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Evaluation of the Efficacy of PRF as a Bone Substitute Material Alternative to Xenograft in Critical-Sized Bone Defects in Periradicular Surgery: an Animal Study.

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Abstract: *The study evaluated platelet-rich fibrin (PRF) alone and in combination with Xenograft as an alternative to bone substitute in the treatment of critical-sized bone defects in dogs.* **Methods:***Eight healthy adult male dogs were included according to the power study. Root canal treatment of the mandibular 2nd and 4th premolar teeth in both sides was done. Two critical-sized bone defects (7 mm × 7 mm × 7 mm each)were createdin each side of the mandible in relation to the distal root of the selected teeth (32 bone defects). According to the bone cavity filling materials used there were four groups: Xenograft (X), Xenograft and PRF (X and PRF), PRF and Blood Clot groups (BC). The animals were sacrificed at 4 days, 2 weeks, 5 weeks and 9 weeks after the treatment and the mandibles were harvested. Dual-energy X-ray absorptiometry evaluation (DEXA)was done to evaluate the bone density at the defects. A histological evaluation was done to evaluate new bone formation.* **Results:** *At four - day scarification period, the highest bone density was observed in the X group (0.602 ± 0.012) followed by X and PRF (0.521 ± 0.012), PRF (0.163 ± 0.024) and BC (0.102 ± 0.024) groups (p < 0.001). At 9-weeks, the highest bone density was observed in the X and PRF group (1.325 ± 0.029), followed by X (1.04 ± 0.023) and PRF (1.05 ± 0.035) groups (p < 0.001) with no significant difference between X and PRF groups. Histologically X and PRF group showed the highest new woven bone formation at 9 weeks.* **Conclusion:** *PRF is a valuable material that can be used as a grafting material in periradicular surgery to improve bone regeneration.*

Keywords: *Bone regeneration, bone substitutes, bone density, platelet-rich fibrin, xenograft.*

I. Introduction

Root canal treatment aims to completely control root canal infection by creating an adequate environment suitable for periradicular healing. However, despite treatment, bacteria may remain within the root canal, which may affect the healing of periradicular lesion [1].Newly developed equipment have resulted in better treatment outcomes, but if the treatment fails, surgical intervention becomes a choice [2]. Surgical management of periradicular lesions results in bone defects of variable sizes that necessitate bone substitute to enhance bone regeneration [3]. Bone regeneration can be induced through three different mechanisms: osteogenesis, osteoinduction and osteoconduction[4].The primary types of bone graft materials include autogenous bone,

allografts, xenografts and alloplasts[5].The process of bone augmentation has drawbacks ranging from patient morbidity to the possibility of disease transmission[6].

Platelet-rich fibrin (PRF) is a healing biomaterial with great potential for bone and soft tissue regeneration without causing inflammatory reactions. Recently, it has been used alone or in combination with bone grafts to promote haemostasis, bone growth and maturation [7]. However, few studies have used PRF alone or in combination with bone grafts to induce bone regeneration in periradicular area [8], thus, the present study aims to radiographically and histologically evaluate the healing capacity induced by PRF with or without using Xenograft. The authors hypothesized that there is no difference amongst the tested groups.

II.   Materials and methods

2.1 Ethical approval:

The study was conducted in accordance with the ARRIVE guidelines and approved by the ethics committee of the Faculty of Dental Medicine, Al-Azhar University with Ref No: 115/137/-04-2019.

2.2 Animal model:
Eight healthy adult male purpose-bred dogs within the same age range and weighing 15–20 kg were used. The animals had no systemic diseases and were vaccinated. They were bred and housed under similar conditions at 22 °C room temperature, 40% humidity and 12 h daylight cycle with free access to standard laboratory diet and water. The animals underwent clinical and radiographic evaluations in cooperation with the veterinary team of the general veterinary hospital in Alabbassia, Cairo, Egypt. Dogs rather than the animal model were excluded from the study.
2.3 Preparation of dogs

All dogs were fasted for 12 hours before anaesthesia and premedicated with intramuscular injection of atropine sulphate (atropine; ADWIA Co., Cairo, Egypt) at a dose of 0.04 mg/kg followed by xylazine hydrochloride (Xyla-Ject; ADWIA Co., Cairo, Egypt) at a dose of 1 mg/kg.

2.4 Animal anaesthetization:

General anaesthesia was induced by intravenous injection of ketamine hydrochloride (Keiran; EIMC Pharmaceuticals Co., Cairo, Egypt) at a dose of 10 mg/kg body weight and maintained with intravenous injection of thiopental sodium at a dose of 2.5 mg/kg. One third of the estimated dose was injected within 15 s, and the remainder was administered slowly until loss of pedal and corneal reflexes and development of shallow regular respiration.

2.5 PRF preparation

PRF preparation was performed in accordance with the PRF protocol prepared by Choukroun (9). A 10 ml of dog's blood was extracted and placed in two 5 ml sterile pre-vacuumed plain glass tubes. The tubes were centrifuged at 3000 rpm for 10 min. After centrifugation, the blood in the tube was separated into three distinct zones: the upper layer of platelet-poor plasma, PRF in the middle and red blood cells at the bottom. The tubes were maintained until the bony cavities were prepared.

2.6 Preparation of the operative field:

All procedures were conducted under a clean aseptic protocol. Isolation of the field for endodontic treatment of the mandibular 2nd and 4th premolar teeth was conducted on both sides. At the obturation phase, the mesial roots were filled using cold lateral compaction technique, whilst the distal roots were kept free to be filled with orthograde MTA (Angelus, Londrina, Brazil) after critical-sized bone cavity preparation. The access cavity was temporarily packed with sterilized gauze until this step.

2.7 Surgical Procedure:

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Prior to surgical intervention, 1.8 ml of 2% lidocaine with1: 100,000 norepinephrine (Amriya Pharm Industries, Alexandria, Cairo, Egypt) was injected into the surgical area. Two separate three-sided rectangular full-thickness mucoperiosteal buccal flaps were raised over the 2nd and 4th mandibular premolar teeth. Following flap elevation, a 7×7 mm metallic template was fixed opposite to the apical 1/3 of the distal roots of the 2nd and 4th premolar teeth on each side Fig.1.
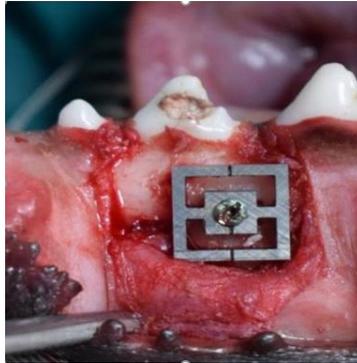

Fig1: A photograph showing the metallic template in place

The position of the metallic template was confirmed radiographically. Then, a modified surgical bur with metallic stopper welded at 8 mm from the tip of the bur was used to create the bone defect through the template. After creating the outlines of the bony cavity, the bone in between, including the apical 3 mm of the root, was removed with the periosteal elevator. Immediately after bony cavity creation, the distal roots were filled with MTA. The tooth was restored with resin-modified glass ionomer filling material (DentsplyMaillefer, Ballaigues, Switzerland). According to the bone cavity filling materials used, there were four groups xenograft group (X), platelet-rich fibrin group (PRF),combination group (X and PRF), and blood clot group (BC). The flaps were repositioned andsutured with 3-0 resorbable suture vicryl (polyglactin 910, E,thicon, Inc 2018).

2.8 Postsurgical care:

The animals were intramuscularly administered with amoxicillin and flucloxacillin (Flummox: E.I.P.I.CO, Tenth of Ramadan City – Industrial Area, Egypt) as antibiotics at a dose of 50 mg/kg lean body weight per day and Xylaject at a dose of 3 ml every 12 h for 5 days to control pain and infection under the supervision of a veterinarian. The animals were fed a soft diet composed of milk, rice, meat, liver and bread for the first postoperative week. On the second postoperative week, they were able to eat the usual diet. The general health of all animals was evaluated clinically until they were sacrificed. In addition, daily examination for signs of infection such as redness and warm skin, mouth-opening ability, eating and swallowing was conducted.

2.9 Scarification and sample harvesting:

Euthanasia was scheduled at 4 days, 2 weeks, 5 weeks and 9 weeks after surgery with two dogs being sacrificed at each time. Euthanasia was performed under general anaesthesia provided by intravenous injection of pentobarbital (Socumb, Butler Company, Columbus, Ohio, USA) at 30 mg/kg. The dogs were euthanized with additional pentobarbital at a dose of 90 mg/kg in the carotid artery. A perfusion with 10% buffered formalin (Fisher Scientific, Fair Lawn, New Jersey, USA) in the carotid artery was done. The mandible was surgically removed and divided at the midline into two halves. The samples were maintained in 10% buffered formalin until the time of bone density measurement.

2.10 Densitometric analysis:

The two mandibular halves of each dog were referred for bone density examination at the bone defect site by using dual-energy X-ray absorptiometry (DEXA) after sacrifice at each examination period. The measurements were performed using three sites axial scanner, bone mineral analyzer (lunar prodigy primo) at Radiology Center of Raba hospital, Nasr City, Cairo, Egypt. The bone mineral density (BMD) of each bone defect was measured at the 7×7 mm^2 region of interest by DEXA.

2.11 Fixation and decalcification:

After completion of densitometric analysis, experimental teeth and their surrounding bone tissue were block-sectioned using an orthopedic electrical saw under copious normal saline irrigation. The specimens were

replaced in 10% buffered formalin for fixation up to 4 days at room temperature. Decalcification was performed by immersing the specimens in equal volumes of 20% sodium citrate and 5% formic acid (Decal Chemical Corporation, Congers, New York) for a period of 2 months. The solution was replaced once per week.

2.12 Histologic preparation and evaluation:

Upon removal from the decalcifying solution, the specimens were washed under running tap water for 20 min followed by immersion in 70% ethyl alcohol. The specimens were then dehydrated in ascending concentrations of ethanol and processed on a Leica TP 1020 dip n' dunk processor (Leica, Wetzlar, Germany) at 45 min per station in the following manner: one cycle of 70% ethanol, two cycles of 80% ethanol, two cycles of 95% ethanol, two cycles of 100% ethanol, two cycles of xylene and two cycles of Paraplast paraffin (Kendall, Mansfield, Massachusetts) at 58 °C. The tissues were removed from the storage cassettes and embedded in paraffin. Next, they were sectioned on a Leica Jung RM 2045 microtome (Wetzlar, Germany). Sections were made longitudinally in mesiodistal direction every 5 μm and placed in ProbeOn Plus slides. Tissues were stained with haematoxylin and eosin (HE), rinsed in cool running double-distilled water for 5 min, dipped in 0.5 eosin for 12 times, dipped in distilled water and dehydrated in ascending concentrations of ethanol. The sections were dipped in xylene several times, mounted on slides covered with a coverslip with Cytoseal (Thermo Fisher Scientific, Waltham, MA) and evaluated under a light microscope up to 400× magnification.

2.13 Histologic evaluation:

Histologic evaluation was conducted on the healing sites, in which three digital images were captured at 100× magnification for each HE-stained slide to describe the stage of new bone formation in the tested groups at each time.

2.14 Data management and analysis:

Data were collected, tabulated and statistically analyzed. The mean and SD values were calculated for each group in each test. Data were explored for normality using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Data showed non-parametric distribution. Friedman test was used to compare more than two groups in related samples. The Kruskal–Wallis test was used to compare more than two groups in non-related samples. The Mann–Whitney U test was used to compare between two groups in non-related samples.

The significance level was set at $p \leq 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

III.   Results:

3.1 Bone density results:

3.1.1 At 4 days:

A statistically significant difference was found between X (0.602 ± 0.012) and each of X and PRF (0.521 ± 0.012), PRF (0.163 ± 0.024) and BC (0.102 ± 0.024) groups ($p < 0.001$). A significant difference was also found between X and PRF (0.521 ± 0.012) and each of PRF (0.163 ± 0.024) and BC (0.102 ± 0.024) groups ($p < 0.001$). Also, a significant difference was found between PRF (0.163 ± 0.024) and BC (0.102 ± 0.024) groups ($p = 0.003$).

3.1.2 At 2 weeks:

A statistically significant difference was found between X (0.674 ± 0.024)   and each of X and PRF (0.745 ± 0.012), PRF (0.235 ± 0.035) and BC (0.174 ± 0.012) groups where (p=0.004), (p<0.001) and ($p < 0.001$). Also, a significant difference was found between X and PRF (0.745 ± 0.012) and each of PRF (0.235 ± 0.035) and blood clot (0.174 ± 0.012) groups where ($p < 0.001$). A statistically significant difference was found between PRF (0.235 ± 0.035) and BC (0.174 ± 0.012) groups where ($p = 0.012$).

3.1.3 At 5 weeks:

A statistically significant difference was found between X (0.868 ± 0.012) and each of X and PRF (1.08 ± 0.046), PRF (0.602 ± 0.012) and BC (0.245 ± 0.024) groups (p <0.001). A significant difference was also found between x and PRF (1.08 ± 0.046) and each of PRF (0.602 ± 0.012) and blood clot (0.245 ± 0.024) groups (p<0.001). Also, a significant difference was found between PRF (0.602 ± 0.012) and BC (0.245 ± 0.024) groups (p < 0.001).

3.1.4 At 9 weeks:

A statistically significant difference was found between X and PRF (1.325 ± 0.029) and each of PRF (1.05 ± 0.035) and BC (0.388 ± 0.024) groups (p < 0.001). A significant difference was also found between blood clot (0.388 ± 0.024) and each of X (1.04 ± 0.023), x and PRF (1.325 ± 0.029) and PRF (1.05 ± 0.035) groups (p < 0.001).

3.2 Histologic results:

Histological examination of sections stained with H&E at different scarification period at 100x magnification revealed different histological pictures as shown in Fig 2,3,4,5 and Table 1.
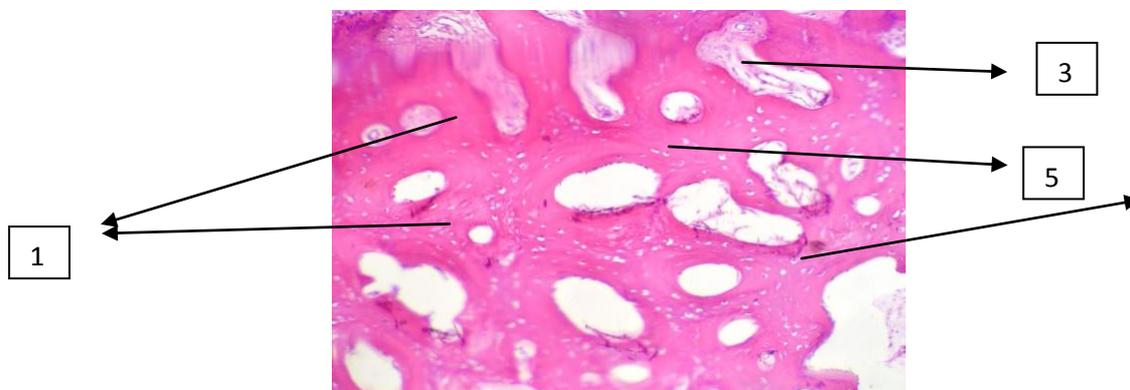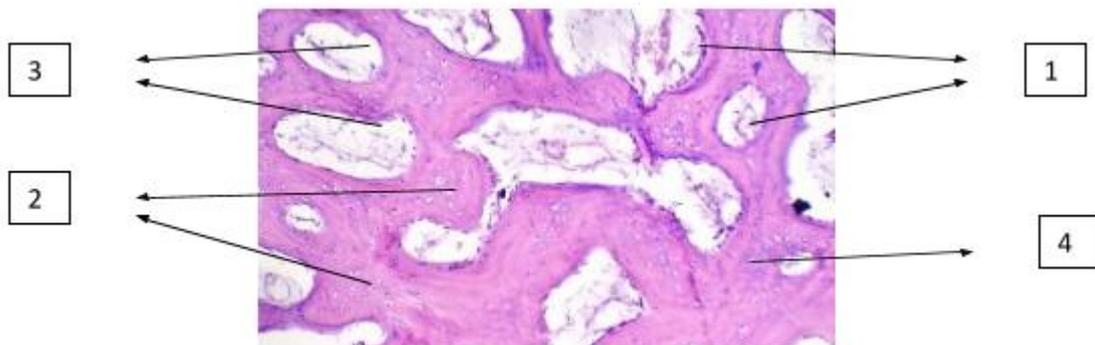

Fig 2: photomicrograph for X group at 9 weeks showing: typically, variable bony trabeculae (1), Osteoblastic cells (2), and dilated BVs (3). (H&E.100x)
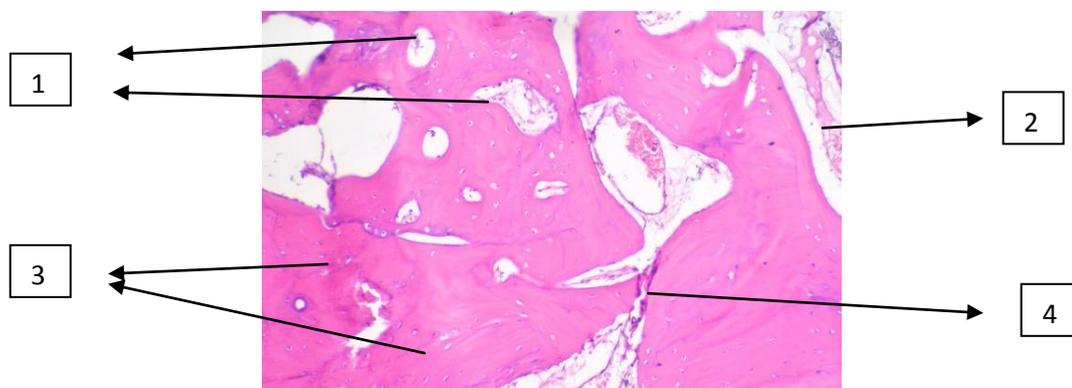
Fig 3:photomicrograph for X and PRF group at 9 weeks showing: large diluted BVs (1), well organized granulation tissue (2), islands of thick bone trabeculae

(3), Osteoblastic activity (4). (H&E.100x)
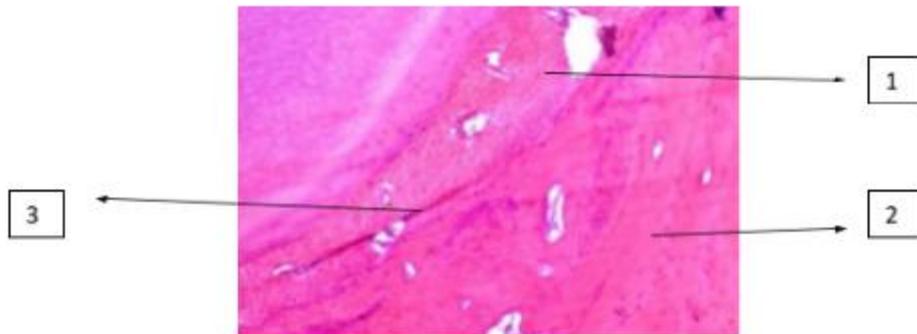
Fig 4: Photomicrograph for PRF group at 9 weeks showing: typically organized GT (1), osteonal bone formation (2), and periodontal ligament(3).

(H&E.100x)

Fig 5: Photomicrograph for BC group at 9 weeks showing: typically organized GT (1), osteonal bone formation (2), and periodontal ligament(3). (H&E.100x)

Scarification Periods — Testing materials	4 days	2 weeks	5 weeks	9. weeks

Xenograft group	- Blood clot - Inflammatory cells - Organized granulation tissue - Mitotic activity - Xenograft particle	- Blood clot - Inflammatory cells - Well organized granulation tissue - Island of very thin bone trabeculae - Xenograft particle	- Remnant of blood clot - Well organized granulation tissue - Woven bone with variable trabeculae	- Healing with new osteon bone in different degree of maturation
Xenograft and platelet-rich fibrin group	- PRF and blood clot - Inflammatory cells - Well organized granulation tissue - Early woven bone formation	- PRF and blood clot remnant - Inflammatory cells - Well organized granulation tissue - Woven bone formation with thin bone trabeculae	- Small remnant of PRF and blood clot - Well organized granulation tissue - Woven bone with thick trabeculae with small marrow spaces	- Healing with new osteon bone in different degree of maturation - Periodontal ligament formation in early stage
Platelet-rich fibrin group	- PRF and blood clot - Inflammatory cells - Well organized granulation tissue - Very early woven bone	- PRF and blood clot remnant - Inflammatory cells - Well organized granulation tissue -Early woven bone formation with thin bone trabeculae	- PRF and blood clot remnant - Inflammatory cells - Well organized granulation tissue - Woven bone with thick trabeculae - Primary osteon	- PRF remnant - Well organized granulation tissue - Newly formed bone of variable thickness trabeculae - Reversal line
Blood clot group	- Large blood clot - Inflammatory cells - Poor granulation tissue - Thin collagen fibrils	- Blood clot - Inflammatory cells - Granulation tissue - Thin collagen fibrils	- Blood clot - Inflammatory cells - Well organized granulation tissue - Very early woven bone	- Well organized granulation tissue -Woven bone with thin trabeculae - Primary osteon

Table 1: Description of the histological sections of the tested groups at each scarification period:

IV.   Discussion:

Recent bone healing studies attempted to accelerate bone regeneration to improve treatment outcome by using different types of bone grafts to fill the bone defect and induce healing [10]. The present study evaluated the effect of PRF as an alternative bone substitute with or without using xenograft.

Dogs were used as a model in the present study due to their anatomical, physiological and biomechechanical similarity to human beings with better experimental manipulation for various diagnostic and functional tests than rodents [11, 12]. Out of 12 dogs, 8 dogs were used depending on the power of the study to provide 95% confidence with a significance level (alpha) of 0.05 (two tailed). Four dogs were excluded: one died immediately after surgery due to massive uncontrolled blood loss during surgery despite life-saving efforts; the other three dogs were excluded preoperatively due to congenital abnormalities. The smallest number of dogs, which did not affect the results, was used depending on the power of the study.

Compassion with dogs was taken into consideration; thus, efforts were made to minimise the number of times the dogs were exposed to general anaesthesia. Exposure to general anaesthesia may affect the general health of the dogs. Thus, root canal treatment and periradicular surgery were performed in a single appointment, which was consistent with a previous study [13].

Disinfection of the operative field followed by rubber dam application was of paramount importance to allow for an aseptic field whilst working.Obturation of the mesial roots was conducted. Distal root obturation was postponed after surgery to be filled with MTA via orthograde way to confirm adequate filling. The bony cavity was compacted with gauze to prevent MTA extrusion during distal root filling. MTA was used to seal the roots in relation to the periradicular surgery as it exhibits the highest apical healing (90.4%) compared with other root-end filling materials [14]. MTA also prevents leakage than other root-end filling materials [15].The distal root of the 2nd and 4th premolars was used rather than other roots as there is a large distance of bone and soft tissue in between and opposite to the 3rd premolar. Separate flaps were elevated in relation to each root to avoid seepage of experimental materials between experimental cavities. In addition, the remaining distance of soft tissue between flaps is adequate and does not affect vitality.The metallic template was used in this study for the purpose of standardisation of the bone cavity, in which different sizes may heal differently. The metallic template was replaced with a new one when the old one was affected by cutting. Critical-sized bone defect was defined as a bone defect that does not heal over the lifetime of the dogs. Later, it was redefined as the size of a defect that will not heal throughout the study. This is because most studies are of limited duration and do not extend over the entire life of the dogs [16]. The goal of this study was to create a bone defect that was unable to heal on its own through the study time, similar to human nonunions. Thus, a 7 mm × 7 mm × 7 mm bone defect was created.

PRF is considered as a new platelet concentrate and a bioactive surgical additive that regulates inflammation and induces healing. Platelets are cytoplasmic fragments of megakaryocytes formed in the bone marrow measuring about 2 μm in diameter. They contain more than 30 bioactive proteins that play a fundamental role in tissue healing. PRF was prepared in accordance with the protocol of Choukroun[7], PRF was constituted by a fibrin network, in which platelets, leucocytes, cytokines and stem cells are enmeshed. PRF contains nearly 97% of platelets and more than 50% of leucocytes [17]in the blood. PRF is also rich in platelet cytokines including transforming growth factor B1, platelet-derived growth factors and insulin-like growth factors. PRF also enmeshes glycosaminoglycans, which have a great capacity to support cell migrations and healing processes[18-20]. PRF was used as a scaffold in other studies. However, few studies have used PRF in bone healing.

Regarding the effect of time on bone mineral content, BMD and woven bone formation, 9 weeks period showed the highest result in relation to other time periods, and this result is logical in which bone density will increased as the time for healing increase. The xenograft group showed the highest mineral content and bone density after 4 days and 2 weeks followed by the xenograft and PRF, PRF and blood clot groups. This finding may be related to the nature of xenograft materials that is evident immediately after surgical intervention, whilst PRF is considered as a soft tissue, which exhibits low-intensity signals on X-ray scanning immediately after application [21]. At 5 weeks, no difference was observed between the xenograft and PRF and xenograft groups. At 9 weeks, the xenograft and PRF group showed the highest mineral content and bone density, and no difference was observed between the xenograft and PRF groups. This result may be due to the effect of PRF, which gradually increases osseous healing at every follow-up period [22]. The results of this study are in agreement with other studies, showing that PRF acts as an appropriate scaffold with a strong fibrin structure that optimally supports the transplanted mesenchymal cells and allows for the gradual release of growth factors over a long period ranging from 7 days to 28 days [23-26]. Combining PRF with bone grafts is advantageous as it stabilises the grafting material at the defect site and allows the release of growth factors, which enhance the healing of hard and soft tissues [27, 28]. Moreover, adding PRF to bone substitute showed a reduction in the time required to promote graft consolidation and maturation and improved trabecular bone density [29]. Besides its low cost, PRF may induce healing in medically compromised patients, particularly those with a history of radiotherapy, because it is able to stimulate natural defense mechanisms[30]. The null hypothesis of the study was rejected as

the tested groups showed different bone regeneration capabilities. A longer evaluation periods are recommended to provide more valuable information about the degree of maturation of the newly formed bone.

V.   Conclusion:

Under limitations of the present study, Platelet-rich fibrin is a promising biomaterial that accelerates bone regeneration of critical sized bone defects especially when combined with xenograft bone substitute in periradicular surgery.

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