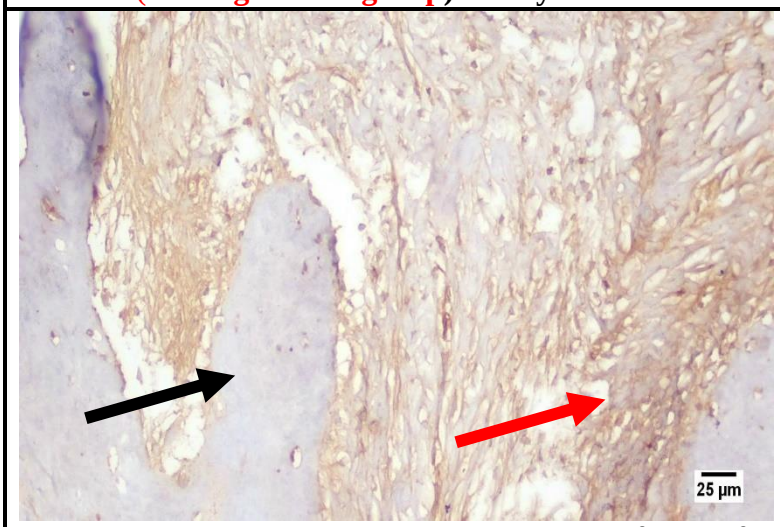

Fig. (5) An immunostained photomicrograph of the defect area, showing moderate expression of proliferating fibrous tissue (red arrow) with ill-defined bone trabeculae (black arrow). (TGF-β1 x200)

Lt. side (PRF. Group) 14 days

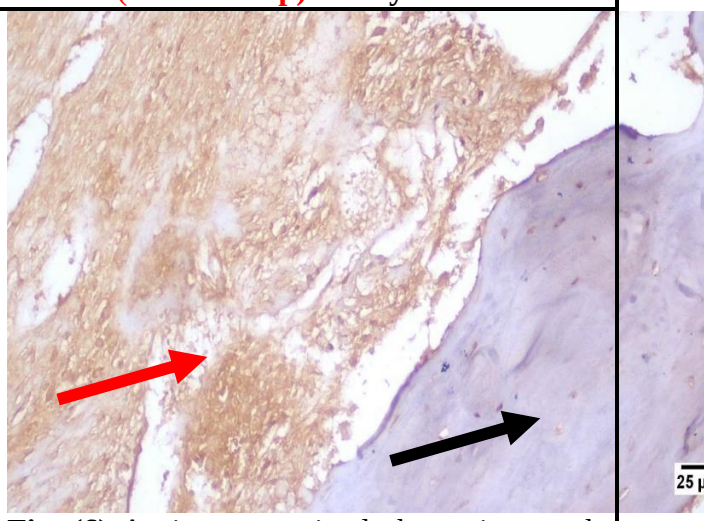

Fig. (6) An immunostained photomicrograph of the defect area, showing mild positive expression of fibrous tissue (red arrow) with ill-defined bone trabeculae (black arrow). (TGF-β1 x200)

Rt. Side (Moringa+ PRF group) 28 days

Lt. side (PRF. Group) 28 days

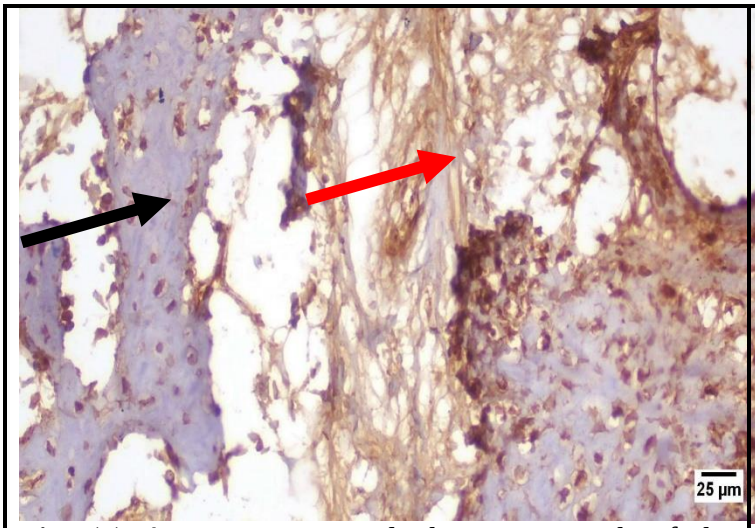

Fig. (7) An immunostained photomicrograph of the defect area, showing severe expression of well-organized fibrous connective tissue (red arrow) with newly formed bone trabeculae (black arrow) (TGF-β1 x200)

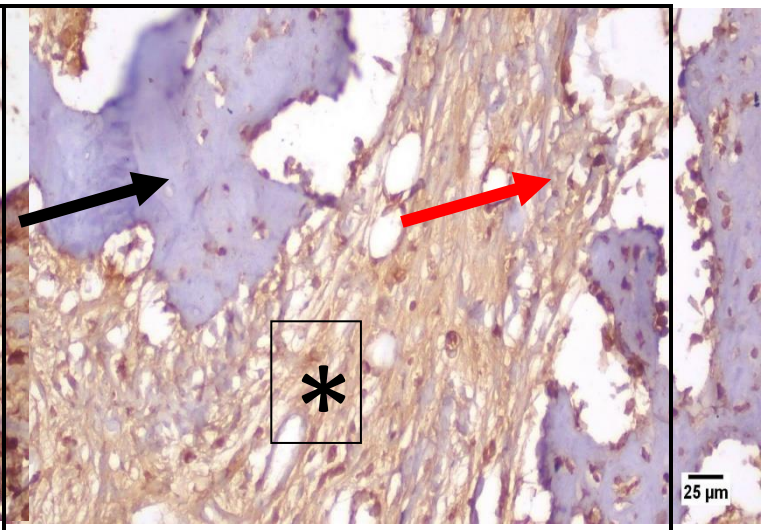

Fig. (8) An immunostained photomicrograph of the defect area,showing moderate expression proliferating fibrous tissue (red arrow) with fibroblasts (asterisk) and well-defined new bone trabeculae (black arrow). (TGF-β1 x200)

Rt. Side (Moringa+ PRF group) 14 days

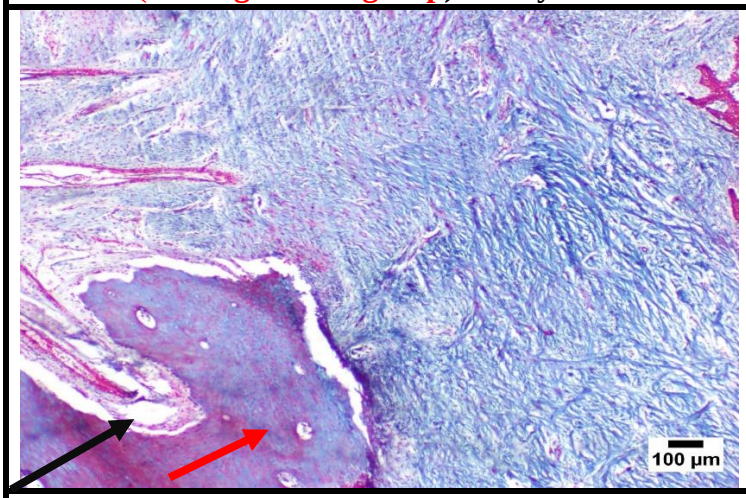

Fig. (9) Masson's trichrome stain photomicrograph of the defect area, **Moringa +PRF** group, showing increased collagen bundles at the defect area (black arrow) with increase amount osteoid tissue (red arrow). (Masson's trichrome stain x200)

Lt. side (PRF. Group) 14 days

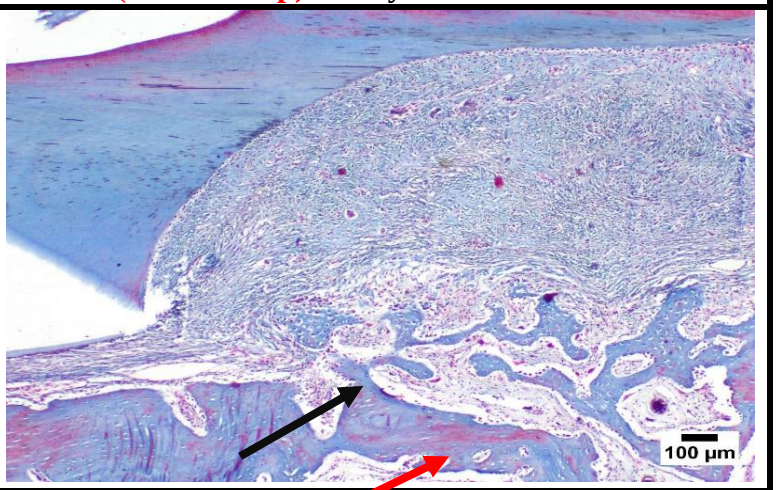

Fig. (10) Masson's trichrome stain photomicrograph of the defect area, **PRF** group, showing moderate amount of fibrous tissue (black arrow) filling the bone defect with small part of osteoid tissue (red arrow). (Masson's trichrome stain x200)

Rt. Side (Moringa+ PRF group) 28 days

Lt. side (PRF. Group) 28 days

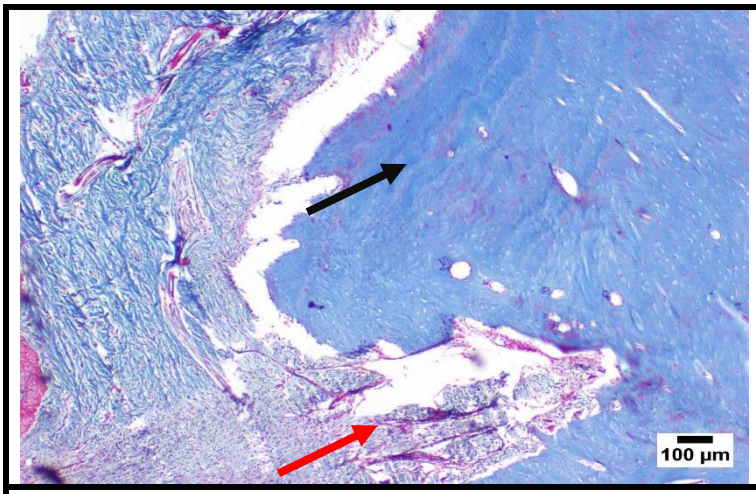

Fig. (11) Masson's trichrome stain photomicrograph of the defect area, showing increased collagen bundles at the defect area (red arrow) with well-defined mature bone (black arrow). (Masson's trichrome stain x200)

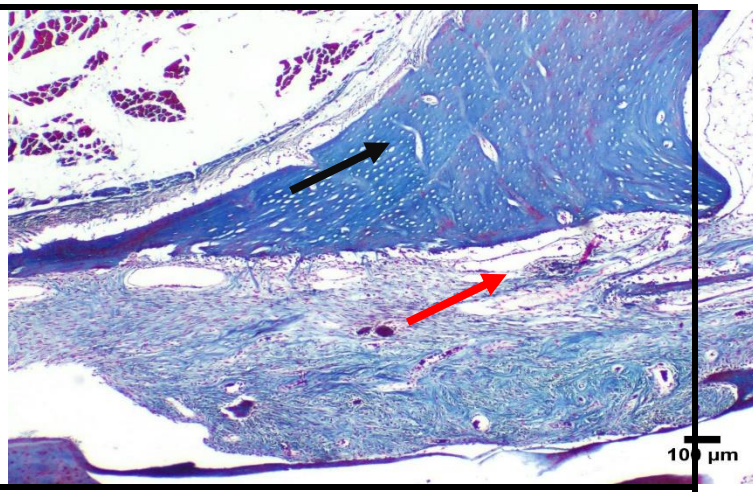

Fig. (12) Masson's trichrome stain photomicrograph of the defect area, showing increased amount of fibrous tissue at the bone defect ((red arrow) with illl-defined mature bone trabeculae ((black arrow). (Masson's trichrome stain x200)

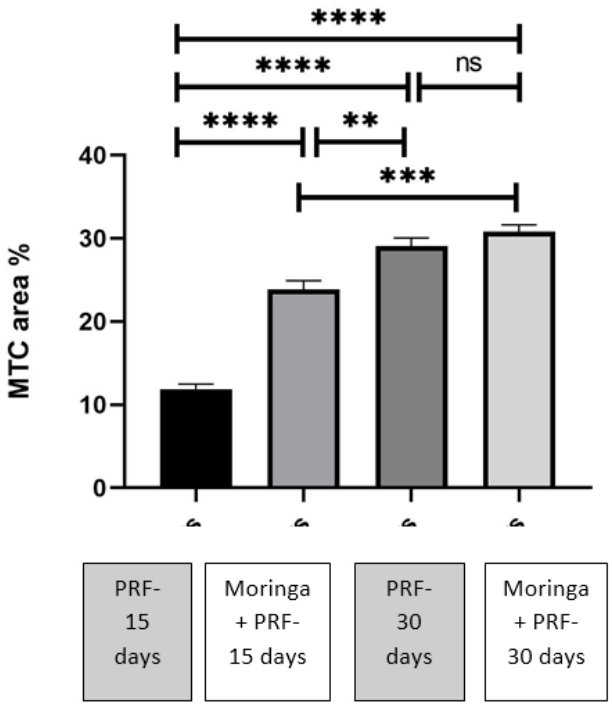

Fig. (13) Quantification of MTC stained area (as area %). Data was presented as means ±SE.

Legend of figures

Figure 1: Photomicrograph of bone, after 14 days, **Moringa + PRF Group1** showing moderate positive expression of proliferating fibrous tissue (red arrow) with organized fibrous connective tissue (asterisks) and ill-defined new bone trabeculae (black arrow) (PCNAx200)

Figure 2: Photomicrograph of bone, after 14 days, **PRF Group2** showing mild expression of proliferating fibrous tissue (red arrow) with ill-defend bone trabeculae (black arrow).(PCNAx200)

Figure 3: : Photomicrograph of bone, after 28 days, **Moringa + PRF Group1** showing severe positive expression of proliferating fibrous tissue (red arrow) and well-defined large new bone trabeculae (black arrow). (PCNAx200)

Figure 4: : Photomicrograph of bone, after 28 days, **PRF Group2** showing severe expression of proliferating fibrous tissue (red arrow) with well-defined new bone trabeculae (black arrow). (PCNAx200)

Figure 5: Photomicrograph of bone, after 14 days, **Moringa + PRF Group1** showing moderate expression of proliferating fibrous tissue (red arrow) with ill-defined bone trabeculae (black arrow).
(TGF-β1 x200)

Figure 6: Photomicrograph of bone, after 14 days, **PRF Group2** showing , showing mild positive expression of fibrous tissue (red arrow) with ill-defined bone trabeculae (black arrow). (TGF-β1 x200) .

Figure 7: Photomicrograph of bone, after 28 days, **Moringa + PRF Group1** showing severe expression of well-organized fibrous connective tissue (red arrow) with newly formed bone trabeculae (black arrow) (TGF-β1 x200) .

Figure 8: Photomicrograph of bone, after 28 days, **PRF Group 2** showing moderate expression proliferating fibrous tissue (red arrow) with fibroblasts (asterisk) and well-defined new bone trabeculae (black arrow). (TGF-β1 x200) .

Figure9 : Photomicrograph of bone, after 14 days, **Moringa + PRF Group1** showing increasedcollagen bundles at the defect area (black arrow) with increase amount osteoid tissue (red arrow). (Masson's trichrome stain x200) .

Figure 10: Photomicrograph of bone, after 14 days, **PRF Group 2** showing moderate amount of fibrous tissue (black arrow) filling the bone defect with small part of osteoid tissue (red arrow). (Masson's trichrome stain x200).

Figure 11: Photomicrograph of bone, after 28 days, **Moringa + PRF Group 1** showing increased collagen bundles at the defect area (red arrow) with well-defined mature bone (black arrow). (Masson's trichrome stain x200).

Figure 12: Photomicrograph of bone, after 28 days, **PRF Group 2** showing increased amount of fibrous tissue at the bone defect (red arrow) with illl-defined mature bone trabeculae (black arrow). (Masson's trichrome stain x200) .

Figure 13: Quantification of MTC stained area (as area %). Data was presented as means ±SE.