The Effect of Platelet Rich Fibrin and Hyaluronic Acid on Bone Regeneration After Periradicular Surgery in Healthy and Diabetic Dogs

(Radiographic and Histological study)

Thesis Submitted in partial fulfillment of the requirements for Doctor Degree in Endodontics

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1445H - 2023G

تاثير الصفائح الغنية بالفيبرين وحمض الهيالورونيك علي التئام العظام بعد الجراحة حول الجذرية في الكلاب السليمة والمصابة بالسكري

(دراسة اشعاعية وهستولوجية)

دراسة مقدمه كجزء من مقومات الحصول على درجة الدكتوراه فى علاج الجذور

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Acknowledgement

I would like to express my deep gratitude to **prof. Dr.Taher Medhat Islam** Professor of endodontics, Faculty of Dentistry, Al-Azhar University for his valuable guidance and effort throughout my academic and clinical work.

My sincere appreciation to **Prof. Dr.Motaz-bellah A. Alkhawas** Professor of endodontics, Faculty of Dentistry, Al-Azhar University for his valuable supervision and unlimited support.

My deep appreciation to Prof. **Dr.Hany Gameil Gobran** Professor of oral biology, Faculty of Dentistry, Al-Azhar University for his support of this project and his expertise in animal research for his sincere help, cooperation and encouragement during this study.

Special thanks to All staff members of the department of Endodontics
Faculty of Dentistry, Alazhar University.

Dedication

I dedicate all this work to the pure soul of my mother, who I hoped would be in our hands today so that my joy would be complete

Also I dedicate this work to my father, my brother, my wife and my little kids for the countless sacrifices they have made throughout the years. Without their love and support, none of my accomplishments, however small, would have been possible.

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2	HA	Hyaluronic acid	1
3	HMW	High molecular weight	1
4	ECM	Extracellular matrix	2
5	TNF	Tumor necrosis factor	2
6	PDGF	platelet-derived growth factor	6
7	TGF-b	Transforming growth factor-b	6
8	EGF	Epidermal growth factor	6
9	PMNL	Poly morpho nuclear leukocyte	6
10	MMPs	matrix metaloproteinases	7
11	EGF	Endothelial grouth factor	8
12	KGF	keratinocyte growth factor	8
13	B FGF	Basic fibroblast growth factor	8
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26	EHA	Esterified hyaluronic acid	25
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Introduction

Periapical pathology occurs as a sequela of microbial insults from the root canal leading into excessive osteoclastic bone resorption circumscribing the root [(1)]. Failure of nonsurgical root canal treatment necessities surgical intervention in the form of periradicular surgery [(2)]. The periradicular surgery aims to remove the pathological and granulation tissues in the periapical area. Removal of such tissues creates a surgical defect in the periapical area that requires bone substitutes to fill the defect. The type of bone substitute is a critical factor that affects the healing of the periradicular area. The best bone substitute is autologous bone which has osteoconductive and osteoinductive properties that accelerate the healing of the periapical tissues[(3)]. Unfortunately, such type of bone substitute requires donor sites rather than the defective surgical one that leads to provoking pain, stress,and delayed surgery for the patent [(4)]. As an alternative to autologous bone, synthetic bone substitutes are used that only have osteoconductive properties that require more time for complete healing To overcome the disadvantages of both autologous and synthetic bone substitutes different alternatives have been developed such as using blood derivatives. Platelet rich in fibrin (PRF) which is an autologous leukocyte and platelet-rich fibrin biomaterial with a specific composition and three-dimensional architecture.it consists of a fibrin three-dimensional polymerized matrix in a specific structure, with the incorporation of almost all the platelets and more than half of the leukocytes along with growth factors and circulating stem cells. It supports and accelerates the healing process which introduces it as a promising blood derivative for bone healing [(5)]. Alternatively, Hyaluronic acid (HA), which is also called hyaluronan, is a high molecular weight (HMW) glycosaminoglycan, composed of repeated nonsulfated

disaccharide units of N-acetyl glucosamine and D-glucuronic acid [6,7]. It is one of the main components of the extracellular matrix (ECM) [8]. It was found that HA has an active role in cellular signaling; morphogenesis and matrix organization in addition to regulating fibroblast and myofibroblast proliferation [9]. Subsequently to tissue injury, HA concentration levels significantly increase to stimulate migration of the ECM cells to the zone and to form a temporary structural skeleton by forming fibrin-clot relations. Therefore, the initial phase of the healing process is regulated by HA [10,11]. Concomitantly with the type of bone substitute, other factors may affect the healing of bone which is the hormonal changes that leads to systemic diseases such as diabetes mellitus. Diabetes mellitus is a chronic metabolic disease with high blood glucose levels [12]. Diabetes results from deficits in the production of insulin or deficit insulin resistance coupled with insufficient insulin production. Diabetes mellitus often leads to serious complications that affect the heart, blood vessels, eyes, kidneys, and nerves. Hyperglycemia may affect bone through enhanced expression of pro-inflammatory cytokines such as Tumor necrosis factor(TNFα), which reduces osteoblast differentiation, and osteoblast activity and increases osteoblast apoptosis[13]. Although there is an abundance in the research where PRF and HA are used in the dental aspect, however little few studies in which PRF and HA are used alone or in combination to induce bone regeneration in periradicular areas.

Review of literature

Section outline:

2.1 Mechanism of wound healing.

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2.2 .1 Traditional Bone grafts.

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2.1 Mechanism of wound healing in periradicular surgery:

A wound is defined as a disruption in the normal anatomic structure and function. Wounds can be classified as acute or chronic based on whether or not they progress through an orderly and timely healing process to restore anatomic continuity and function. [14]

Wound healing is the programmed tissue response to injury of a living organism that involves complex cellular and molecular biological processes [15]

2.1.1 Types of surgical wounding:

A-Incisional wound: Incisional wound is a type of wound made with a scalpel and involves the mucoperiosteal tissues, permits proper approximation of similar tissues (epithelium to epithelium and fibrous connective tissue to fibrous connective tissue) which allows primary healing [16]

B-Blunt dissection wound: Blunt directional wound is a type of wound made with a periosteal elevator, separating mucoperiosteal tissues from cortical bone during the flap reflection procedure [17]

C- Excisional wound: Excisional wound is a type of wound made with a rotary instrument during bone removal and root-end resection, and this type of wounding is associated with the secondary type of healing[17]

2.1.2 Types of wound healing:

A- Primary intentional wound healing:

This type of healing Occurs when wound edges are closely reapproximated, and separated by a thin clot layer. The result of this

healing mechanism is known as regeneration, and the wounded tissues are ultimately restored to a normal anatomical pattern and function [17]

B- Secondary intentional wound healing:

This type of healing occurs when wound edges are not properly approximated. Granulation tissue formation is necessary for cells to bridge the hiatus. The end result of this healing mechanism is known as repair and the normal anatomy and function are not restored. This type of healing is associated with scar formation [4]

Periradicular surgeries vary from just apical curettage to apicoectomy, the procedure which implies bony defect creation with variability in the size of the defect owing to the case [18,19]. Bone healing occurs either by the primary type of healing when the defect is small as 0.1mm, or by the secondary type of bone healing when the defect is larger. So, the secondary type of bone healing is the predominant type in endodontic periradicular surgery [20]. Bone healing may occur with repair which is defined as the healing of a wound by tissues that does not fully restore the architecture or the function of the part [21].

Regarding healing in periradicular surgery, histological studies revealed that healing can occur through one of three types [22]:

a) Healing with the re-formation of the periodontal membrane and with no or mild periapical inflammation

b) Healing with fibrous tissue (scar tissue) in communication with or adjacent to the periodontal membrane, and with varying degrees of inflammation,

c) Moderate or severe periapical inflammation without scar tissue

2.1.3 The physiological processes of normal wound healing

Wound healing involves a variety of cellular activities including chemotaxis, phagocytosis, mitogenesis, migration, and ECM synthesis/remodeling. Wounds of the oral mucosa and skin proceed through the same stages of the healing process: hemostasis, inflammation, proliferation, and remodeling [23]

A-Hemostasis (clotting) begins with platelet aggregation and clot formation to limit blood loss, clot is formed at the wound site and originates from the extra vasated blood from injured blood vessels through intrinsic and extrinsic mechanisms. Blood clot seals the wound from the outer environment, fixating the wound edges in relation to each other, Platelets degranulate, releasing granules that secrete several growth factors, including platelet-derived growth factor (PDGF), transforming growth factor-b (TGF-b), insulin-like growth factor-1, epidermal growth factor (EGF) and platelet factor IV. These proteins initiate the wound-healing cascade by attracting/activating neutrophils Poly morpho nuclear leukocyte(PMNL), endothelial cells, and macrophages (Mø). The clot comprising fibrin, fibronectin, vitronectin, von Willibrand factor, and thrombospondin, provides the provisional matrix which acts as a scaffold for cellular migration either inflammatory or regenerative cells between wound sides. Platelet degranulation also releases vasoactive amines, for example, serotonin which increases microvascular permeability. This leads to the exudation of fluid into the extravascular space resulting in tissue edema, although this feature is evident clinically in the inflammatory phase [24] When a blood clot is thin, wound healing is directed toward the primary type of healing, while a large blood clot leads to the secondary type of wound healing (associated with scare formation), as it prevents adequate approximation of wound edges and should be completely removed from the site before healing [25].

B-Inflammation begins with the activation of the complement and the initiation of the classical molecular cascade. This leads to PMNL migration stimulated, within 24 h of injury, by a number of agents including platelets, TGF-b, complement components such as C5a, and bacteria. PMNL rapidly adheres to the endothelial
cells in blood vessels by a process called margination and actively moves through the vessel wall. In the wound environment, they phagocytose exogenous material including bacteria by their generation of proteolytic enzymes and reactive oxygen species. PMNL therefore, acts to rapidly minimize bacterial contamination of the wound and prevent infection.

In the late inflammatory phase, monocytes (Mo) are attracted to the wound by a variety of chemoattractants. These will also now include immunoglobulin, ECM breakdown products, cytokines/ growth factors, and other inflammatory mediators (eg. leukotriene B4, platelet factor IV, PDGF, and TGF-b). Circulating monocytes (Mo) in the wound undergo a phenotypic change to become tissue macrophages Approximately 3 days after injury. MF, in addition to their function as phagocytic cells by performing phagocytosis of pathogens and cell debris [(26)], is a major producer of growth factors responsible for the production of ECM, and the proliferation of smooth muscle and endothelial cells required for angiogenesis. In addition, MF releases proteolytic enzymes, such as matrix metalloproteinases (MMPs), that debride the wound. Depletion of Mo and MF consequently causes severe impairment of wound healing. [(27)]

The inflammatory response to injury is essential for supplying growth factor and cytokine signals that are responsible for cell and tissue movements, which are crucial for the subsequent repair mechanisms in adult mammalians [(28)]

C- Proliferation occurs rapidly in response to injury. Where there is a breach in the surface barrier (skin or mucosa), keratinocytes at the wound

edge proliferate and migrate over the dermis. A single layer of keratinocytes migrate from the wound edges within a few hours of wounding and at 12 h after wounding, there is a marked increase in mitotic activity in the basal cells from the wound edges or around skin appendages. These cells migrate across the provisional matrix, this migration being facilitated by their MMP expression. When advancing keratinocytes meet, further movement is halted by 'contact inhibition' and a new basement membrane is generated. Further epithelial cell growth and differentiation re-establishes the stratified epithelium. Several growth factors such as Endothelial grouth factor EGF, keratinocyte growth factor (KGF), and basic fibroblast growth factor (bFGF), modulate epithelialization by stimulating keratinocyte mitogenesis and proliferation. Fibroblasts appear in the wound between 2 and 4 days after wounding, attracted to the wound by a number of factors, including PDGF and TGF-b5,6. In uninjured tissue, fibroblasts occupy a quiescent state but are able to actively proliferate in response to mitogenic stimuli, such as growth factor signaling following tissue injury [(29)]. Fibroblasts are ubiquitous cells that play a number of crucial roles in various stages of the healing process: forming the granulation tissue, reorganizing the provisional ECM, and remodeling theresulting scar [(30)].

D- Remodeling:

Remodeling is the last phase of wound healing and occurs from day 21 to up to 1 year after injury. The formation of granulation tissue stops through apoptosis of the cells. A mature wound is, therefore, characterized as avascular as well as a cellular [(31)]. During the maturation of the wound, the components of the ECM undergo certain changes. Collagen III, which was produced in the proliferative phase, is now replaced by the stronger collagen I, This type of collagen is oriented in small parallel bundles and is, therefore, different from the basket-weave

collagen in the healthy dermis[32]. Later on, the myofibroblasts cause wound contractions through their multiple attachments to collagen and help to decrease the surface of the developing scar[5]. Furthermore, the angiogenic processes diminish, the wound blood flow declines and the acute wound metabolic activity slows down and finally stops.

Oral mucosal healing

In contrast to the 'scar-forming' skin wounds, wounds in healthy oral mucosa, like early-gestational foetal skin [33], are characterized by rapid re-epithelialization and re-modeling)[34]. This reduced scarring phenotype was, like foetal wound healing, initially, simply attributed to a moist wound environment and the presence of cytokines/ growth factors in saliva; a number of important candidate molecules are present in saliva, including EGF and secretory leukocyte protease inhibitor (SLPI; a cationic serine protease inhibitor with antimicrobial and anti-inflammatory properties) [35].

Dento alveolar wound healing:

The inflammatory and proliferative stages of healing for bone tissue are similar to those for soft tissue,However, the maturation phase of bone healing differs totally from that for soft tissues because of the tissues involved: cortical bone, cancellous bone, alveolar bone proper, endosteum,PDL, cementum, dentin, and inner mucoperiosteal tissue. Bone formation can be categorized into two types woven bone and lamellar bone In both types , osteoblasts produce the bone matrix. In the formation of woven bone, which occurs by the matrix vesicle‐based process, osteoblasts produce matrix vesicles through exocytosis (the release of substances contained in a vesicle within a cell by a process in which the membrane surrounding the vesicle unites with the membrane forming the outer wall of the cell) of their plasma membrane resulting in

formation of hydroxyapatite crystals which amalgamate to form structures known as spherulites. Union of the separate spherulites results in mineralization.[36] . During lamellar bone formation Osteoblast secrete an organic matrix composed of longitudinally arranged collagen matrix fibrils (mainly type I collagen). Mineralization occurs by mineral deposition directly along the collagen fibrils[37.38]The gradual osteoclastic resorption of immature woven bone with osteoblastic bone formation and maturation to lamellar bone is known as creeping substitution.[39]

2.2 Bone grafts.

2.2 .1 Traditional Bone grafts.

Periradicular surgery may lead to a critical bone defect which defined as "the smallest osseous defect in a particular bone and species of dogs that will not heal spontaneously during the lifetime of the dogs" [40,41] . In this situation bone grafts can aid wound healing through preservation of morphologic contour, restoration of mechanical strength and function, elimination of dead space, prevention of ingrowth of soft tissue, and enhancement of retention of prosthetic devices in such bone defects[42,43]. Bone graft induce new bone formation by one or more of the following mechanisms: osteoconduction, osteoinduction and osteogenesis[44,45].

Osteoconduction: refers to the ability to support the attachment of osteoblast and osteo-progenitor cells, and allow the migration and ingrowth of these cells within the three-dimensional architecture of the graft.

Osteoinduction: describes that the graft can induce the primitive, undifferentiated and pluripotent stem cells to develop into the bone-forming cell lineage, by which osteogenesis is induced.

Osteogenesis: means the osteo-differentiation and subsequently new bone formation by donor cells derived from either the host or grafts.

2.2.1 Types of Traditional Bone grafts. :

A -Autologous bone grafts:

Autologous bone graft is an osseous graft that is transplanted from site to another site in the same individual, having osteoconductive, osteoinductive and osteogenic properties and considered as the gold standard in treating bone defects. The osteoinduction property of autograft is related to its high level content of proteins, which are preserved when the autografts are appropriately treated. In the early phase of autograft transplantation, hematoma and inflammation are formed rapidly with the recruitment of mesenchymal stem cells (MSCs) to lay down fibrous granulation tissue. In human being new bone formation usually takes about 6 to12 months to replace grafted bone[46,47], while in dogs it takes from 6 to 12 –week.

b-Allogeneic bone grafts:

Allograft is a type of natural bone graft that harvested and transplanted between individuals belonging to the same species. Allograft induce new bone formation by osteoconduction property, and considered as an alternative to the outograft that can be provided in different forms. allografts takes longer times to be incorporated into the recipient site that may be extended to several years due to host inflammatory responses that occasionally leads formation of fibrous tissue around the graft[48,49].

C-Xenogeneic bone grafts:

Xenografts are type of grafts that originate from a species other than human, such as bovine bone, porcine bone or from special management to a specific type of corals and termed ‘‘coral derived granules’’[50]. They

induce new bone formation by osteoconduction property, and can be prepared by freeze drying or demineralization and deproteinization[51].

D- Bone substitute:

1- Calcium sulphate (plaster of Paris or gypsum):

The first publication on the use of calcium sulphate as a bone substitute was reported early in 1892 and it revealed that it can be introduced in different forms, such as hard pellets or injectable viscous fluids that harden on application[52].

2-Ceramics:

They are calcium based materials and composed mainly from a mixture of Hydroxyapatite (HA) and Tricalcium Phosphate(TCP). HA is a relatively inert substance that is retained for prolonged periods of time, whereas the more porous TCP typically undergoes biodegradation within six weeks of its introduction into the area of bone formation. Moreover, HA achieves very high mechanical strength, while TCP has poor mechanical qualities[53].

3-Hydroxyapatite (HA):

It forms about 50% of the bone and teeth by weight and present naturally as mineral form of calcium salts which accounts for its excellent osteoconductive and osteointegrative properties. HA is unstable structure that allow the exchange of ions, a property that stands behind its use as a bone substitute. HA is available in one of two forms, either a particulate nonresorbable ceramic form or a particulate resorbable nonceramic form[54]

4- Bioactive glass: It was firstly developed in 1970s and was originally constituted by silicon dioxide (SiO2) as the main component of 45-52% in addition to some other mineral oxide[55]. Bioactive glass bonds

chemically with host bone through the bioactivity[56] in which silicon ions leach on the surface of the material on implantation and exposure to body fluid with formation of hydroxyapatite layer which absorb proteins and attract oseoprogenator cells then become replaced with new bone formation.

2.2.2 Recent alternative Bone grafts.

2.2.2.1 Platelet rich fibrin (PRF)

The platelet concentrates have been used for the improvement of reparation and regeneration of the soft and hard tissues after various periodontal surgical procedures. Using platelet concentrates is a way to accelerate and enhance the body's natural wound-healing mechanisms [57]

Platelet-Rich Plasma (PRP) is the first generation platelet concentrate that was introduced for the first time by Marx et al. in 1998[58] who reported that PRP addition accelerated the rate and degree of bone formation because PRP contains high concentrations of platelet-related growth factors and normal concentrations of plasma-derived fibrinogen, both of which contribute synergistically to the regenerative process. However, current disadvantages of PRP include a relatively complicated preparation procedure, the use of bovine thrombin, an animal-derived biological coagulant[59] These factors limited PRP usage and its incorporation into daily practice become rare.

Joseph Choukroun[60] simplified the platelet-rich fibrin preparation procedure and improved handling efficiency without the aid of animal-derived factors.

PRF is a second-generation platelet concentrate widely used to accelerate soft and hard tissue healing and is a strictly autologous fibrin matrix containing a large number of platelets and leukocyte cytokines [61] Growth factors are released after activation from the platelets trapped

within the fibrin matrix and have been shown to stimulate the mitogenic response in the periosteum for bone repair during normal wound healing[62]

A- Advantages drawbacks and of PRF:

The whole process of PRF preparation is natural, without any external manipulation since there is no need to use bovine thrombin and anticoagulants leading to the absence of any immunological reaction[**63**] . It is strictly an autologous leukocyte-platelet-rich fibrin matrix[64]. Which acts as a biodegradable scaffold[65] that allow the development of micro vascularization and are able to guide epithelial cell proliferation and migration to its surface[6]. It has a natural fibrin mesh with growth factors within that may keep their activity for a relatively long period and stimulate tissue regeneration[66] when used as a membrane, it avoids a donor site surgical procedure and results in a reduction in patient discomfort during the early wound-healing period[67].

In addition, It is an economical option, and its preparation is easier and quick due to the single-stage centrifugation. However there it has some drawbacks as the final amount available is low because it is autologous blood [68] and for the process a glass-coated tube is needed to achieve clot polymerization[69]

B- Classification of PRF:

The protocol for PRF preparation is very simple that requires only collecting blood specimens and a centrifuge device, however, the type of PRF results depends on the speed and time of centrifugation as following:

1- leukocyte-rich PRF (L-PRF):

The blood sample was collected without anticoagulant in glass-coated plastic tubes and immediately subject to centrifugation at 2,700 rpm

(around 400 g) for 12 min. The obtained PRF is usually termed Choukroun's PRF or leukocyte and PRF (L-PRF) [70]

2-Advanced PRF (A-PRF):

Advanced PRF was provided by using a reduced centrifugal force of 1,500 rpm (230 g) for 14 min and glass-based vacuum tubes [71], Also A-PRF may also be obtained by using the same time of centrifugation (14 min) but with a centrifugation speed of 1,300 rpm (200 g), as was suggested later[72].

3-Advanced PRF plus:

Obtained by By lowering the centrifugal speed to1,300 rpm (200 g) and centrifugal time to 8min[15]

4- **Injectable PRF (i-PRF):**

This injectable form of PRF is produced by using blood without anticoagulant and centrifuged at 700 rpm (60 g) for 3 min in plastic tubes without any coatings [73] The obtained PRF by decreasing centrifugation speed is richer in the total number of viable cells compared to the L-PRF. Among them, an increase in the number of neutrophils, lymphocytes, and platelets was observed, The presence of immune cells influences the differentiation and maturation of macrophages. This may lead to bone and soft tissue regeneration, mainly through the growth factors released from macrophages[14], since macrophages are responsible for osteoblast differentiation, and bone generation is absolutely limited without these cells [74]

C- Growth factors in PRF:

1-Transforming growth factor-β (TGF-β) :

Released from α-granules of platelets, Stimulates angiogenesis, Synthesis of collagen type I and fibronectin, Enhanced woven bone formation, Enhanced chemotaxis of osteoblast, and stimulates its

formation[75], inhibits osteoclast formation and bone degeneration so it has a very important role in the process of bone healing.

2- Platelet-derived growth factor (PDGF) :

Induces TGF-β secretion from macrophages. Provokes migration and proliferation of mesenchymal cells[76] and has an angiogenic effect on endothelial cells[77]

3- Insulin growth factor-1 (IGF-1):

Has Chemotactic effects on human osteoblasts, Increased expression of osteocalcin, Enhances wound healing, and Stimulates osteoblast proliferation [20]

4- Vascular endothelial growth factor (VEGF):

Initiates angiogenesis; enhances the permeability of the vessels; induces endothelial cell proliferation and migration[78]

D- Studies on PRF:

Yilmaz D et al[79]. Investigate the effect of platelet-rich fibrin (PRF) and beta-tricalcium phosphate (β-TCP), alone or in combination, on bone regeneration in pig tibial defects. Since four bony defects were prepared in both tibias of three adult male pigs. The first defect was left unfilled as a Blood clot; the others were filled with either PRF, β-TCP, or PRF mixed with β-TCP, Histologic and stereological examination revealed more new bone formation in the defects filled with PRF mixed β-TCP than in the defects filled with either β-TCP or PRF alone so the study concluded that The combination of platelet-rich fibrin and beta-tricalcium phosphate effectively induces new bone formation.

In another study [80] study aimed to compare the potential effect of L-PRF in a standardized model in rabbits sacrificed at three distinct time points: 1 week, 5 weeks, and 12 weeks followed by Histological and histomorphometric which revealed that at the early phase of bone regeneration (1 week), from a descriptive analysis, a higher proportion of

connective tissue colonized the regeneration chamber in the two groups containing bovine hydroxyapatite particles. Nevertheless, no statistical differences were found within the four groups in terms of bone quantity and quality at each time point so the study concluded that L-PRF does not seem to provide any additional effect on the kinetics, quality, and quantity of bone in this model of guided bone regeneration.

A study was done to histologically and radiographically evaluate the effect of platelet-rich fibrin matrix as bone filler for dental sockets after tooth extraction,Twenty-four rabbits were used for the extraction of upper central incisors under general anesthesia. The left side was filled with platelet-rich fibrin matrix material and the right side was left for normal healing as Blood clot group Results: The results were studied histologically after 1,2,3,4 weeks postoperatively. Histological examination showed rapid bone formation and accelerated healing process in the socket filled with PRFM than in the empty socket. Radiographical examinations showed that the ossification of the socket filled with PRFM begins after 2 weeks and is completely filled with radiopacity after 4 weeks. They concluded that PRFM material was osteoinductive material that enhances of osteogenesis process in the extraction tooth socket in comparison to the normal physiological healing process.

contrary to the previous study another study was done to assess the improvement of healing of extraction sockets after the use of platelet-rich fibrin, A total of 20 patients with bilateral soft tissue impacted mandibular third molars were included in this study. The left and right third molars were extracted during the same session. Subsequently, the PRF membrane was randomly administered to one of the extraction sockets, whereas the contralateral sockets were left without treatment as a Blood clot. On postoperative 1. and 3. months, panoramic images and

bone scintigrams were taken to evaluate the bone healing between PRF-treated and non-PRF-treated sockets. Results showed that The average increase in technetium-99m methylene diphosphonate uptake which is an indication of improved bone healing did not differ significantly between PRF-treated socket and Blood clot socket1. and 3. Months postoperatively, also Radioopacity that can show bone healing on panoramic images were measured by the Image J program and they did not differ significantly. so the study concluded that PRF might not lead to enhanced bone healing in impacted mandibular third molar extraction sockets at different periods.[81]

Clinically sometimes there is a need for a sinus elevation procedure during implant placement so Choukroun et al[(82)]. Evaluate the effect of PRF mixed with freeze-dried bone allograft (FDBA) to enhance bone regeneration in sinus floor elevation. Nine sinus floor augmentations were performed in 6 sites, PRF was added to FDBA particles (test group), and in 3 sites FDBA without PRF was used (Blood clot group)., bone specimens were harvested from the augmented region during the implant insertion procedure after Four months for the test group and 8 months for the Blood clot group. These specimens were treated for histologic analysis. Which revealed the presence of residual bone surrounded by newly formed bone and connective tissue. After 4 months of healing time, the histologic maturation of the test group appears to be similar to that of the Blood clot group after a period of 8 months. Moreover, the quantities of newly formed bone were equivalent between the 2 groups. They concluded that Sinus floor augmentation with FDBA combined with PRF leads to a reduction of healing time prior to implant placement.

Jain S. et al[(83)] managed a case with grade II furcation defect by platelet-rich fibrin (PRF) and β-Tricalcium phosphate with a coronally advanced flap And concluded that Platelet-rich fibrin and β-Tricalcium

phosphate with coronally advanced flap has been shown to be a promising and successful approach for the treatment of furcation defect simultaneously.

Another case report is About bone augmentation with a combination of Platelet-Rich Fibrin (PRF) and β-TCP for the treatment of chronic periapical cysts. The patient presented with a chronic periapical lesion in maxillary anterior teeth with a history of old trauma. Radiographically, a periapical cyst was seen in relation to maxillary left central and lateral incisors. Due to the failure of Conventional root canal treatment Apical surgery was performed. Bone augmentation was done using PRF in combination with β-TCP bone graft to achieve rapid healing of the periapical region. Regular follow-ups at 3, 6, 9, and 12 months were done. Results revealed progressive, significant, and predictable clinical and radiographic bone regeneration/healing without any clinical symptoms they concluded that Combined use of PRF and β-TCP for bone augmentation in the treatment of periapical defects is a potential treatment alternative for faster healing than using these biomaterials alone[84].

2.2.2.2. Hyaluronic acid . Hyaluronic acid (HA), also known as hyaluronate or hyaluronan, is an endogenous high molecular weight linear polysaccharide of a repeating disaccharide unit that has a number of embryologic and wound healing properties, including the facilitation of cell migration and differentiation during tissue formation and repair[85,86].HA is naturally occurring non-sulfated glycosaminoglycans with a high molecular weight of 4000-20,000,000 daltons. HA structure consists of polyanionic disaccharide units of glucuronic acid and N-acetyl glucosamine connected by alternating β1-3 and β1-4 bond[87].It is located in the extracellular space of all tissues with the same chemical structure in all species and is synthesized in the cellular plasma membrane [88,89].

Hyaluronic acid has an important role in cell–cell interaction, cell–matrix adhesion, cell motility, and the ordering of extracellular matrix. Free HA concentration is relatively low but is dramatically elevated immediately after tissue injury [(90)].

A- The molecular weight of HA:

1- Low-MW HA (<103 kDa) was mostly reported to increase cell proliferation, stimulate angiogenesis, and induce inflammatory effects[(91)].

2- High-MW HA917 (>103 kDa) with a debatable effect on cell proliferation, exhibits higher viscosity, longer residence time, and higher biocompatibility. High-MW HA also shows anti-inflammatory effects and inhibits angiogenesis and interleukin (IL)21b, IL-6, tumor necrosis factor-a, and prostaglandin E2 production[(92,93)].

B- Properties and physiochemical characteristics:

1-Hygroscopic nature: There are many hygroscopic molecules known in nature. HA is one of them, when incorporated into an aqueous solution, hydrogen bonding occurs between the adjacent carboxyl and N acetyl groups; this feature allows HA to maintain conformational stiffness and retain water. 1 g of HA can bind up to 6 L of water. it has functions in space-filling, lubrication, and shock absorption[(94)].

2-Viscoelastic properties: Hyaluronan has a role in the process of periodontal regeneration by maintaining spaces and protecting surfaces because it is a viscoelastic substance that may also slow the penetration of viruses and bacteria, an important feature in the treatment of periodontal

disease. [95]. anti-inflammatory Hyaluronan act as a scavenger by draining prostaglandins, metalloproteinases, and other bioactive molecules so has an anti-inflammatory effect [96].

3-Anti oedematous : Due to the osmotic activity of HA, it has an anti-oedematous effect. also, it accelerates tissue healing properties so it could be used as an adjunct to mechanical therapy[97].

4-Antioxidant: Scavenging reactive oxygen species by Hyaluronan help in regulating the inflammatory response. Thus, hyaluronan may help to stabilize the granulation tissue matrix[98].

5-Bacteriostatic effect : The high concentration of medium and lower molecular weight HA has the greatest bacteriostatic effect, particularly on Aggregatibacter actinomycetemcomitans, Prevotella oris, and Staphylococcus aureus strains, which are commonly found in oral gingival lesions and periodontal wounds. during the surgical therapy application of HA membranes, gels, and sponges may reduce the bacterial contamination of the surgical wound site, thereby, lessening the risk of postsurgical infection and promoting more predictable regeneration[99].

6-Carrier function :HA may act as a biomaterial scaffold for other materials, such as bone morphogenic protein-2 and platelet-derived growth factors used in guided bone regeneration techniques and tissue engineering research[100].

7-Osteoconductive potential: HA accelerates bone regeneration by means of chemotaxis, proliferation, and successive differentiation of mesenchymal cells. HA shares bone induction characteristics with osteogenic substances such as bone morphogenetic protein-2 and osteopontin[101]. These are completely biodegradable and support the growth of fibroblasts, chondrocytes, and mesenchymal stem cells.

C- Role Of Hyaluronan In Wound Healing Processes

Many of the biological processes mediated by hyaluronan are also central to the wound-healing process. Following injury, wound healing follows a series of tightly regulated, sequential events. These are inflammation, granulation tissue formation, reepithelization, and remodeling.

1-Inflammatory phase : Inflammation generates many of the factors required for the subsequent steps of wound healing. These include growth factors, cytokines, eicosanoids, etc. which promote the migration of inflammatory cells, fibroblasts, and endothelial cells into the wound site. The wound tissue in the early inflammatory phase of wound repair is rich in hyaluronan, probably a reflection of increased synthesis[102]. Hyaluronan has multiple roles in inflammation as Enhancement of cell infiltration[103] .Increase of proinflammatory cytokines TNF-a, IL-1b and IL-8 via a CD44- mediated mechanism[104] Facilitates primary adhesion of cytokine-activated lymphocytes to the endothelium[105]

2-Granulation phase: The granulation tissue matrix is rich in hyaluronan[106]The hyaluronan-rich matrix may contribute to a variety of cell functions that are essential for tissue repair. These include the facilitation of cell migration into the provisional wound matrix, cell proliferation, and organization of the granulation tissue matrix.

3-Hyaluronan and cell migration: Cell migration is essential for the formation of granulation tissue. In the former, hyaluronan provides an open, hydrated matrix that facilitates cell migration whereas in the latter, through specific cell interaction via cell surface hyaluronan receptors, directed migration and Blood clot of the cell locomotory mechanisms are mediated[107]

4-Hyaluronan and Cell proliferation: Cell proliferation is also an essential part of tissue repair. It has been shown that increased hyaluronan occurs and is essential for fibroblast detachment from the matrix and

mitosis[108]. Although hyaluronan has been shown to facilitate cell detachment has not been shown to have direct mitogenic activity. However, through facilitating cell mitosis in response to mitogenic factors, which are abundant during the early phases of tissue repair, hyaluronan may have an important, albeit indirect, role in cell proliferation too.

5-Hyaluronan and Angiogenesis Hyaluronan may also have a role in the Blood clot of angiogenesis. High molecular weight hyaluronan in the extracellular matrix has been shown to inhibit angiogenesis[109]. However, low molecular weight hyaluronan oligosaccharides have been shown to promote angiogenesis in several experimental models[110], and enhance the production of collagens by endothelial cells[111].

6-Reepithelization : Hyaluronan-rich matrix is associated with proliferating basal keratinocytes[112] and Facilitates keratinocyte migration via a CD44-mediated mechanism[113].

7-Remodeling: Hyaluronan-rich matrix may reduce collagen deposition, leading to reduced scarring as seen in fetal wound healing[114]

D- Studies on HA:

M. ASLAN ET AL[115]. Evaluate the effect of Hyaluronic Acid-supplemented Bone Graft in Bone Healing in an experimental study in rabbits In this study, two cavities of 3mm diameter and depth have been created in the right tibia of 30 mature rabbits. One of the cavities is filled with HA and bone graft and the other is filled with only spongiosal bone graft as a Blood clot group. On the 20th, 30th, and 40th days, rabbits have been sacrificed three different periods in equal numbers and defective areas have been subjected to histopathological examination they concluded that the cavities that have been filled with HA and bone graft have shown higher scores than the Blood clot group during every period of the study**.**

Mendes, R. M ET AL [116]evaluated the effects of sodium hyaluronate (HY) in the healing process of tooth sockets of rats: following the extraction of the upper first molars of male Holtzman rats, right sockets were treated with 1% HY gel (0.1 ml), while left sockets were lifted empty as Blood clot (blood clot). The rats were sacrificed at 2, 7, and 21 days after tooth extraction and upper maxillaries were processed for histological and morphometric analysis of the apical and medium thirds of the sockets. Carbopol, an inert gel, was used to evaluate the mechanical effect of gel injection into sockets. Expression of bone morphogenetic protein-2 (BMP-2) and osteopontin (OPN) was determined by immunohistochemistry at 1, 2, 3, 4, 5, and 7 days after tooth extraction. Histological analysis revealed that HY treatment induced earlier trabecular bone deposition at 7 and 21 days after tooth extraction. Additionally, the expression of BMP- 2 and OPN was enhanced in HY-treated sockets compared with Blood clot sockets. So they concluded that HY accelerates the healing process in tooth sockets of rats stimulating the expression of osteogenic proteins.

Ballini [117] evaluate the osteoinductive effect of the hyaluronic acid (HA) by using an esterified low-molecular HA preparation (EHA) as a coadjuvant in the grafting processes to produce bone-like tissue in the presence of employing autologous bone obtained from intra-oral sites, to treat infra-bone defects without covering membrane. 9 patients with periodontal defects were treated by EHA and autologous grafting with a mean depth of 8.3 mm of the infra-bone defects, as revealed by intra-operative probes. Data were obtained at baseline before treatment and after 10 days, and subsequently at 6,9, and 24 months after treatment. Clinical results showed a mean gain high clinical attachment (*gCAL)* of 2.6mm of the treated sites, confirmed by radiographic evaluation. Such results suggest that autologous bone combined with EHA seems to have

good capabilities in accelerating new bone formation in the infra-bone defects.

Nguyen [118] evaluated the effect of the Combination of Biphasic Calcium Phosphate Scaffold with Hyaluronic Acid-Gelatin Hydrogel as a new tool for Bone Regeneration. A New Zealand white rabbit (weight 3 kg) was used and 4 holes with a diameter of 5mm were drilled in the right-hand side of the sagittal border using a trephine drill, with continuous saline washing to prevent tissue dehydration. The HyA-Gel/ BCP scaffolds were placed over the drilled parietal bone after sterilization and sterile saline washing. The subcutaneous tissue was closed, and the overlying skin was resutured. he rabbits were sacrificed 1 and 3 months after implantation, and the entire portion of the defected femur was sectioned for Histological analysis which confirmed a rapid new bone formation and a high rate of collagen mineralization and In vivo expression of extracellular matrix proteins demonstrated that this novel bone substitute holds great promise for use in stimulating new bone regeneration.

Sadikoglu, T. B [119]evaluated the effects of different molecular weight hyaluronic acid on bone formation in rats after expansion of the inter pre maxillary suture since Twenty-four male Sprague Dawley rats were divided into three groups. Each group was subjected to expansion for 5 days and retention for 10 days. Group 1 received 50 ml of high molecular weight hyaluronic acid (HMWHA), group 2 received 50 ml of low molecular weight hyaluronic acid (LMWHA), and the Blood clot group received the same amount of saline solution to the inter-premaxillary suture. Ten days after injection, the rats were killed and their maxillas dissected. For the histomorphometric evaluation, Results showed that – HMWHA showed a statistically higher ratio of osteoblast and capillary cell scores compared with the LMWHA and Blood clot groups so they concluded that Local injection of HMWHA in the inter-premaxillary

suture after rapid maxillary expansion stimulated new bone formation, which may shorten the retention period and may reduce the risk of relapse.

Sandhu GK [120] assessed the regenerative capacity of Gengigel® in conjunction with bioactive PRF in a patient with grade II furcation defect in the mandibular left first molar area. After administration of local anesthesia, buccal and lingual crevicular incisions were made, and the mucoperiosteal flap was reflected to access the underlying bone morphology in the furcation area After debridement with Gracey curettes the furcation area was assessed using Q2N Naber's probe to assess the defect dimensions horizontally and vertically, the debrided defect was slightly overfilled with Gengigel®. Autologous PRF of the required size was filled into the furcation defect and the remaining part was used as a GTR membrane by squeezing out the fluids to cover the furcation followed by repositioning and suturing of the flap. Six months postoperative furcation defect assessment through surgical re-entry using Q2N Naber's probe showing a significant percentage of bone formation

Gupta, S [121]evaluated the role of Gengigel® (0.8% hyaluronic acid) as a potential material for the regeneration of lost attachment apparatus. A total of 20 sites with Grade II furcation defects from 10 patients were selected using a random sampling technique. These were divided into Group A (placement of hyaluronic acid) and Group B (without placement of hyaluronic acid) according to treatment modality. Furcation defect assessment was done in vertical and horizontal depth preoperatively and postoperatively at six months through surgical re-entry. The study concluded that placement of Gengigel® with coronally positioned flap and coronally positioned flap without placement of Gengigel are effective in the treatment of Grade II furcation defects but The combination of

Gengigel® with coronally positioned flap leads to better results in hard tissue measurement as compared to coronally positioned flap alone.

2.3 Diabetes Mellitus

Diabetes mellitus (DM) is a group of complex multisystem metabolic disorders due to a deficiency in insulin secretion caused by pancreatic β-cell dysfunction and/or insulin resistance in the liver and muscle. Diabetes affects more than 9% of the adult population and has a dramatic impact on the healthcare system through high morbidity and mortality among affected individuals[(122)]

2.3.1 Types of Diabetes Mellitus:

Type-1 diabetes :

is also called insulin-dependent diabetes mellitus because this disease is characterized by an absolute deficiency of insulin. Beta cells are destructed due to invasion by viruses, the action of chemical toxins, or due to action of autoimmune antibodies. This beta cell necrosis is caused insulin deficiency and caused Type-1 diabetes [(123)].

Type-2 diabetes :

Non-insulin-dependent diabetes mellitus or Type-2 diabetes is frequently accompanied by target organ insulin resistance that limits responsiveness to both endogenous and exogenous insulin [(124)].

Type-3 diabetes:

This type of diabetes is caused by chronic pancreatitis or chronic drug therapy with glucocorticoids, thiazide diuretics, diazoxide, growth hormone, and with some protease inhibitors (e.g. saquinavir).

Type-4 diabetes: This type of diabetes is observed in approximately 4-5% of all pregnancies, due to placental hormones that promote insulin resistance[125].

2.3.2 Diagnosis of diabetes mellitus:

Diagnostic tests of diabetes mellitus aim mainly to evaluate either insulin secretion or insulin insensitivity:

1- Insulin secretion:

- An estimate of stimulated insulin secretion:

In which beta cells stimulated to secret insulin through the administration of intraoral or intravenous glucose

- C-peptide:

Used as a marker of insulin secretion through the measurement of C-peptide concentration 6 minutes after intravenous injection of a bolus of glucagon.

2- Insulin insensitivity:

-Hyperinsulinaemic euglycaemic clamp

It is a test in which the patient is exposed to a predetermined amount of insulin while the plasma glucose is maintained within the euglycaemic range by infusion of glucose. The amounts of insulin and glucose administered are used to calculate the insulin sensitivity.

-Frequently specimens intravenous glucose tolerance test:

It is a test in which glucose is administered to patients and specimens for glucose and insulin are analyzed at numerous time points. It is very important to understand the normal mechanism by which blood glucose is regulated and the role of insulin in the process of glucose regulation[126].

2.3.3 Induction of diabetes for research:

The five major diabetogenic agents are chemicals, biological agents, peptides, potentiators, and steroids but the most commonly used

chemicals agents are alloxan and streptozotocin [127] , Alloxan is one of the most prominent chemical compounds used in diabetes induction for research. Alloxan is a urea derivative that induces selective necrosis of the β- cells of pancreatic islets [128].so it is used for induction of Type 1. The chemical name of alloxan is 2,4,5,6 tetraoxypyrimidine; 2, 4, 5, 6-pyrimidinetetrone, which is an oxygenated pyrimidine derivative that is present as alloxan hydrates in aqueous solution. Brugnatelli originally isolated alloxan in 1818 and the name was given by Wohler and Liebig in 1838[129]. Alloxan was prepared by the oxidation of uric acid by nitric acid and the monohydrate form is simultaneously prepared by the oxidation of barbituric acid by chromium trioxide. The diabetogenic action of alloxan has been noted when administered parenterally, i.e., intravenously, intraperitoneally, or subcutaneously. The dose of alloxan required for inducing diabetes depends on the animal species and route of administration [130]. Moreover, alloxan has been demonstrated to be safe for the human beta-cells, even invery high doses, because humans have different mechanisms for glucose uptake as compared to rodents [131]

2.3.4 Effect of Diabetes on Wound Healing

Impaired healing in diabetes is the result of a complex pathophysiology involving vascular, neuropathic, immune, and biochemical components [132]. Hyperglycemia correlates with stiffer blood vessels which cause slower circulation and microvascular dysfunction, causing reduced tissue oxygenation [133]. Blood vessel alterations observed in diabetic patients also account for reduced leukocyte migration into the wound, which becomes more vulnerable to infections [25].

The hyperglycemic environment itself can compromise leucocyte function. The healing process in diabetes is mainly characterized by chronicization of the inflammatory conditions, disrupted angiogenic

process, reduction of endothelial progenitor cells, and an imbalance in extracellular matrix regulation.

In diabetes, the production of several growth factors involved in initiating and sustaining the healing process is compromised. For instance, reduced levels of insulin-like growth factor-1 (IGF-1) and transforming growth factor-_ (TGF_) have been reported in wound tissue in both diabetic animals and humans. IGF-1 is implied in cell granulation and wound re-epithelization[134] . while TGF_ recruits immune cells, keratinocytes, fibroblast, and vascular cells and is involved in angiogenesis and formation of the ECM [135]. However, in diabetes, the balance between the promotion of new vessel formation and maturation is perturbed.

Angiogenesis is dysfunctional in endothelial cells exposed to high glucose levels and in the wound area, capillary density is insufficient. Hyperglycemia affects hypoxia-inducible factor HIF-1_ stability and activation and consequently, it suppresses HIF-1_ target genes like VEGF [136] Furthermore, in diabetes animal models, macrophages, which are the main source of VEGF, exhibit impaired phagocytic activity and altered phenotype, resulting in failure of tissue repair[137].

Additionally, the maturation phase of wound healing appears impaired in diabetes. The production of factors leading to vascular mature phenotype (including angiopoietin (ANG) 1 and 2, PDGF) is compromised [138].and topical application of ANG1 and PDGF increased wound healing in a mouse model of diabetes induced by streptozotocin or in db/db mice, respectively[139]. Finally, an impairment in the regulation of ECM, whose build-up is modulated by metalloproteinase (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) is observed in diabetes. Higher MMPs levels have been reported in diabetic wounds, due to high

glucose that may directly induce the production of MMPs and the reduction of TIMPs, thus contributing to disruption of the healing process [140].

MMPs are involved in various stages of wound healing like cell migration through the degraded ECM, leukocyte invasion, processing of multiple cytokines, and growth factors involved in the healing process The balance between MMPs and TIMPs is essential to avoid the disruption of the scaffolding structures necessary for a proper wound healing [141].

2.3.5 Diabetes and Its Effect on Bone Healing

In diabetes mellitus, Pro-inflammatory mediators including TNF-α, IL-1β, IL-6, and IL-18 are increased locally which is thought to contribute to diabetic complications [142,143]. Diabetics have difficulty in down-regulating inflammation once induced [144,145]. Increased levels of TNF may decrease the ability of diabetics to down regulate other inflammatory genes and increase apoptosis, which has been shown to reduce bone coupling in diabetic animals[146]

Un controlled diabetes increases and prolongs inflammation, which may lead to enhanced osteoclastogenesis. Diabetes increases osteoclast formation in a number of conditions including periodontal disease, fracture healing and osteoporosis[147]. Diabetes decreases osteoblast formation and function and reduces the number of osteoblasts.

Bone formation is reduced in diabetics as reflected by reduced levels of osteocalcin in type 2 diabetic patients compared to non-diabetic Blood clots, reflecting a decrease in osteoblast activity, which is inversely related to IL-6 and C reactive protein (CRP)[148]. Elevated levels of glucose enhance protein glycation (nonenzymatic glycosylation), with the formation of advanced glycation end-products (AGEs)which are non-enzymatic chemical modifications of proteins by aldose sugars, formed

by the oxidation of products generated during the Maillard reaction. The accumulation of AGEs has been associated with diabetic complications as well as degenerative diseases that occur with aging. AGEs bind to a number of receptors including the receptor for AGEs (RAGE) and stimulate inflammatory cytokines [149]. AGEs and hyperglycemia are linked to increased osteoclast formation[150,151]and RAGE is expressed in osteoclasts and stimulates osteoclastogenesis[152].

Hyperglycemia may affect bone through enhanced expression of proinflammatory cytokines such as TNFα, which reduces osteoblast differentiation, and osteoblast activity and increases osteoblast apoptosis [153].In addition, AGEs inhibit differentiation of osteoblasts as reflected by reduced expression of alkaline phosphate and collagen 1α1 and inhibited the formation of a mineralized matrix [138] Moreover, there is evidence that AGEs induce osteoblast apoptosis to reduce osteoblast numbers and impair bone formation[154]. High levels of glucose stimulate the generation of reactive oxygen species which in turn can increase osteoclast formation and activity[155].

Insulin binds to receptors on osteoblasts and stimulates anabolic effects [156]. It is possible that the reduced insulin levels or reduced insulin signaling in osteoblasts negatively affects bone and contributes to reduced bone formation caused by diabetes[157]

2.4 Bone density

Bone-mineral density (BMD) is a measure of the inorganic mineral content in bone and is one of the more informative assessments of bone quality in both clinical studies and forensic investigations. Several factors, such as age, sex, disease, genetics, and lifestyle, affect BMD measurements, there are several technological modalities for BMD quantification including Single- and dual-energy photon absorptiometry, quantitative ultrasound computed tomography, cone beam computed

tomography,dual-energy X-ray absorptiometry (DXA), and magnetic resonance imaging, and each presents distinct advantages and limitations, depending on the purpose of the analysis, the specific characteristics of the individual, the bone site under examination, and the equipment and trained personnel available. [158]

2.4.1 Magnetic resonance imaging (MRI):

With this procedure, several parameters of bone density and microarchitecture (bone volume/total volume, vBMD, trabecular number, trabecular thickness, and trabecular separation) are quantified. The main advantage is the lack of exposure to ionizing radiation. Although notable advances in this type of imaging have occurred in the last few decades and fully automated protocols for structural analysis have been developed, the difficulties in standardizing image quality and quantification, combined with the high cost of the technique[159].

2.4.2 Cone Beam Computed Tomography(CBCT) Is a simple device used for the acquisition of tridimensional images of oral and maxillofacial regions with a low cost and reduced radiation emission also it has been proposed as a diagnostic method for the determination of bone mineral density and was developed by Mozzo et al[160]. Bone mineral density with CBCT is described in Hounsfield units (HU) which represents the relative density of a body tissue according to a calibrated gray-level scale based on HU values of the air (-1000 HU), water (0 HU), and dense bone (+1000 HU)[161]. HU values are directly related to the mass absorption coefficient of different tissues([162]) and, these values may be used for the determination of the density of tissues with a high degree of accuracy([163]) and sensitivity, detecting density differences of 1% or less with some exceptions.

The results of CBCT showing bone mineral density may be associated with some errors due to:

1- Phenomenon of X-ray beam hardening:

This results from the inclusion of the X-ray beam on broad-spectrum photons with different energies that affect their behavior when reach an object. This phenomenon will generate different readings of the attenuation coefficient of this point and may produce dark streaks in the images obtained.

2- An underestimation of the attenuation coefficient:

Darker gray values may occur as a consequence of scattered radiation which is added to the primary radiation of the x-ray beam overestimating the intensity measured by the system and underestimating the attenuation coefficient of the object which will affect the obtained values of density.

3- Projection data discontinuity artifact:

This artifact occurs when the field of view is smaller than the scanned object, the X-ray beam strikes the areas outside the field and creates bright bands near the field

4- Variation in the devices and image-acquisition settings:

This may influence the images obtained because alterations of these variables are associated with low reproducibility of gray values.

2.4.3 Dual-energy X-ray absorptiometry (DXA):

Commercial DXA systems first became available in the late 1980s[(164)].DXA is an X-ray imaging technique primarily used to derive the mass of one material in the presence of another through knowledge of their unique X-ray attenuation at different energies. Two images are made from the attenuation of low and high average X-ray energy. DXA is a special imaging modality that is not typically available with general-use X-ray systems because of the need for special beam filtering and near-perfect spatial registration of the two attenuations.

2.4.3.1 MEASUREMENTS FROM DXA SCANS

Bone mineral content (BMC). BMC is the mineral mass component of bone in the form of hydroxyapatite, Ca10(PO4)6(OH)2. BMC is typically measured in grams.

Bone area (BA). BA is the projected area of the bone onto the image plane, typically in cm2.

Areal bone mineral density (BMD). aBMD is the mineral mass of bone per unit image area in g/cm2.

BMC divided by the area or volume of the bone estimates BMD

$$aBMD = BMC/BA \ (g/cm2)$$

DXA measures areal BMD (aBMD) in g/cm2 by using ionizing radiation with photon beams of two different energy levels. The differences in attenuation of the beams passing through body tissues of variable composition allow the instrument to provide a quantitative measurement of bone density. Bone is composed of minerals, principally calcium hydroxyapatite, embedded in type I collagen and specialized proteins that make up the bone matrix. Bone mineral absorbs much more radiation than soft tissue. The amount of X-ray energy that is absorbed by bone mineral calcium in a section determines the measured bone mineral content (BMC). [165]

2.4.3.2 Advantages of DXA:

1-DXA can calculate body mass and distinguishes between lean and fat mass, due to differences in tissue-attenuation properties. This allows monitoring of the patient's general health conditions that could affect BMD loss[166].

2-Biomechanical studies have shown a strong correlation between mechanical strength and BMD measured by DXA[167].

3- Excellent accuracy and precision of DXA[168].

4- Very low Radiation exposure with DXA[169]

Aim of the study

The present study was directed to evaluate the effect of platelet rich fibrin and hyaluronic acid on bone regeneration after peri radicular surgery in healthy and diabetic dogs in comparison with blood clot.

The null hypothesis stated that there will be no significant difference among the tested groups.

Materials & Methods

Section outline:

4. MATERIALS AND METHODS

4.1. Ethical consideration:

The study was done according to the Animal Research Reporting of In Vivo Experiments guidelines (ARRIVE) and approved by the institutional Ethics committee of the Faculty of Dental Medicine, Al-Azhar University, Cairo (boys) on 15-4-2019 with EC Ref No:115/ ١٣٧/ -٠٤-٢٠١٩.

4.2. Animal model:

Based on the power analysis of the study 16 (Out of 17) Adult healthy male purpose-bred mongrel dogs aged 1 year to 2 years old were selected to be used in this study. The weight of the dogs ranged from 15 to 20 Kg and had their vaccines throughout the course of the study. All dogs were bred and housed under similar conditions since all dogs were placed separately in cages that protected them from the elements (heat, sun, rain, cold, and other bad weather) **(Fig 1)**. Cage dimensions were large enough for the dog to turn around but not so large that they can't be warmed by body temperature, the cages were provided with a comfortable, solid floor with a smooth, non-slip finish composed of sand for resting and sleeping. Proper nutrition was an important aspect to provide complete, balanced nutrition for dogs where the veterinarian helped to estimate the dog's daily caloric needs. Dogs were fed raw bones regularly as a part of a balanced diet and for good dental health. Meals were provided in a separate food bowl for each dog and maintained in a clean condition.

Dogs always had access to clean drinking water at all times. Water containers were checked daily and maintained in a clean condition(Fig1).

Fig (1): A photograph showing dogs' housing

4. 3. Preoperative assessment:

In cooperation with the veterinary team of the general veterinary hospital in Al Abbasya, Cairo, Egypt. for proper standardization of the animal model clinical examination of all included doges was done under sedation with a subcutaneous injection of 0.04 mg/Kg atropine immediately before surgery (atropine sulfate; ADWIA Co., Cairo, Egypt), followed by intramuscular injection of 1mg/Kg Xylaject (xylazine hydrochloride; ADWIA Co., Cairo, Egypt.) and intramuscular injection of ketamine HCL 15mg/Kg body weight (EIMC. Pharmaceuticals co, Egypt).in which dogs with intraoral soft tissue laceration, fractured teeth, and teeth with periodontal diseases were excluded. A radiographic examination was done and dogs that had teeth with open apex, pulp stones, and root resorption were excluded.

4.4. **Grouping of the selected dogs**

Randomization of the dogs was done in which each dog was given a number from 1 to 16 then using arandomizer software (www.randomizer.org) the selected dogs were assigned blindly and

randomly into 2 main groups according to their medical status into two main groups:

Group A: Healthy dogs

Group B: Diabetic dogs

Diabetes mellitus was induced in 8 of these 16 dogs while the other 8 dogs were kept in healthy condition.

4.5. Induction of diabetes in the diabetic group:

Diabetes mellitus was induced in 8 of the selected dogs by intraperitoneal injection of a solution of alloxan (Research-Lab Fine Chem, Industries, Mumbai, India) 50 mg/kg body weight, given on three consecutive days according to the protocol[(170)]. The preparation was used immediately after dissolution in 0.1 M acetate buffer at pH 4.4. Diabetes mellitus was confirmed by measuring blood glucose level using a blood glucose meter and the dogs were considered diabetic when blood glucose level was above 220 mg/ml as the normal blood glucose level is about 88-120mg/dL.

4. 6. **Preparation of the dogs**

For good hygiene and reduced chances of infection, the dogs were bathed the day before surgery and all dogs were fasted 12 hours before anesthesia and were premedicated with a subcutaneous injection of 0.04 mg/Kg atropine immediately before surgery (atropine sulfate; ADWIA Co., Cairo, Egypt), followed by intramuscular injection of 1mg/Kg Xylaject (xylazine hydrochloride; ADWIA Co., Cairo, Egypt.)

4. 7. Animal anesthetization:

The dogs were placed on the operating table in a supine position with tilted heads to provide an unblocked air-way, after that A cannula, 18-20 gauges, was fixed in the radial vein, and general anesthesia of the dogs was induced by intravenous injection of Ketamine HCl 5mg/1kg body weight (EIMC. Pharmaceuticals co., Egypt). anesthesia was maintained

by 25mg/Kg intravenous incremental doses of 2.5% solution of thiopental sodium (EIPICO, Cairo, Egypt). One-third of the estimated dose was injected within 15 seconds, the remainder was administered slowly until loss of pedal and corneal reflexes, and development of shallow regular respiration.

4. 8. PRF preparation:

According to Choukroun's protocol [171] 10 mm of the dog's blood was collected without anticoagulant in glass-coated plastic tubes and immediately subjected to centrifugation at 2,700 rpm (around 400 g) for 12 min. The resultant product separated into 3 distinct zones**(Fig 2)**

- Top-most layer consisting of platelet-poor plasma (PPP).
- PRF in the middle.
- RBC at the bottom.

The tube was maintained until the bony cavities were prepared

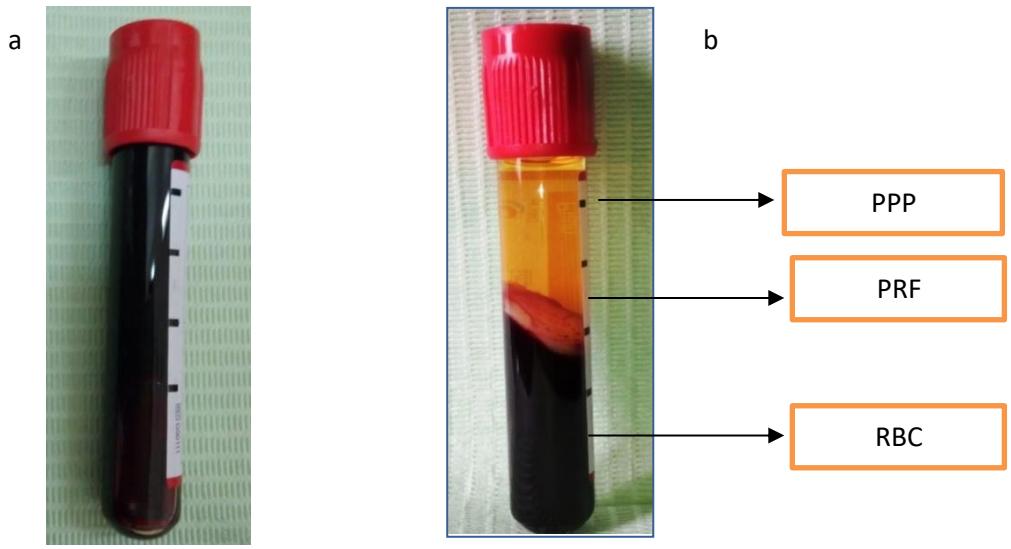
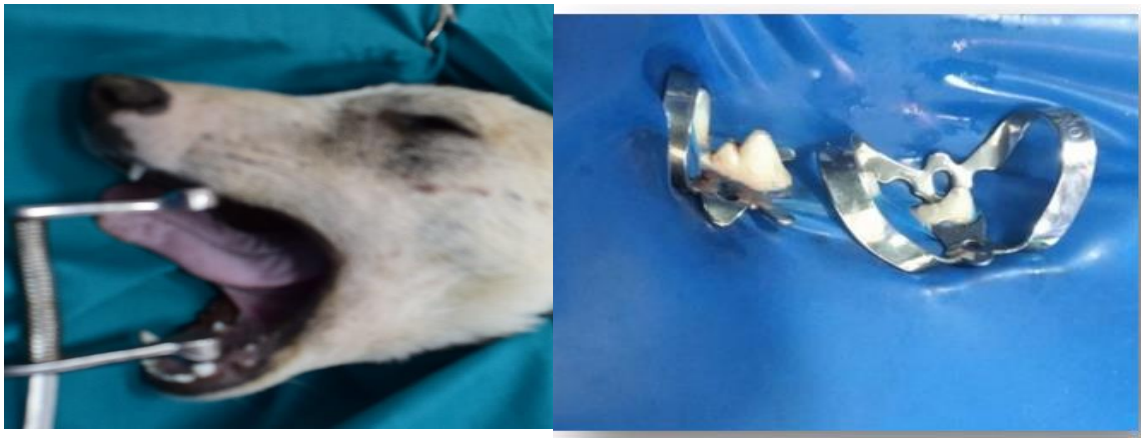


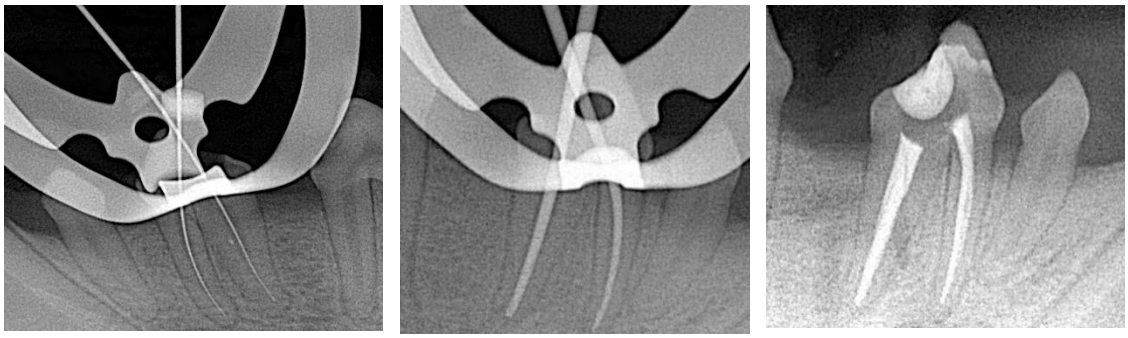
Fig (2) Photographs showing collected dog blood samples before (a) and after centrifugation (b).

4. 9. Preparation of Operative Field and Endodontic procedure:

All procedures were conducted under a clean aseptic protocol with the use of sterile materials and equipment in which the dog's mouth, head, and neck were swapped with betadine solution (Nile Company for Pharmaceuticals and Chemical Industries, Cairo, Egypt) followed by wrapping the head and neck by sterile napkins. After the rubber dam application, Root canal treatment for the second and fourth mandibular premolar teeth of the dogs was done on the right side and the fourth mandibular premolar on the left side **(Fig 3)**. Access cavity preparation using a # 2 round bur mounted on a high-speed handpiece with coolant (Foshan wenjian medical instrument co, china). The working length was measured using an apex locator and confirmed radiographically (Fig) followed by mechanical preparation of the root canals using a pro taper rotary system until size F3. Irrigation after each file was done with 5 ml of 5.25% NaOCl(Clorox, Alexandria detergent of a chemicals company, Alexandria, Egypt.) using a side vented irrigating needle mounted on a 5 ml plastic syringe. The canals were dried by paper point size 30.02, The master cone gutta-percha size 30 taper 0.04 was placed in each canal and verified radio graphically followed by obturation of root canals using cold lateral compaction technique. The access cavity was filled with resin-modified glass ionomer filling material (Dentsply Maillefer, Ballaigues, Switzerland).



a **b**



c **d** **e**

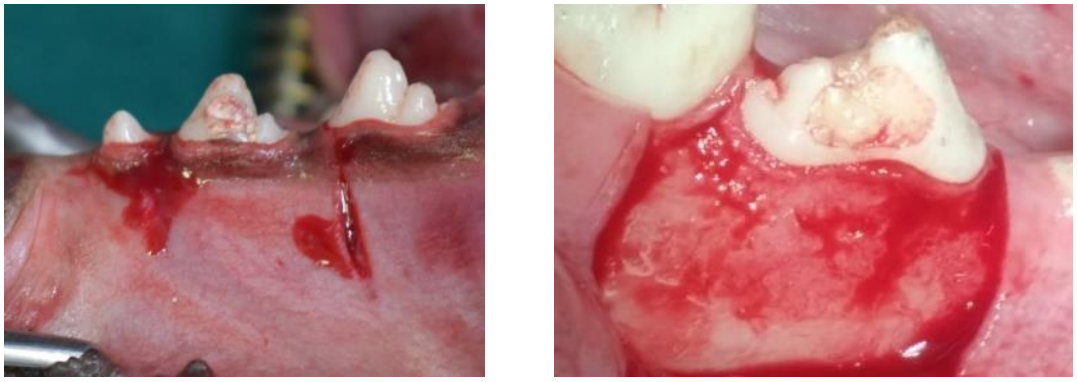
Fig 3: Photographs showing the preparation of the field and endodontic treaatment of the teeth showing. A) Mouth gauge in place b) Rubber dam isolation, c) working length radiograph d) master cone radiograph e) obturation radiograph.

4. 10. Surgical Procedure:

4. 10. 1. Flap Design and Elevation

Before the beginning of the surgical procedure local infiltration of 1.8ml 2% lidocaine with nor- epinephrine (1:100,000) was administrated for hemostasis and hydrodisection.On the right side, two separate rectangular flaps were performed opposite to the second and fourth premolars while the distance between the two flaps was maintained not less than 1 cm, and one flap on the left side was opposite to the fourth premolar **(Fig 4)**. For the second premolar, vertical releasing incisions were placed mesial to the inter-dental papilla between the first and second premolar on the mesial side, and distal to the interdental papilla between

the second and third premolars on the distal side. For the fourth premolar, vertical releasing incisions were placed mesial to the interdental papilla between the third and fourth premolars on the mesial side and distal to the interdental papilla between the fourth premolar and 1st molar tooth.



a **b**

Fig 4: Photographs showing rectangular mucoperiosteal flap a) position of vertical incisions b) flap refletion.

4. 10.2. Creation of Bone Defect.

After flap elevation, A metallic template measuring 7×7 mm **(Fig 5)** was fixed using a mini screw of 4mm into a predetermined place opposite to the apical 1/3 of the distal roots of the second and fourth premolars on the right side and distal roots of the fourth premolars in the left side. The position of the metallic template was confirmed radiographically, one hole in each corner was created by surgical bur mounted in high-speed contra in the presence of sufficient coolant followed by the connection of all holes together using the surgical bur, the depth of the hole was adjusted by fixing a metallic stopper welded at 8 mm from the tip of the bur(Fig), so finally a bone defect measuring of 7×7×7 mm was created and resulted in exposing the apical part of the distal root of the tooth, the apical 3mm of the root was resected by fissure surgical bur with no bevel. Retrograde cavity preparation using an ultrasonic tip was done followed by retrograde filling of the root with MTA material.

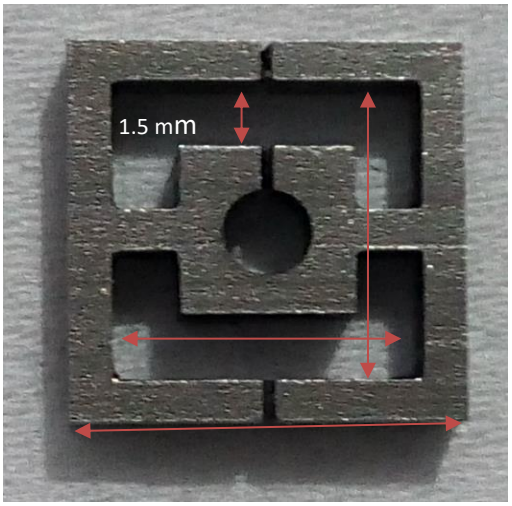
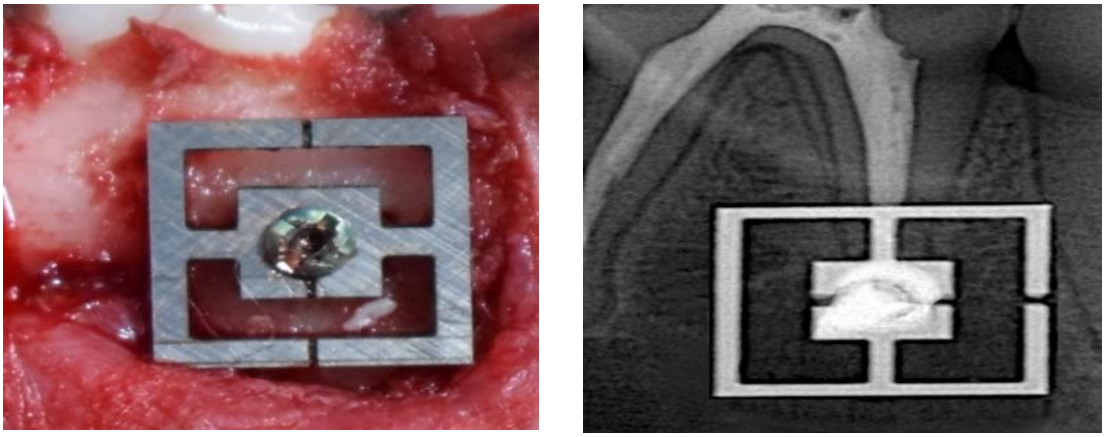
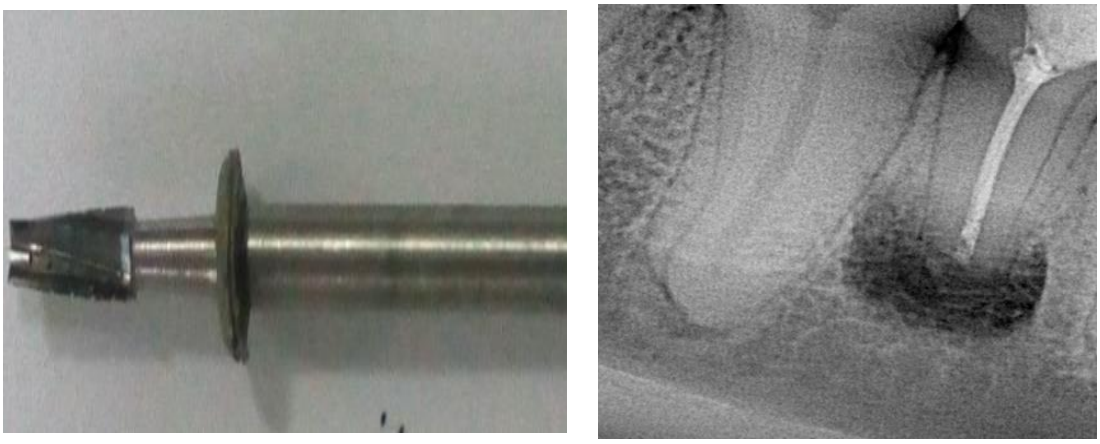


Fig 5: A photograph showing a metallic template for bony cavity creation with its dimensions.



a **b**



c **d**

Fig 6: Photographs showing bone cavity creation a) template in place b)radiograph confirm template position c) modified surgical bur d) postoperative radiograph after MTA filling.

4. 10.3. Sub-Grouping of the Selected Dogs (Fig 9):

After grouping the selected dogs into 2 main groups (group A: healthy dogs and group B: diabetic dogs) further subgrouping of each main group was done according to the bony cavity-filling materials into 3subgroups:

Group 1: High-Molecular-weight hyaluronic acid **(** GENGIGEL PROF**.** RICERFARMA S.R.L.MILANO-ITALY) on the bone cavity related to the second premolar on the right side

Group2: PRF on the bone cavity related to the fourth premolar on the right side

Group 3: Blood clot on the bone cavity related to the fourth premolar on the left side

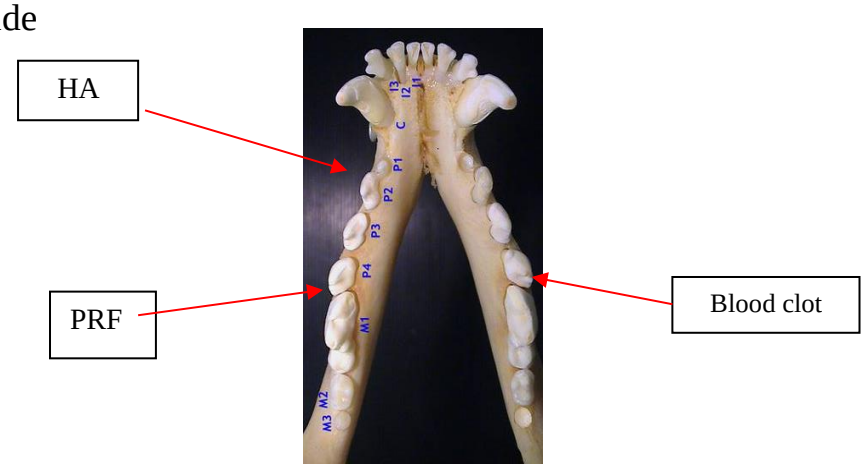
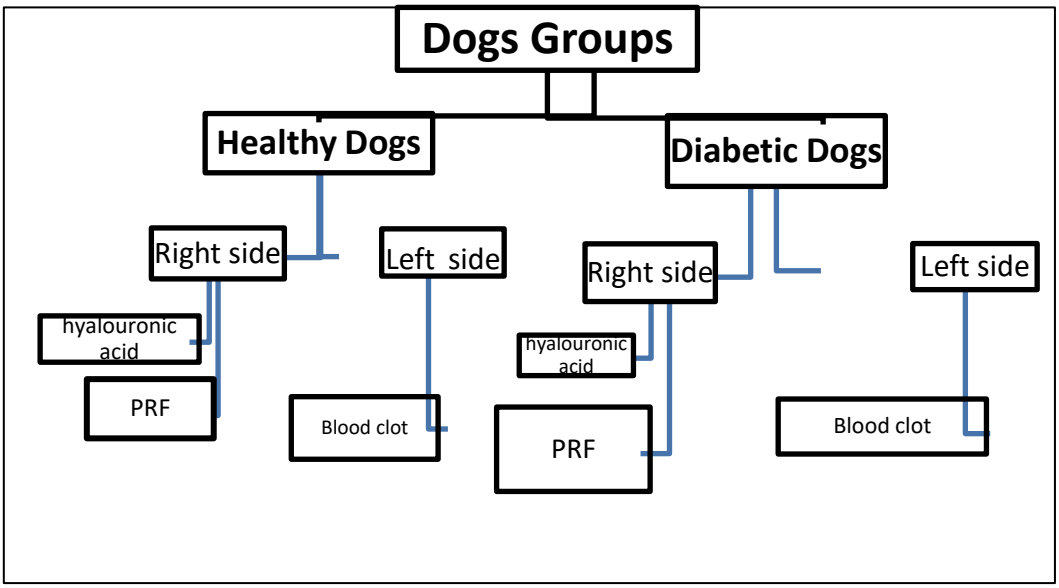


Fig (7): A photograph showing a live model of the mandible of a dog illustrating the materials used for each tooth.



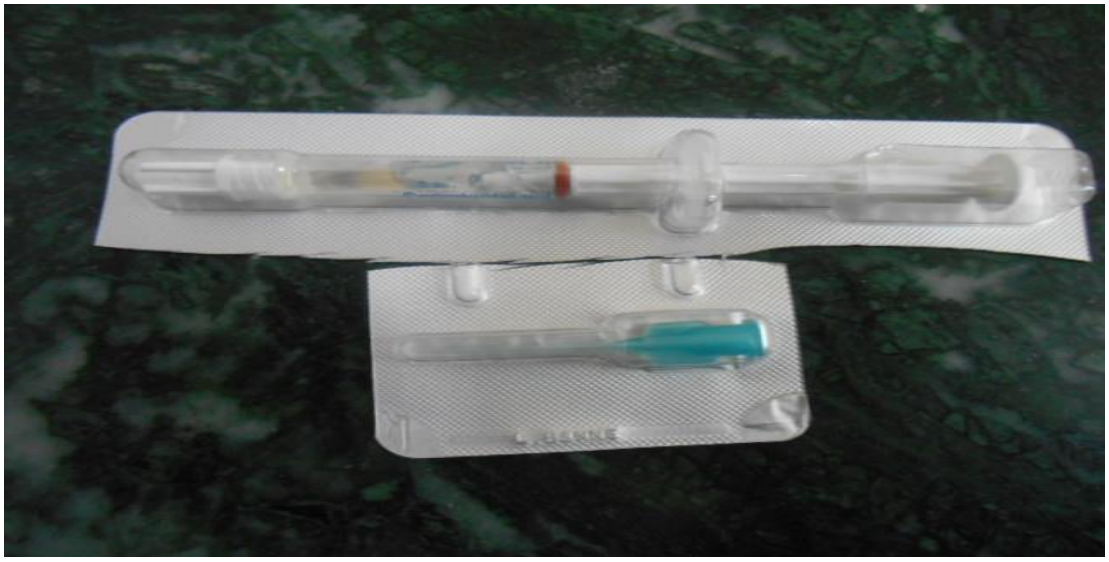
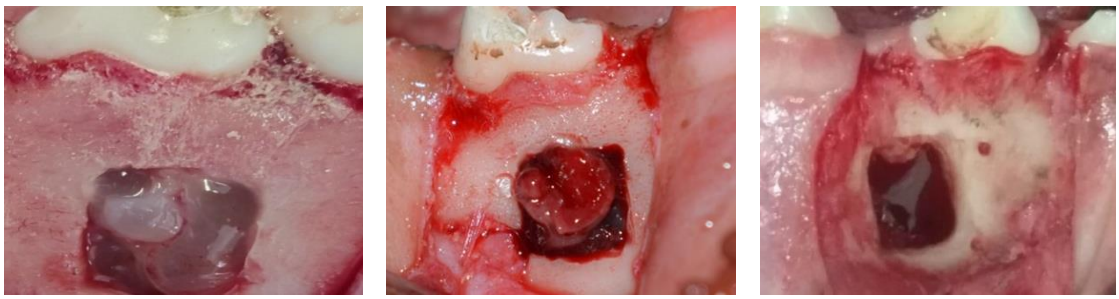


Fig 8: Photographs showing HA kit including pre filled syringe with their tips



a **b** **c**

Fig 9: Photographs showing the tested materials placed in the bone defect after periradicular cavity creation a) hyaluronic acid b) PRF C) Blood clot

4. 10.4. Flap repositioning, compression, and suturing (Fig 9)

Following the procedure of filling the bony defect, the flap was repositioned and sutured with 3-0 resorbable suture vicryl (polyglactin 910, Ethicon, Inc 2018) mounted on 19mm 3/8c reverse cutting needle was used to reposition the flap .

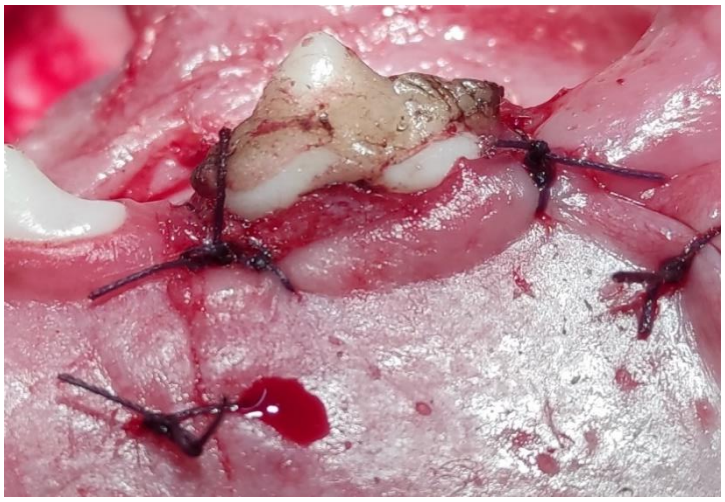


Fig 10: Photographs showing Flap repositioning and suturing

4. 11. Postoperative Care

Non-steroidal, anti-inflammatory medications and antibiotics were recommended to decrease inflammation and pain so the dogs received amoxicillin and flucloxacillin (flummox: E.I.P.I.co, 10th of Ramadan city-industrial area, Egypt) as antibiotics at a dose of 50 mg/ kg per lean body weight per day and Zyleject, 3ml intramuscularly every 12 hours, for 5 days to control pain and infection under supervision of the vet physician. For the first postoperative week, dogs were kept on a soft diet composed of milk, rice, meat, liver, and bread. On the second postoperative week, dogs were able to eat the usual diet. All the dogs were evaluated clinically for assessments of their general health until sacrification. Also, a daily examination was carried out for the presence of signs of infection such as redness, hotness, and the ability of the mouth to open, eat, and swallowing was conducted.

4.12. **Sacrification, Specimens Harvesting.**

Euthanasia was scheduled at the timetable of ١, ٥, ٩, and ١٣ weeks after surgery in which four dogs (two healthy and two diabetics) were sacrificed at each time. Euthanasia was done under general anesthesia provided by intravenous injection of pentobarbital (Socumb, Butler Company, Columbus, Ohio) at 30 mg/kg. The carotid arteries were exposed and cannulated then the dogs were euthanized with additional pentobarbital at a dose of 90 mg/kg. The dogs were perfused with 10% buffered formalin (Fisher Scientific, Fair Lawn, New Jersey). The mandible was surgically removed and divided at the midline into two halves right and left then maintained in 10% buffered formalin until the time of bone density measurement.

4.13 Densitometric Analysis (DEXA) (Fig 10) .

The two mandibular halves of each dog were referred freshly for bone density examination at the bone defect site, by using dual-energy X-ray absorptiometry (DEXA) after sacrification at each examination period. DEXA measures bone mineral content (BMC) which represents minerals mass in each region of interest (ROI) in grams. Bone mineral density (BMD) represents the value of BMD in each ROI. The mandibular half was placed on the apparatus stage and the (ROI) was marked by a red light mark that emanate from the apparatus then the scanning process was started. The technique measures bone density as area density in units of gm/cm2. These measures were done using a three-site axial scanner, bone mineral analyzer (lunar prodigy primo), and its software using small dogs mode at the Radiology Center of Rabaa Hospital, Naser City, Cairo, Egypt. This instrument was calibrated with a phantom of known mineral content. On the monitor, partial acquisition of images was shown while the scanner precedes the analysis mode. When the scan finished the

densitometric images were saved then the effect of time, materials, and health status of the dogs on both BMC and BMD were measured. All data were collected, tabulated, and statistically analyzed.

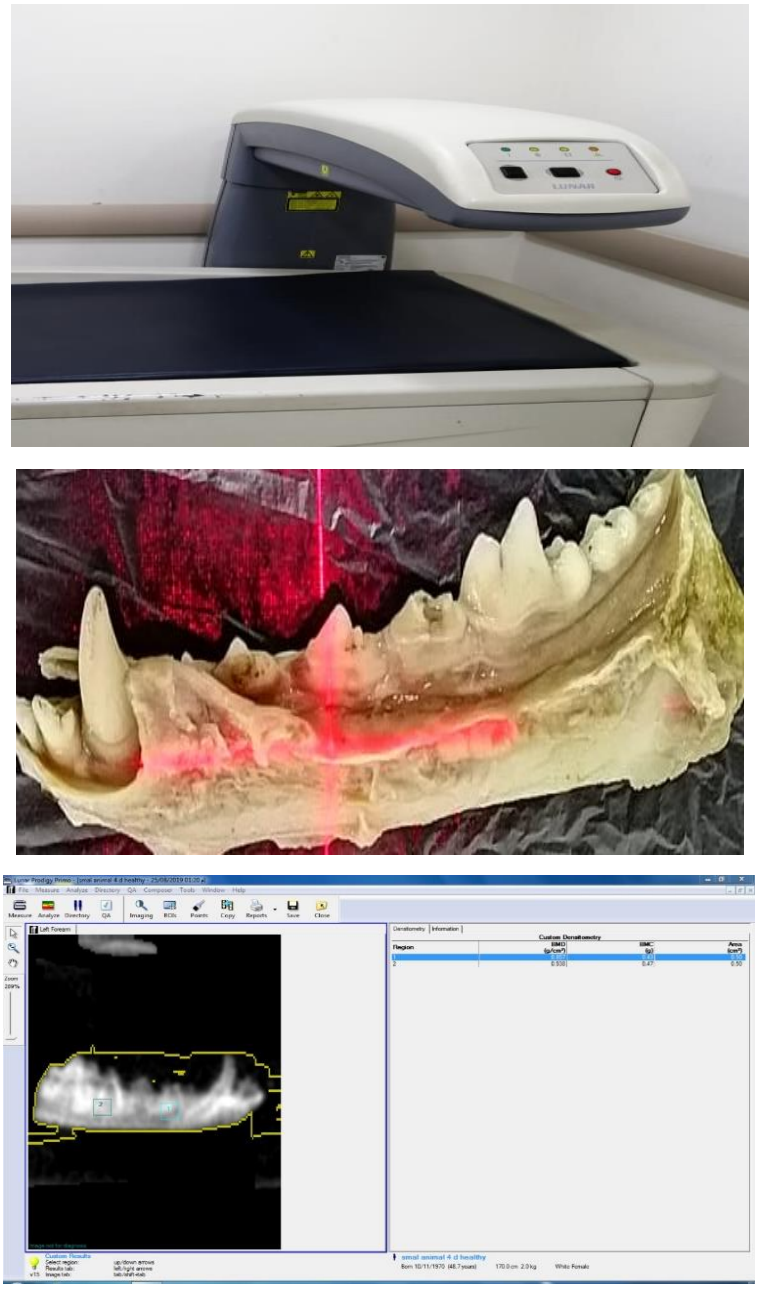


Fig 11: Photographs showing a) Norland densitometer b) specimen during scanning c) software window.

4.14 Fixation and Decalcification:

After completion of the densitometric analysis, specimens of mandibular blocks were sectioned and collected including the experimented teeth and half of the neighboring teeth on each side with the full thickness of the mandibular bone using an orthopedic electrical saw (tech, UK) under copious irrigation with normal saline. Specimens were replaced in 10%buffered formalin for fixation for up to 5 days at room temperature. Decalcification was performed by immersion of the bony specimens in equal volumes of 20% sodium citrate and 5% formic acid (Decal Chemical Corporation, Congers, New York) for a period of 2 months and the solution was renewed once per week.

4.15 Histologic Preparation and Evaluation:

Upon removal from the decalcifying solution, the specimens were placed under a running tap water wash for 20 minutes followed by immersion in 70% ethyl alcohol. The specimens were dehydrated through ascending gradations of ethanol and processed on a Leica TP 1020 dip n' dunk processor (Leica, Wetzlar, Germany) at 45 minutes 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, embedded in paraffin, and sectioned on a Leica Jung RM 2045 microtome (Wetzlar, Germany). Sections were made longitudinally in mesio distal direction every 5 μm starting from the occlusal surface to the inferior border of the mandible, then placed on the probe on plus slides. Tissues were stained with Hematoxylin and Eosin (H &E), then rinsed in cool running double-distilled water for 5 minutes, 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, and covered with a coverslip with Cytoseal (Thermo Fisher Scientific, Waltham, MA). Finally, the slides were evaluated under light microscopy at up to 400 X magnification.

4.16 Data Management and Analysis

Data were collected, tabulated, and statistically analyzed using One way ANOVA test, and Duncan's multiple ring test was used for Comparisons between the groups. The mean and SD values were calculated for each group in each test The significance level was set at $P \leq 0.05$. Statistical analysis was performed using SPSS for Windows 25 softw

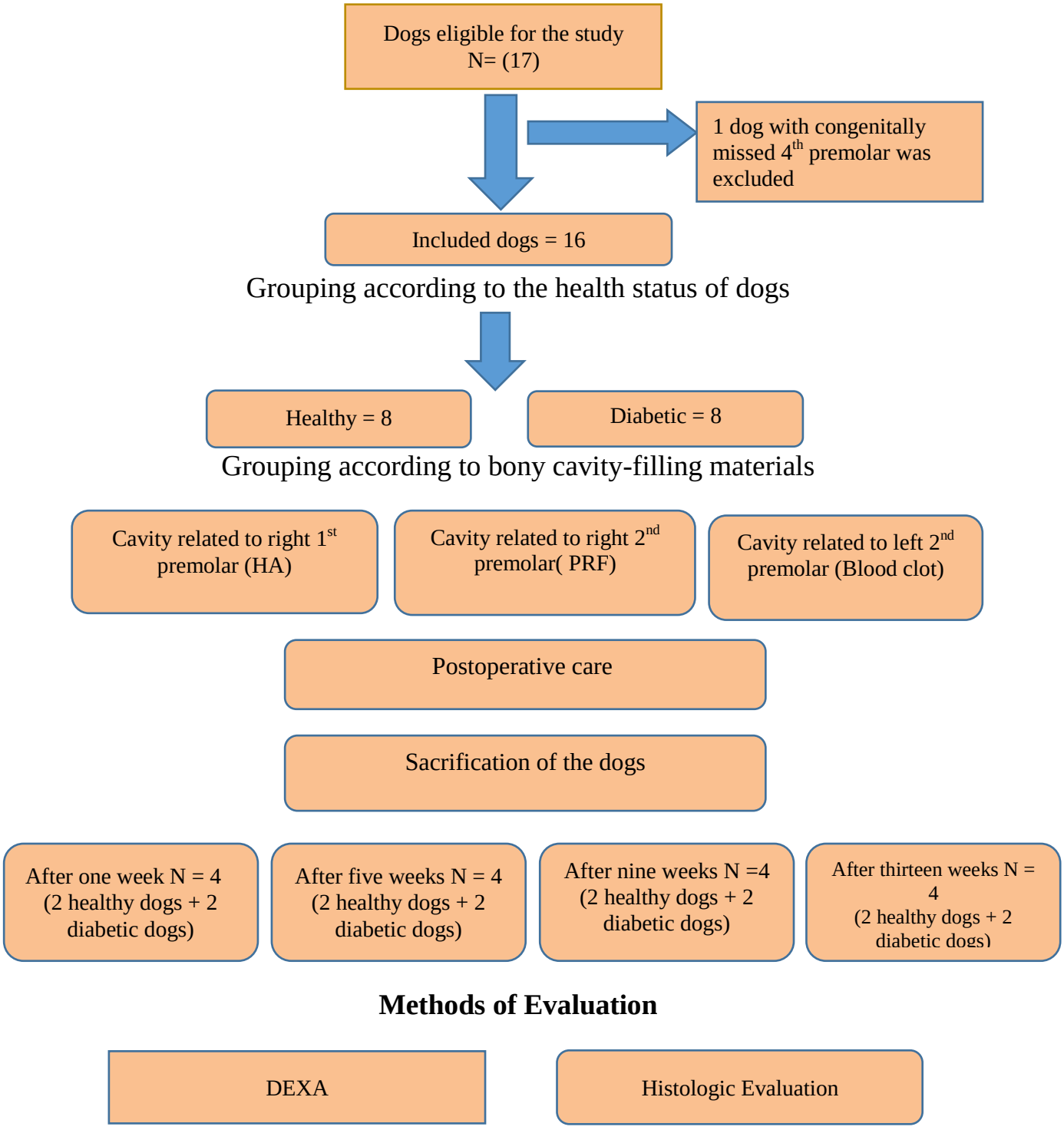


Fig 12: A flow chart representing a review of materials and methods used in the study

RESULTS

Section outline:

5.1 Evaluation of bone mineral density:

5.1.1 Effect of time of sacrification.

5.1.2 Effect of the testing materials.

5.1.3 Effect of general health of the dogs.

5.2 Evaluation of Bone mineral content :

5.2.1 Effect of time of sacrification.

5.2.2 Effect of the testing materials.

5.2.3 Effect of general health of the dogs.

5.3. Descriptive observation of the study.

RESULTS

Data in this section were and statistically analyzed using One way ANOVA test, and Duncan's multiple ring test was used for Comparisons between the groups.

5.1 Evaluation of bone mineral density:

5.1.1 Effect of time of sacrification

A- Healthy animal:

1- **Platelet rich fibrin (PRF):** There was a statistically significant difference between 1 week (0.173± 0.003), 5 weeks (0.566 ± 0.008), 9 weeks (0.830 ± 0.003), and 13 weeks (1.087± 0.037) for platelet-rich fibrin (PRF) where (p=0.000).Table 1.

2-Blood clot: There was a statistically significant difference between 1 week (0.111± 0.001), 5 weeks (0.279 ± 0.006), 9 weeks (0.372 ± 0.009), and 13 weeks (0.433 ± 0.015) for Blood clot where (p=0.000). Table 1.

3-Hyaluronic acid (HA): There was a statistically significant difference between 1 week (0.105± 0.004), 5 weeks (0.281 ± 0.013), 9 weeks (0.363 ± 0.011), and 13 weeks (0.427± 0.027) for Hyaluronic acid (HA) where (p=0.000). Table 1.

Table (1): The mean and standard deviation (SD) values representing the effect of sacrification time on bone mineral density for healthy animals.

Bone Density						
Healthy animal						
Treatment	platelet rich fibrin (PRF)		Blood clot		Hyaluronic acid (HA)	
Time	Mean	SD	Mean	SD	Mean	SD
1 week	0.173 d	0.003	0.111 d	0.001	0.105 d	0.004
5 weeks	0.566 c	0.008	0.279 c	0.006	0.281 c	0.013
9 weeks	0.830 b	0.003	0.372 b	0.009	0.363 b	0.011
13 weeks	1.087 a	0.037	0.433 a	0.015	0.427 a	0.027
Sig.	**		**		**	
P-value	0.000		0.000		0.000	

Asympol * and **represents statistically significant differences at p ≤ 0.05 and p ≤ 0.01,respectively.

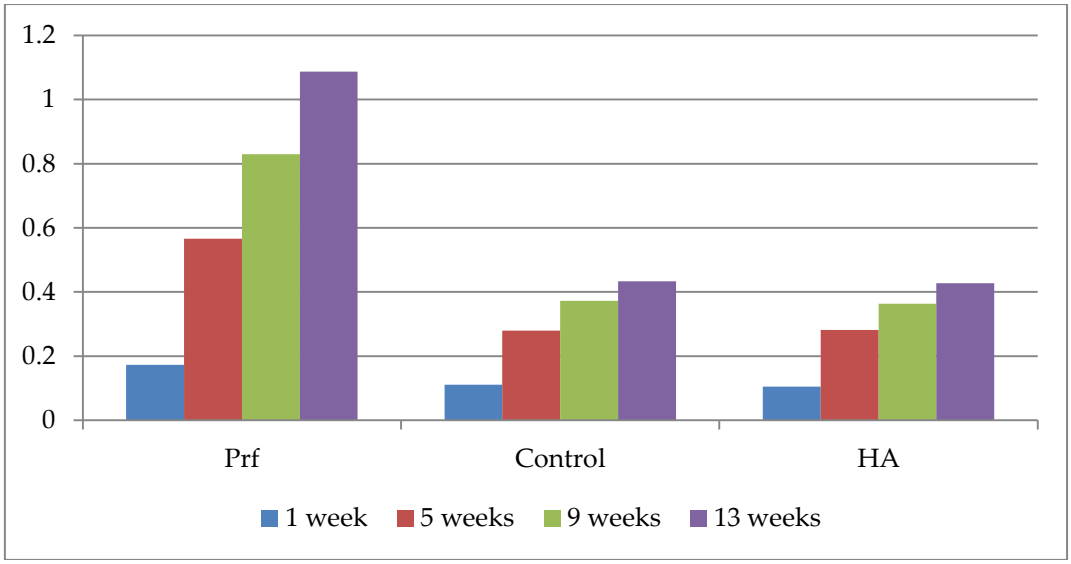


Fig 13. Bar chart representing the effect of sacrification time on bone mineral density in healthy animals

B-Effect of time in Diabetic animal:

1- Platelet rich fibrin (PRF): There was a statistically significant difference between 1 week (0.147± 0.009), 5 weeks (0.432 ± 0.012), 9 weeks (0.614 ± 0.007), and 13 weeks (0.751± 0.010) for platelet-rich fibrin (PRF where (*p*=0.000).Table 2.

2-Blood clot: There was a statistically significant difference between 1 week (0.089± 0.003), 5 weeks (0.204 ± 0.013), 9 weeks (0.254 ± 0.008), and 13 weeks (0.396± 0.005)for Blood clot where (p=0.000). Table 2.

3- Hyaluronic acid (HA): There was a statistically significant difference between 1 week (0.088 ± 0.004), 5 weeks (0.206± 0.022), 9 weeks (0.250 ± 0.009), and 13 weeks (0.388± 0.004) for Hyaluronic acid (HA) where (p=0.000). Table 2.

Table (2): The mean, standard deviation (SD) values representing the effect of sacrification time on bone density for diabetic animals. -

Bone Density						
Diabetic animal						
Treatment	platelet rich fibrin (PRF)		Blood clot		Hyaluronic acid (HA)	
Time	Mean	SD	Mean	SD	Mean	SD
1 week	0.147 d	0.009	0.089 d	0.003	0.088 d	0.004
5 weeks	0.432 c	0.012	0.204 c	0.013	0.206 c	0.022
9 weeks	0.614 b	0.007	0.254 b	0.008	0.250 b	0.009
13 weeks	0.751 a	0.010	0.396 a	0.005	0.388 a	0.004
Sig.	**		**		**	
P-value	0.000		0.000		0.000	

A symbol * and **represents statistically significant differences at $p \leq 0.05$ and $p \leq 0.01$,respectively.

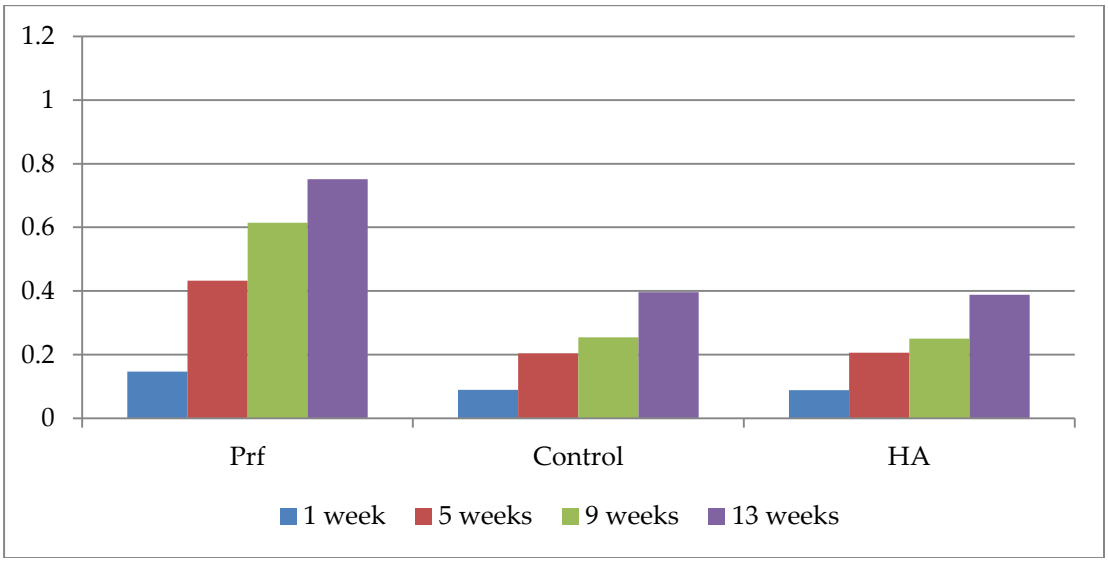


Fig 14. Bar chart representing the effect of sacrification time on bone mineral density in diabetic animals

5.1.2 Effect of the testing materials

A-Healthy animal:

At 1 week: The PRF group showed more values of bone density (0.173± 0.003) followed by Blood clot (0.111± 0.001) and hyaluronic acid(0.105 ± 0.004) with no significance difference both groups where (p=0.000). Table 3.

At 5 weeks: The PRF group showed more values of bone density (0.566 ± 0.006) followed by Blood clot (0.279 ± 0.006) and hyaluronic acid(0.281 ± 0.013) with no significance difference between both groups where (p=0.000). Table 3.

At 9 weeks: The PRF group showed more values of bone density (0.830 ± 0.003) followed by Blood clot (0.372 ± 0.009) and hyaluronic acid(0.363 ± 0.011)with no significance difference between both groups where (p=0.000).Table 3.

At 13 weeks: The PRF group showed more values of bone density (1.087 ± 0.037) followed by Blood clot (0.433 ± 0.015) and hyaluronic acid(0.427 ± 0.027)with no significance difference between both groups where (p=0.000).Table 3.

Table (3): The mean, and standard deviation values representing the effect of the test materials on bone density for healthy animals

		Treatments		1	5 weeks	9 weeks	13
Bone Density	**Healthy animal**	platelet rich fibrin (PRF)	Mean	0.173[a]	0.566 [a]	0.830 [a]	1.087 [a]
			SD	0.003	0.008	0.003	0.037
		Blood clot	Mean	0.111 [b]	0.279 [b]	0.372 [b]	0.433 [b]
			SD	0.001	0.006	0.009	0.015
		Hyaluronic acid (HA)	Mean	0.105 [b]	0.281 [b]	0.363 [b]	0.427 [b]
			SD	0.004	0.013	0.011	0.027
Sig.				**	**	**	**
P-value				0.000	0.000	0.000	0.000

A symbol ** represents statistically significant differences at $p \leq 0.01$

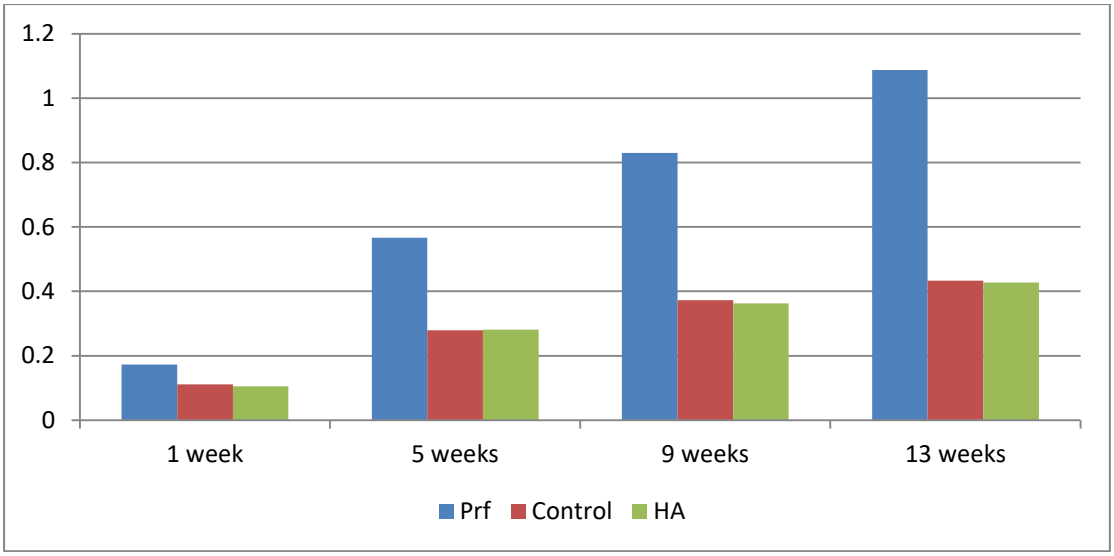


Fig 15. Bar chart representing the effect of the test materials on bone mineral density in healthy animals

B- **Diabetic animals:**

1- 1 week: The PRF group showed more values of bone density (0.147 ± 0.009) followed by Blood clot (0.089 ± 0.003) and hyaluronic acid(0.087 ± 0.004)with no significance difference between both groups where (p=0.000). Table 4.

2- 5 weeks: The PRF group showed more values of bone density (0.432 ± 0.012) followed by hyaluronic acid (0.204 ± 0.013) and Blood clot (0.206 ± 0.022)with no significance difference between both groups where (p=0.000). Table 4.

.**3- 9 weeks:** The PRF group showed more values of bone density (0.614± 0.007) followed by Blood clot (0.254± 0.009) and hyaluronic acid(0.250 ± 0.009)with no significance difference between both groups where where (p=0.000). Table 4.

4- 13 weeks: The PRF group showed more values of bone density (0.751 ± 0.010) followed by Blood clot (0.396 ± 0.005) and hyaluronic acid(0.388 ± 0.004)with no significance difference between both groups where where (p=0.000). Table 4.

Table (4): The mean, and standard deviation values representing the effect of the test materials on bone density for diabetic animals

		Treatments		1	5 weeks	9 weeks	13
Bone Density	Diabetic animal	platelet rich fibrin (PRF)	Mean	0.147	0.432 a	0.614 a	0.751 a
			SD	0.009	0.012	0.007	0.010
		Blood clot	Mean	0.089	0.204 b	0.254 b	0.396 b
			SD	0.003	0.013	0.009	0.005
		Hyaluronic acid (HA)	Mean	0.087	0.206 b	0.250 b	0.388 b
			SD	0.004	0.022	0.009	0.004
Sig.				**	**	**	**
P-value				0.000	0.000	0.000	0.000

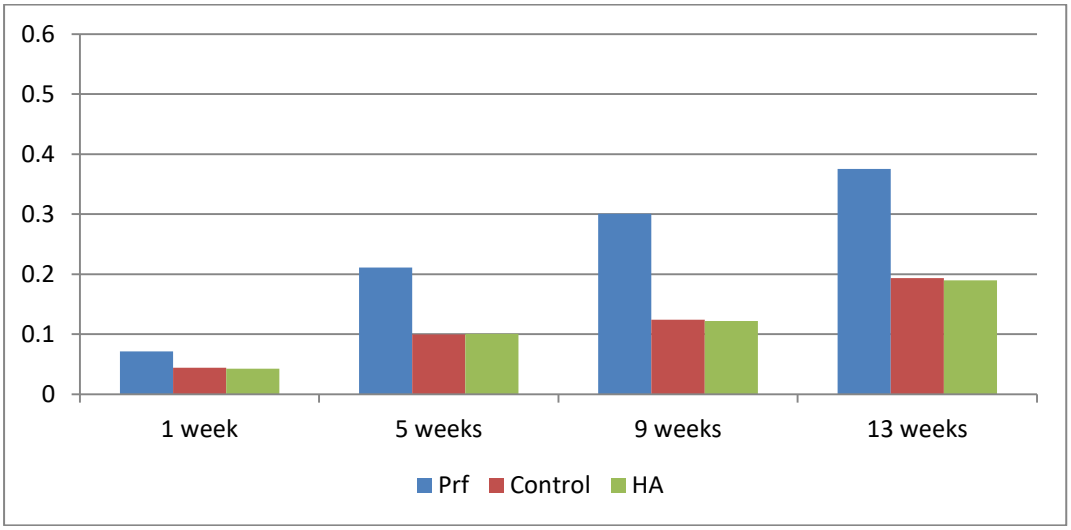


Fig 16. Bar chart representing the effect of the test materials on bone mineral density in diabetic animals

5.1.3 Effect on the general health of the animals:

In platelet rich fibrin (PRF):

At 1 week : There was no statistically significant difference between Healthy animals (0.173±0.003) and Diabetic animals (0.147±0.004)

At 5 weeks: There was a statistically significant difference between the Healthy animals (0.566±0.012) and Diabetic animals (0.432±0.012) groups.

At 9 weeks: There was a statistically significant difference between Healthy animals (0.830 ±0.003)and Diabetic animals (0.614±0.007)

At 13 weeks: There was a statistically significant difference between Healthy animals (1.087 ±0.037)and Diabetic animals (0.751±0.010)

Table (5): The mean, and standard deviation values representing the effect of general health on bone density in platelet-rich fibrin group

Bone Density									
platelet rich fibrin (PRF)									
Variable	1week		5 weeks		9 weeks		13 weeks		Mean
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Healthy	0.173 g	0.003	0.566 e	0.008	0.830 b	0.003	1.087 a	0.037	0.664 a
Diabetic	0.147 g	0.009	0.432 f	0.012	0.614 dc	0.007	0.751 c	0.010	0.486 b
Sig.	*								**
P-value	0.010								0.005

A symbol* and **represents statistically significant differences at $p \leq 0.05$ and $p \leq 0.01$,respectively.

In Blood clot:

At 1 week : There is no statistically significant difference between Healthy animals (0.111±0.001) and Diabetic animals (0.089±0.003)

At 5 weeks: There was a statistically significant difference between the Healthy animals (0.279±0.006) and Diabetic animals (0.204±0.013) groups.

At 9 weeks: There was a statistically significant difference between Healthy animals (0.372 ±0.009)and Diabetic animals (0.254±0.008)

At 13 weeks: There was a statistically significant difference between Healthy animals (0.433 ±0.015)and Diabetic animals (0.395±0.005)

Table (6): The mean, and standard deviation values representing the effect of general health on bone density in Blood clot group.

Bone Density									
Blood clot									
Variable	1week		5 weeks		9 weeks		13 weeks		Mean
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Healthy	0.111 g	0.00	0.279 d	0.006	0.372 c	0.009	0.433 a	0.015	0.299
Diabetic	0.089 h	0.00	0.204 f	0.013	0.254 e	0.008	0.395 b	0.005	0.236
Sig.	**								**
P-value	0.000								0.000

A symbol* and **represents statistically significant differences at $p \leq 0.05$ and $p \leq 0.01$,respectively.

In Hyaluronic acid (HA)

At 1 week : There was no statistically significant difference between Healthy animals (0.105±0.004) and Diabetic animals (0.088±0.004)

At 5 weeks: There was a statistically significant difference between the Healthy animals (0.280±0.013) and Diabetic animals (0.205±0.022) groups.

At 9 weeks: There was a statistically significant difference between Healthy animals (0.362 ±0.011)and Diabetic animals (0.25±0.009)

At 13 weeks: There was a statistically significant difference between Healthy animals (0.427 ±0.027)and Diabetic animals (0.388±0.004)

Table (7): The mean, and standard deviation values representing the effect of general health on bone density in Hyaluronic acid group.

Bone Density									
Hyaluronic acid (HA)									
Variable	1week		5 weeks		9 weeks		13 weeks		Mean
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Healthy	0.105 e	0.004	0.280 c	0.013	0.362 b	0.011	0.427 a	0.027	0.294 a
Diabetic	0.088 e	0.004	0.205 d	0.022	0.250 c	0.009	0.388 b	0.004	0.233 b
Sig.	**								**
P-value	0.000								0.000

A symbol* and **represents statistically significant differences at $p \leq 0.05$ and $p \leq 0.01$,respectively.

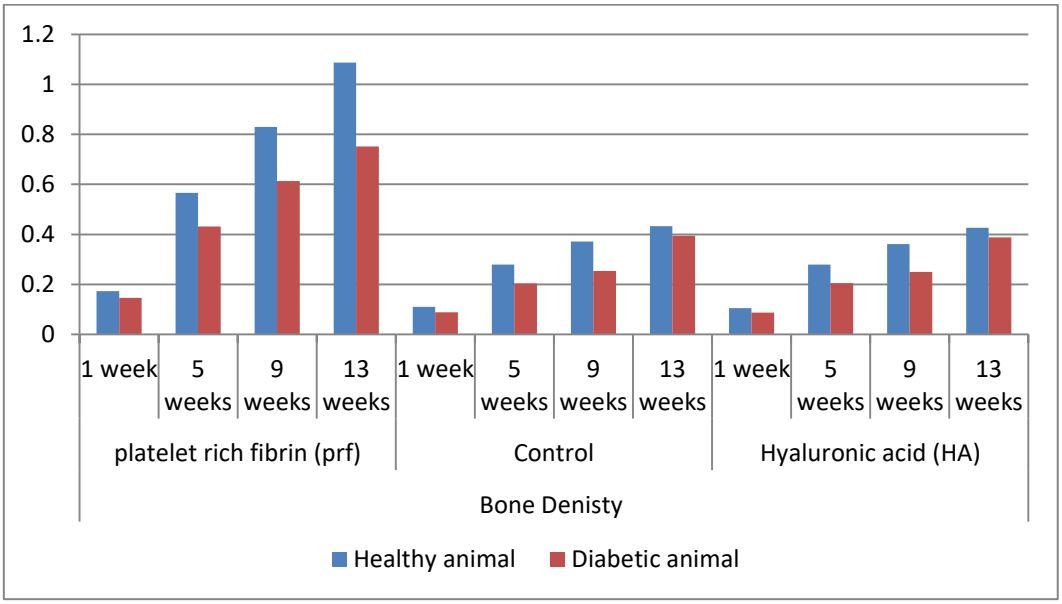


Fig 17. Bar chart representing the effect of general health on bone mineral density

5.2-Evaluation of Bone mineral content:

5.2.1-Effect of the testing time:

A - Healthy animal:

1- Platelet rich fibrin (PRF): There was a statistically significant difference between 1 week (0.084 ± 0.001), 5 weeks (0.276 ± 0.004), 9 weeks (0.406 ± 0.001), and 13 weeks (0.532 ± 0.018) for platelet-rich fibrin (PRF) where (*p*=0.000). Table 8.

2-Blood clot: There was a statistically significant difference between 1 week (0.054± 0.001), 5 weeks (0.136 ± 0.003), 9 weeks (0.182 ± 0.005), and 13 weeks (0.212 ± 0.008) for Blood clot where (p=0.000). Table 8.

3- Hyaluronic acid (HA): There was a statistically significant difference between 1 week (0.051 ± 0.001), 5 weeks (0.137 ± 0.007), 9 weeks (0.177 ± 0.006), and 13 weeks (0.209 ± 0.013) for Hyaluronic acid (HA) where (p=0.000). Table 8.

Table (8): The mean and standard deviation (SD) values representing the effect of sacrification time on bone mineral content for healthy animals

Bone mineral content						
Healthy animal						
Treatment	platelet rich fibrin (PRF)		Blood clot		Hyaluronic acid (HA)	
Time	Mean	SD	Mean	SD	Mean	SD
1 week	0.084 d	0.001	0.054 d	0.001	0.051d	0.002
5 weeks	0.276 c	0.004	0.136 c	0.003	0.137 c	0.007
9 weeks	0.406 b	0.001	0.182 b	0.005	0.177 b	0.006
13 weeks	0.532 a	0.018	0.212 a	0.008	0.209 a	0.013
Sig.	**		**		**	
P-value	0.000		0.000		0.000	

A symbol =* and ** representing statistically significant differences at p ≤ 0.05 and p ≤ 0.01,respectively.

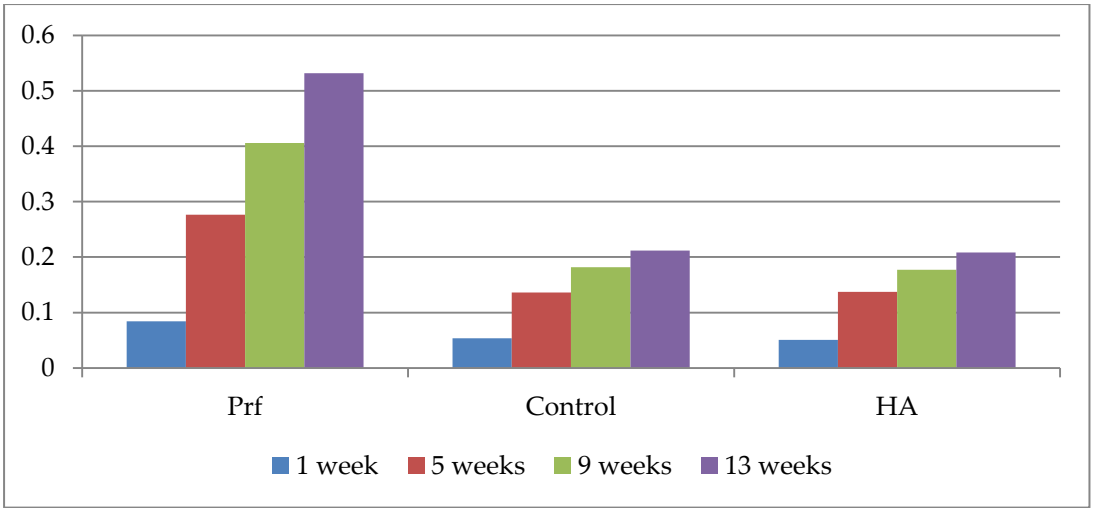


Fig 18. Bar chart representing the effect of sacrification time on the bone mineral content in healthy animals

B- Diabetic animal:

1- Platelet rich fibrin (PRF): There was a statistically significant difference between 1 week (0.071± 0.004), 5 weeks (0.211 ± 0.006), 9 weeks (0.301 ± 0.004), and 13 weeks (0.375± 0.006) for platelet-rich fibrin (PRF) where (p=0.000).Table 9

2-Blood clot: There was a statistically significant difference between 1 week (0.044 ± 0.001), 5 weeks (0.099 ± 0.006), 9 weeks (0.124 ± 0.004), and 13 weeks (0.194 ± 0.002) for Blood clot where (p=0.000).Table 9.

3- Hyaluronic acid (HA): There was a statistically significant difference between 1 week (0.043 ± (0.189 ± 0.002) for Hyaluronic acid (HA) where (p=0.000). Table 9.

Table (9): The mean and standard deviation (SD) values representing the effect of sacrification time on bone mineral content for diabetic animals

Bone mineral content						
Diabetic animal						
Treatment	platelet rich fibrin (PRF)		Blood clot		Hyaluronic acid (HA)	
Time	Mean	SD	Mean	SD	Mean	SD
1 week	0.071 d	0.004	0.044 d	0.001	0.043 d	0.002
5 weeks	0.211c	0.006	0.099 c	0.006	0.101 c	0.011
9 weeks	0.301 b	0.004	0.124 b	0.004	0.122 b	0.004
13 weeks	0.375 a	0.006	0.194 a	0.002	0.189 a	0.002
Sig.	**		**		**	
P-value	0.000		0.000		0.000	

Symbol * and ** represents statistically significant differences at $p \leq 0.05$ and $p \leq 0.01$,respectively.

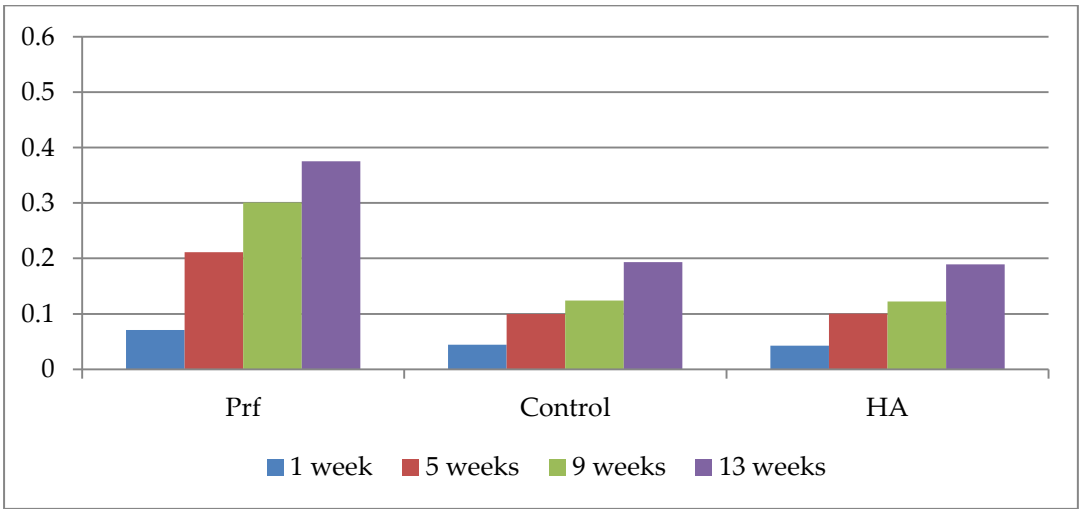


Fig 19. Bar chart representing the effect of sacrification time on the bone mineral content in diabetic animals .

Effect of the testing materials:

Healthy animal:

At 1 week: The PRF group showed more values of bone mineral content (0.084 ± 0.001) followed by Blood clot (0.054 ± 0.001) and hyaluronic acid(0.051 ± 0.002) with no significance difference both groups where (p=0.000). Table 10.

At 5 weeks: The PRF group showed more values of bone mineral content (0.277 ± 0.004) followed by hyaluronic acid (0.137 ± 0.007) and Blood

clot (0.136 ± 0.003) with no significance difference both groups where (p=0.000).Table 10.

At 9 weeks: The PRF group showed more values of bone mineral content (0.406 ± 0.001) followed by Blood clot (0.181 ± 0.005) and hyaluronic acid(0.177 ± 0.006) with no significance difference both groups where (p=0.000). Table 10.

At 13 weeks: The PRF group showed more values of bone mineral content (0.532 ± 0.018) followed by Blood clot (0.212 ± 0.008) and hyaluronic acid(0.208 ± 0.013) with no significance difference both groups where (p=0.000). Table 10

Table (10): The mean, and standard deviation values representing the effect of the test materials on healthy animals

Bone mineral content	Healthy animal	Treatments		1 week	5 weeks	9 weeks	13 weeks
		platelet rich fibrin (PRF)	Mean	0.084[a]	0.277 [a]	0.406 [a]	0.532 [a]
			SD	0.001	0.004	0.001	0.018
		Blood clot	Mean	0.054 [b]	0.136 [b]	0.181 [b]	0.212 [b]
			SD	0.001	0.003	0.005	0.008
		Hyaluronic acid (HA)	Mean	0.051 [b]	0.137 [b]	0.177 [b]	0.208 [b]
			SD	0.002	0.007	0.006	0.013
Sig.				**	**	**	**
P-value				0.000	0.000	0.000	0.000

A symbol ** represents statistically significant differences at $p \leq 0.01$

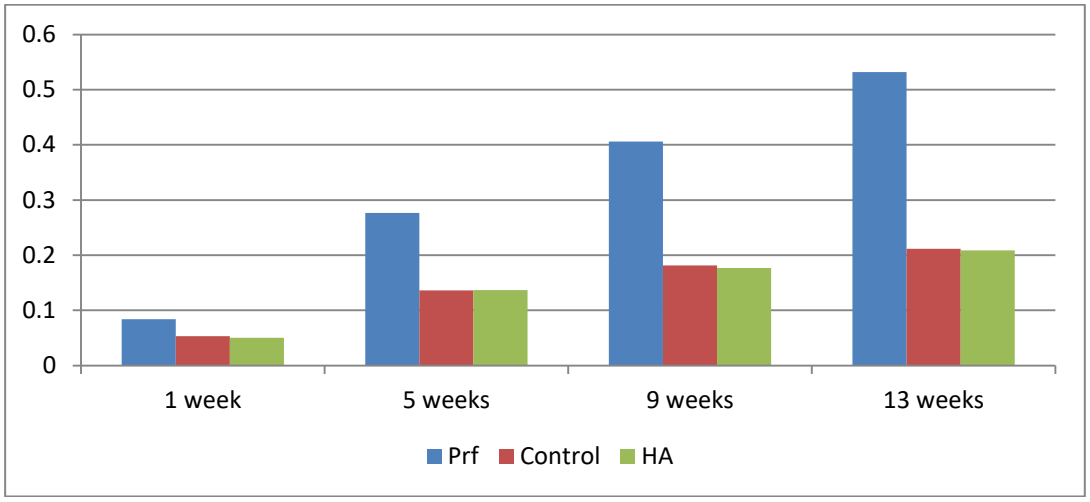


Fig 20. Bar chart representing the effect of the test materials on the bone mineral content in healthy animals

B- **Diabetic animals:**

At 1 week: The PRF group showed more values of bone mineral content (0.071 ± 0.004) followed by Blood clot (0.044 ± 0.001) and hyaluronic acid(0.043 ± 0.002) with no significance difference both groups where (p=0.000). Table 11.

At 5 weeks: The PRF group showed more values of bone mineral content (0.211 ± 0.006) followed by hyaluronic acid (0.101± 0.011) and Blood clot (0.099± 0.006) with no significance difference both groups where (p=0.000).Table 11.

At 9 week: The PRF group showed more values of bone mineral content (0.301 ± 0.004) followed by Blood clot (0.124 ± 0.004) and hyaluronic acid(0.122 ± 0.004) with no significance difference both groups where (p=0.000). Table 11.

4- 13 weeks: At 13 week: The PRF group showed more values of bone mineral content (0.375 ± 0.006) followed by Blood clot (0.194 ± 0.002) and hyaluronic acid(0.189 ± 0.002) with no significance difference both groups where (p=0.000). Table 11.

Table (11): The mean, and standard deviation values representing the effect of the test materials on diabetic animals.

Bone mineral content	Diabetic animal	Treatments		1	5 weeks	9 weeks	13
		platelet rich fibrin (PRF)	Mean	0.071	0.211 a	0.301 a	0.375 a
			SD	0.004	0.006	0.004	0.006
		Blood clot	Mean	0.044	0.099 b	0.124 b	0.194 b
			SD	0.001	0.006	0.004	0.002
		Hyaluronic acid (HA)	Mean	0.043	0.101 b	0.122 b	0.189 b
			SD	0.002	0.011	0.004	0.002
Sig.				**	**	**	**
P-value					0.000	0.000	0.000

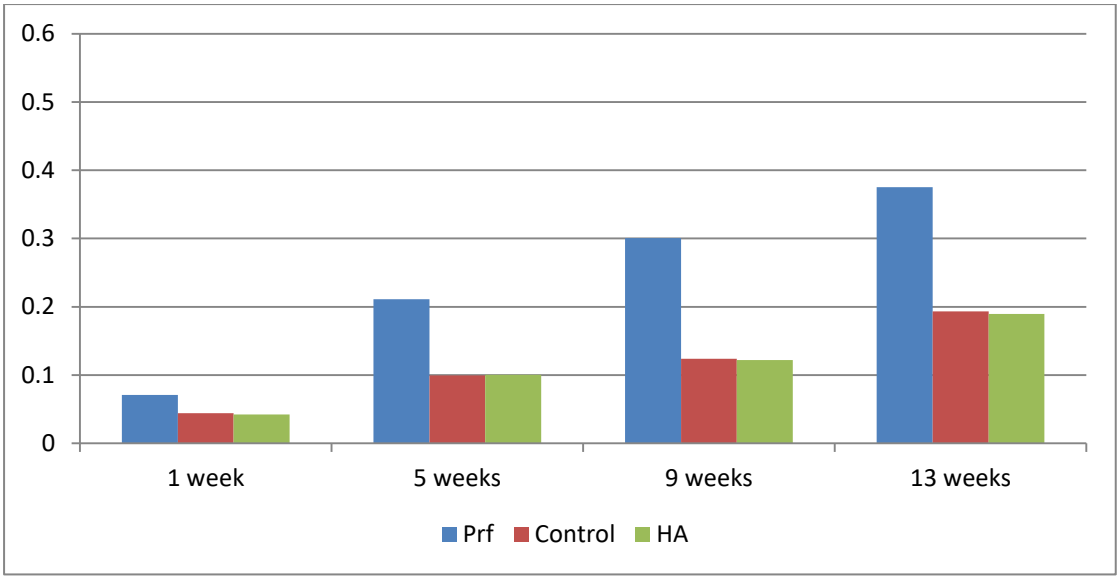


Fig 21. Bar chart representing the effect of the test materials on the bone mineral content of diabetic animals' .

2.3 Effect on the general health of the animals:

A- platelet rich fibrin (PRF):

At 1 week : There was no statistically significant difference between Healthy animals (0.084±0.001) and Diabetic animals (0.071±0.001)

At 5 weeks: There was a statistically significant difference between the Healthy animals (0.277±0.004) and Diabetic animals (0.211±0.004) groups.

At 9 weeks: There was a statistically significant difference between Healthy animals (0.406 ±0.004)and Diabetic animals (0.301±0.018)

At 13 weeks: There was a statistically significant difference between Healthy animals (0.532 ±0.006)and Diabetic animals (0.375±0.006)

Table (12): The mean, and standard deviation values representing the effect of general health of animals on bone mineral content for platelet-rich fibrin group.

Bone mineral content									
platelet rich fibrin (PRF)									
Variable	1week		5 weeks		9 weeks		13 weeks		Mean
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Healthy	0.084 g	0.00	0.277 e	0.004	0.406 b	0.004	0.532 a	0.00	0.325 a
Diabetic	0.071 g	0.00	0.211 f	0.004	0.301	0.018	0.375 c	0.00	0.239 b
Sig.	*								**
P-value	0.010								0.005

A symbol * and **: represents statistically significant differences at $p \leq 0.05$ and $p \leq 0.01$,respectively

B- Blood clot:

At 1 week : There was no statistically significant difference between Healthy animals (0.054 ±0.001) and Diabetic animals (0.044 ±0.005)

At 5 weeks: There was a statistically significant difference between the Healthy animals (0.136±0.001) and Diabetic animals (0.100 ± 0.004) groups.

At 9 weeks: There was a statistically significant difference between Healthy animals (0.182 ±0.003) and Diabetic animals (0.124 ± 0.008)

At 13 weeks: There was a statistically significant difference between Healthy animals (0.212 ±0.006) and Diabetic animals (0.194 ± 0.002)

Table (13): The mean, and standard deviation values representing the effect of health of animals on mineral content for Blood clot

Bone mineral content									
Blood clot									
Variable	1week		5 weeks		9 weeks		13 weeks		Mean
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Healthy	0.054 g	0.00	0.136 d	0.001	0.182 c	0.003	0.212 a	0.006	0.146 a
Diabetic	0.044 g	0.00	0.100 f	0.004	0.124 e	0.008	0.194 b	0.002	0.115 b
Sig.	**								**
P-value	0.000								0.000

A symbol * and **: represents statistically significant differences at $p \leq 0.05$ and $p \leq 0.01$,respectively

C- Hyaluronic acid (HA)

At 1 week : There was no statistically significant difference between Healthy animals (0.051±0.002) and Diabetic animals (0.043±0.006)

At 5 weeks: There was a statistically significant difference between the Healthy animals (0.137 ±0.002) and Diabetic animals (0.101± 0.004) groups.

At 9 weeks: There was a statistically significant difference between Healthy animals (0.177 ±0.007)and Diabetic animals (0.122±0.013)

At 13 weeks: There was a statistically significant difference between Healthy animals (0.209 ±0.011)and Diabetic animals (0.190 ± 0.002)

Table (14): The mean, and standard deviation values representing the effect of health of animals on mineral content for Hyaluronic acid

Bone mineral content									
Hyaluronic acid (HA)									
Variable	1week		5 weeks		9 weeks		13 weeks		Mean
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Healthy	0.051 e	0.00	0.137 c	0.002	0.177 b	0.007	0.209 a	0.011	0.143 a
Diabetic	0.043 e	0.00	0.101 d	0.004	0.122 c	0.013	0.190 b	0.002	0.114 b
Sig.	**								**
P-value	0.000								0.000

A symbol * and **: represents statistically significant differences at $p \leq 0.05$ and $p \leq 0.01$,respectively

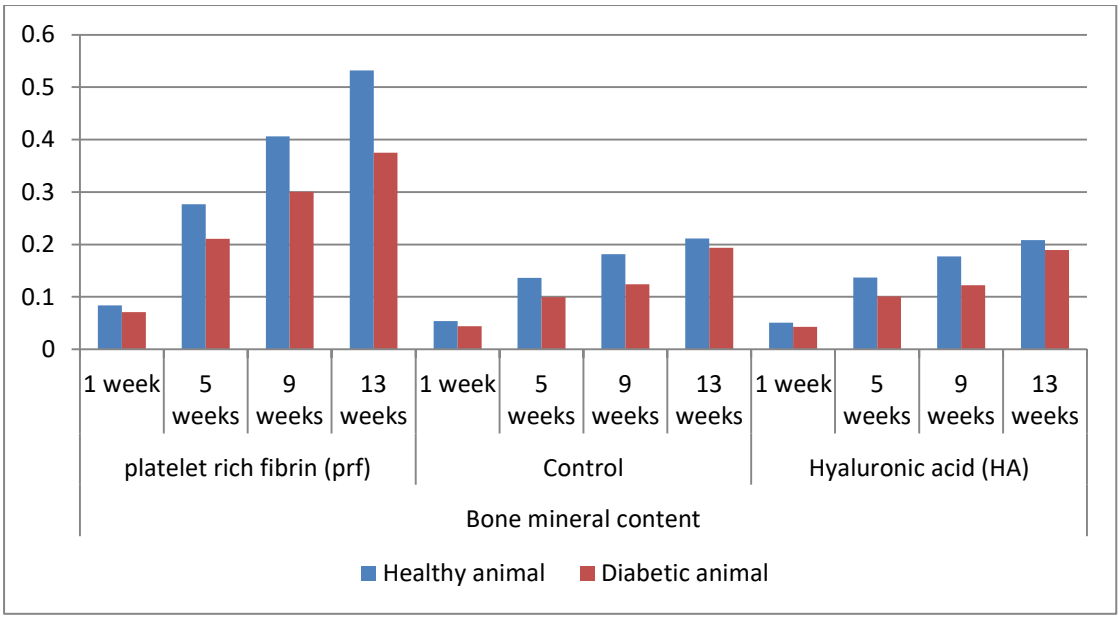


Fig 22. Bar chart representing the effect of general health on bone mineral content

5.3 Descriptive observation of the study

Observation in this section showed Histological finding at each scarification period in both healthy and diabetic dogs including all tested groups

At one week scarification period:

A- HA group:

Healthy dogs :-

Histological examination of sections stained with H&E (at 1 week) showed that presence of large dilated blood vessels ,blood clot, these features reflect angiogenesis ,infiltrated by inflammatory cells , ,organized granulation interspersed with fibroblast , and osteoblast cells were demonstrated.

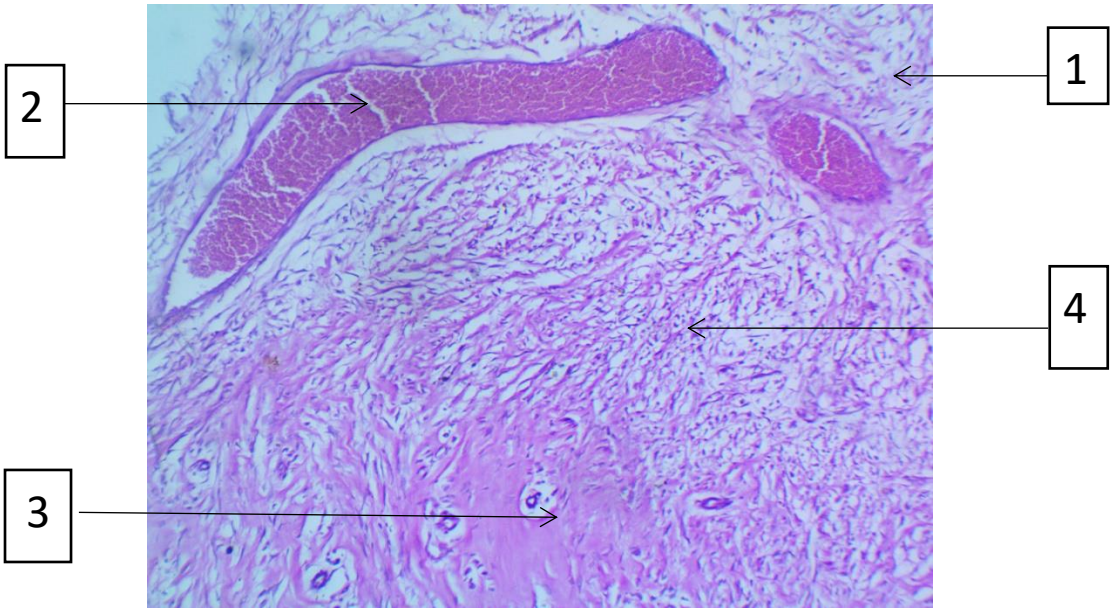


Fig 23: photomicrograph for hyaluronic acid group in healthy dogs at 1 week scarification period showing:- inflammatory cells(1),large dilated blood vessels(2), organized granulation tissue(3), osteoblast(4)

(H&E.100x)

Diabetic dogs: -

Histological examination of sections stained with H&E (at 1 week) showed that,, the presence of:- dilated blood vessels (B.Vs), these feature's reflect low grade of angiogenesis compared to healthy one, with inflammatory cells infiltration, organized granulation interspersed with fibroblast , and osteoblast cells were demonstrated

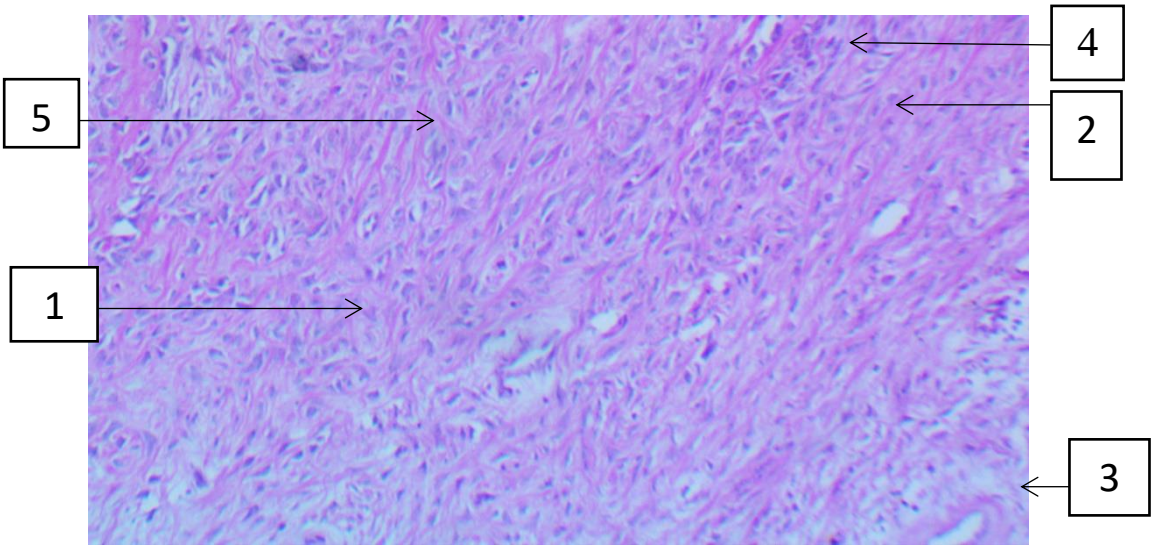


Fig 24: photomicrograph for hyaluronic acid group in diabetic dogs at 1 week scarification period showing:-inflammatory cells(1), fibroblast(2),dilated blood vessels(3),granulation tissues (4), osteoblast(5) . (H&E.100x).

<u>B-Blood clot group:</u>

Healthy dogs: -

Histological examination of sections stained with H&E (at 1 week) showed that, the presence of blood clot infiltrated with inflammatory cell mainly PNL and macrophages, well organized granulation tissues which characterized by dilated blood capillaries. There was recruitment of osteoblast with early formation of small of very thin bone trabeculae, and highly mitotic activity.

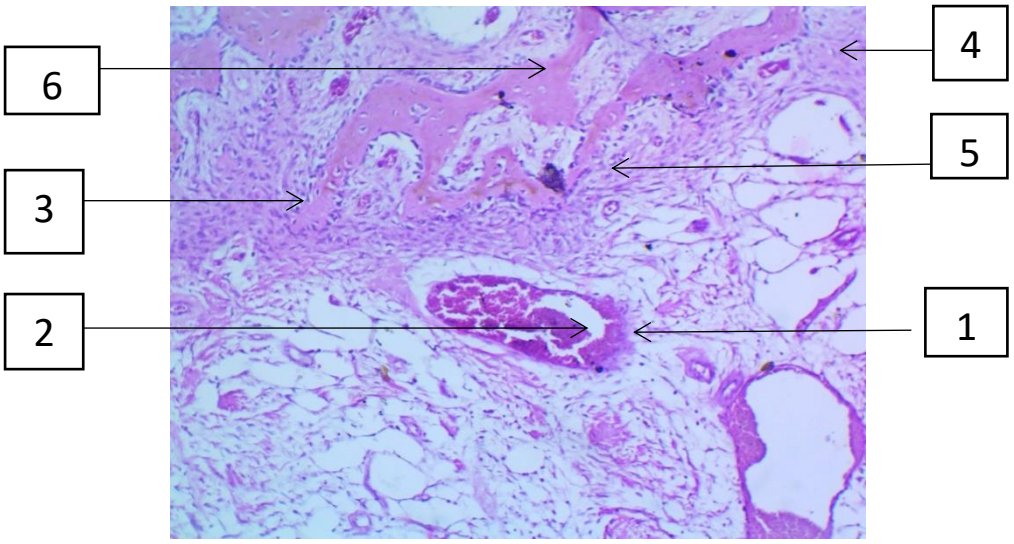


Fig 25: - photomicrograph for Blood clot group in healthy dogs at 1 week scarification period showing:- dilated blood vessels(1), blood clot(2), inflammatory cells(3), fibroblast(4),osteoblast(5),very thin bony trabeculae (6). (H&E.100x).

Diabetic dogs: -

Histological examination of sections stained with H&E (at 1 week) showed that,, the presence of blood clot infiltrated with inflammatory cells, organized granulation tissues, dilated blood vessels. early formation of very thin bone trabeculae with low rate ,fibroblast.

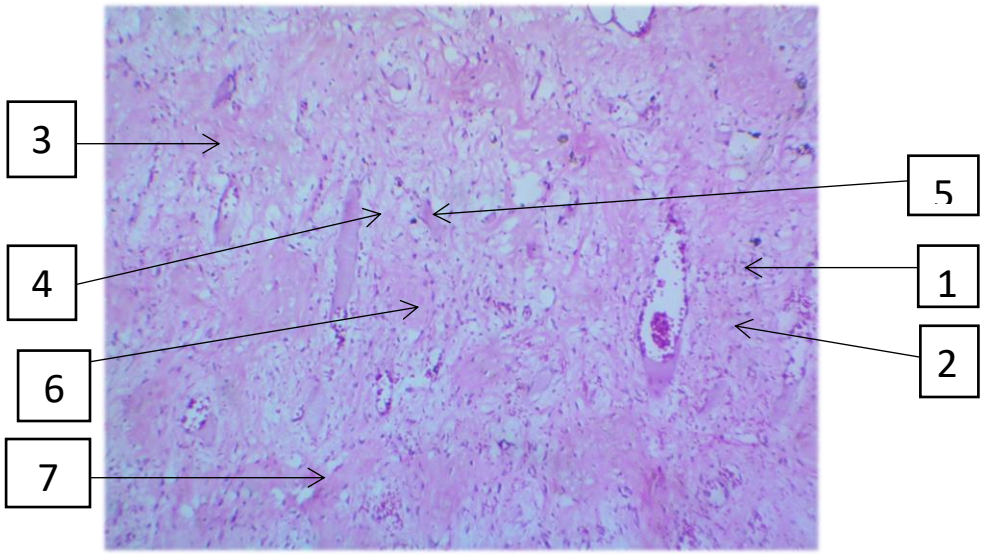


Fig 26: photomicrograph for Blood clot group in diabetic dogs at 1 week scarification period showing:- dilated blood vessels(1), blood clot(2), inflammatory cells(3) , granulation tissue(4), fibroblast(5), osteoblast(6), low very thin bony trabeculae(7). (H&E.100x)

C- PRF group:

Healthy dogs: -

Histological examination of sections stained with H&E (at 1 week) showed that, the presence of blood clot infiltrated with inflammatory cell mainly PNL and macrophages, well organized granulation tissues which characterized by multiple dilated blood capillaries regarding highly angiogenic activity. There was recruitment of osteoblast with early formation of islands of thin bone trabeculae.

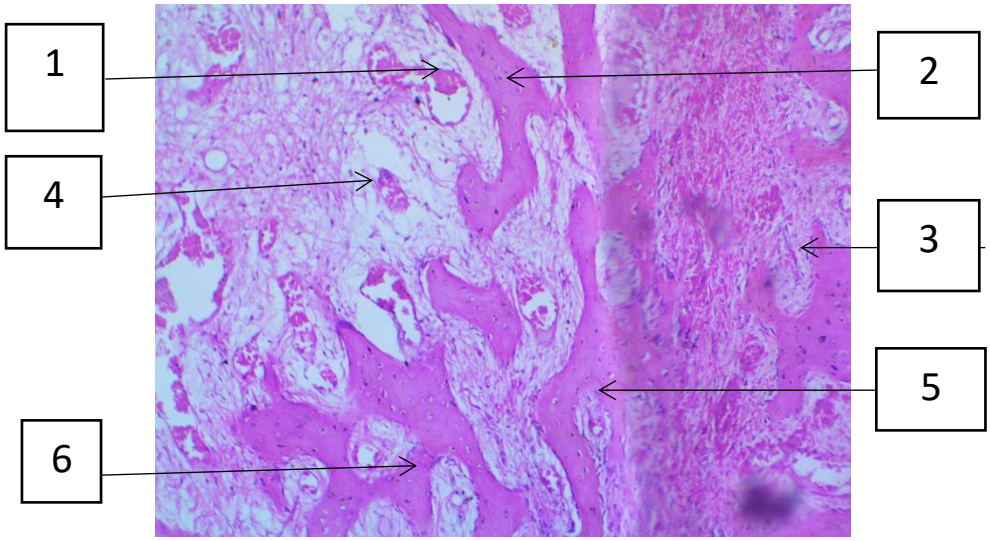


Fig 27: photomicrograph for PRF group in healthy dogs at 1 week scarification period showing:- multiple dilated blood vessels, (1)blood clot(2), inflammatory cells (3),fibroblast(4), osteoblast(5), bony trabeculae with variable thickness(6). (H&E.100x).

Diabetic dogs:-

Histological examination of sections stained with H&E (at 1 week) showed that, the presence of blood clot infiltrated with inflammatory cell infiltration, well organized granulation tissues which characterized by multiple small blood capillaries regarding low angiogenic activity compared to healthy one. There was recruitment of osteoblast with early formation of islands of very thin bone trabeculae

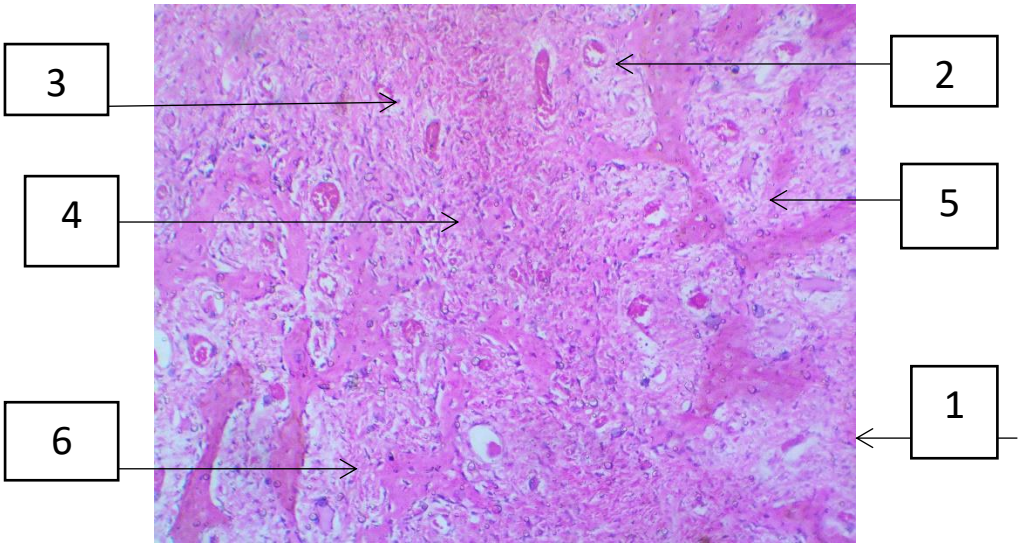


Fig 28 - photomicrograph for PRF group in diabetic dogs at 1 week scarification period showing:- multiple blood vessels(1), blood clot(2), inflammatory cells (3), fibroblast(4), osteoblast(5), very thin bony trabeculae(6).

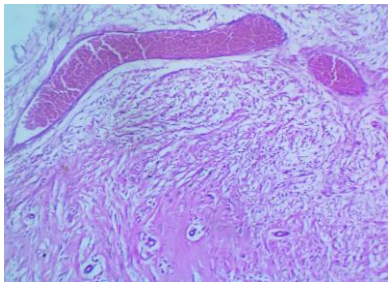
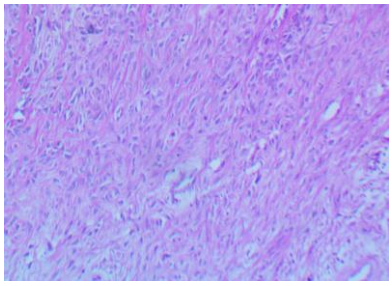
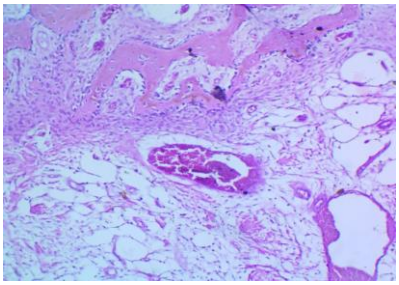
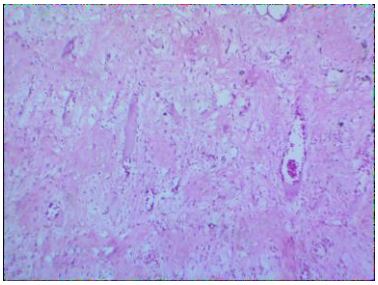
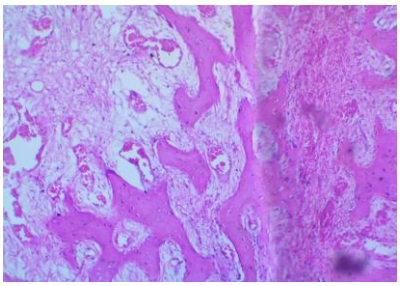
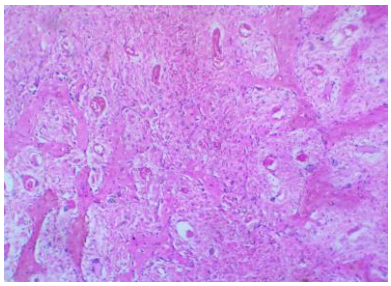
ne week	**Healthy**	**Diabetic**
HA		
Blood clot		
PRF		

Fig 29: A photomicrograph at one week sacrification period in healthy and diabetic dogs.

2 - Five weeks scarification period:

A- HA group:

Healthy dogs: -

Histological examination of section stained with H&E(at 5 weeks) revealed the presence of blood clot infiltrated by inflammatory cells, well organized granulation tissue with tightly packed collagen bundles infiltrated by fibroblastic cells and large dilated blood vessels with hematopoietic tissue .There were recruitment of multiple dividing osteoblast. Woven bone with thin bony trabecular starting to be coalescent with each other, the trabeculae were lined by osteoblasts and osteogenic cells

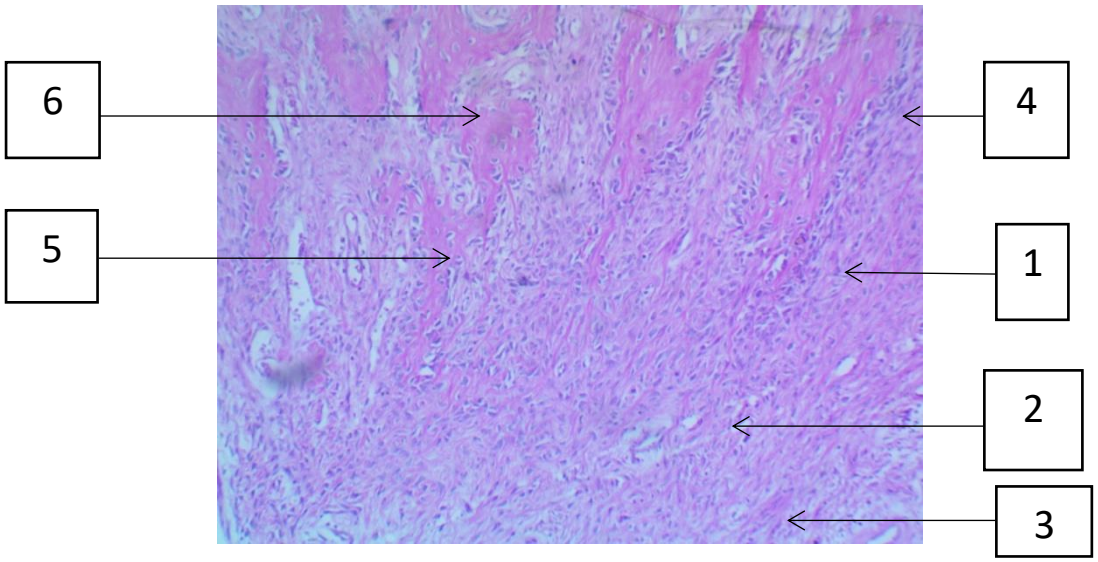


Fig 30:photomicrograph for HA group in healthy dogs at 5 week scarification period showing;- inflammatory cells(1) , Remnant of blood clot (BC) **(2)**,large dilated blood BVs**(3)**, well organized granulation tissue**(4)** , thin collagen fibrils**(5)**, osteoblast**(6)** . (H&E.100x)

Diabetic dogs:

Histological examination of section stained with H&E(at 5 weeks) revealed the presence of blood clot infiltrated by inflammatory cells, well organized granulation tissue infiltrated by fibroblastic cells , large dilated blood vessels .There were osteoblast cells. Very thin fibrils starting to be coalescent with each other.

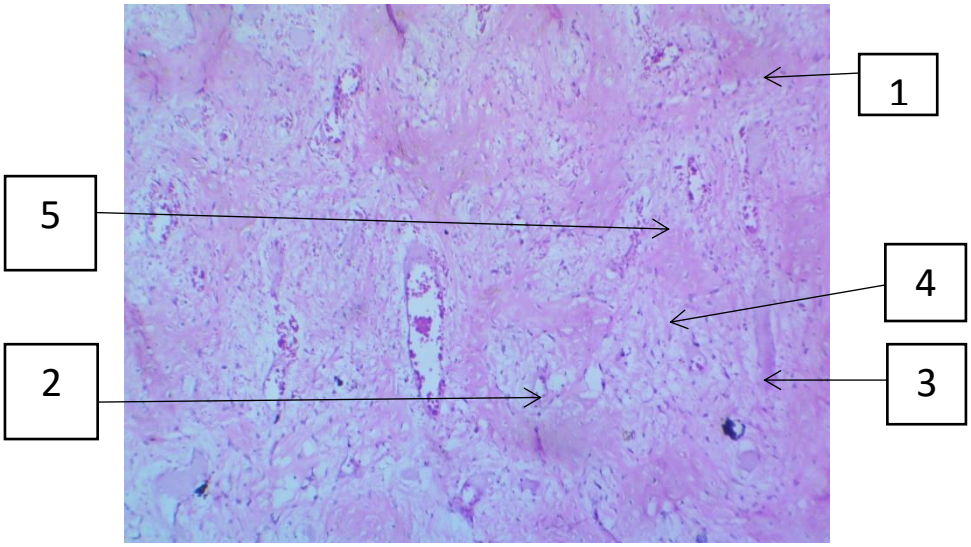


Fig 31: photomicrograph for HA group in diabetic dogs at 5 week scarification period showing:- inflammatory cells(1) , large dilated blood BVs(2) ,granulation tissue(3) , thin collagen fibrils(4), osteoblast(5) . (H&E.100x)

B- Blood clot group:

Healthy dogs:

Histological examination of section stained with H&E(at 5 weeks) revealed that infiltrated with inflammatory cell , well organized granulation tissues which characterized by dilated blood capillaries . There was recruitment of osteoblast with early formation of small islands of bone trabeculae with variable thickness, very wide bone marrow spaces, reflects woven bone formation.

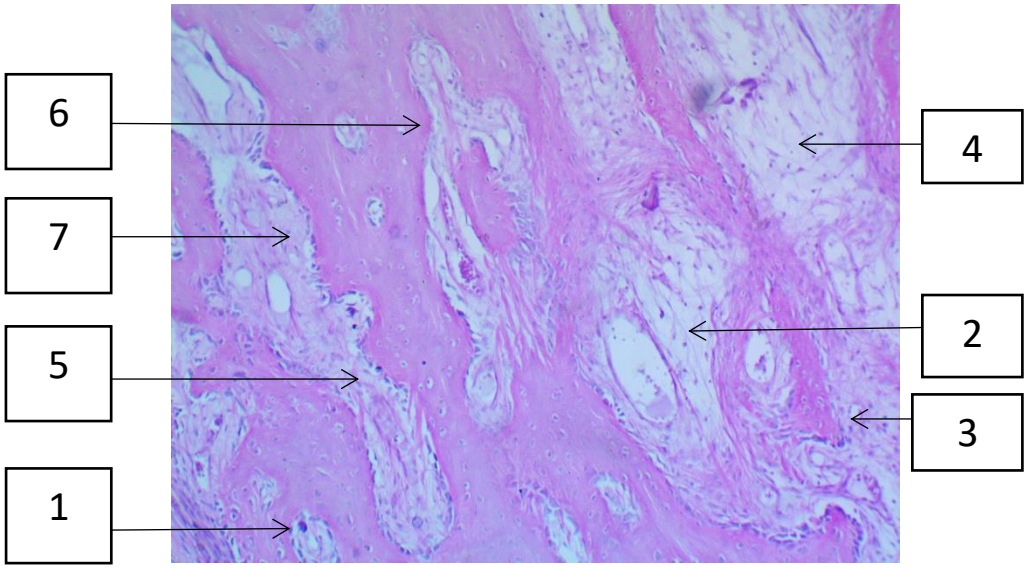


Fig 32: photomicrograph for Blood clot group in healthy dogs at 5 week scarification period showing;- inflammatory cells(1) , large dilated blood BVs(2) ,well organized granulation tissue (3), thin collagen fibrils(4), bony trabeculae, (5)woven bone(6), osteoblast(7) . (H&E.100x)

Diabetic dogs:

Histological examination of section stained with H&E(at 5 weeks) showed that, inflammatory cell , well organized granulation tissues which characterized by blood capillaries . There was recruitment of osteoblast with early formation of small islands of bone trabeculae with very thin thickness, very wide bone marrow spaces, reflects .condensed fibrils.

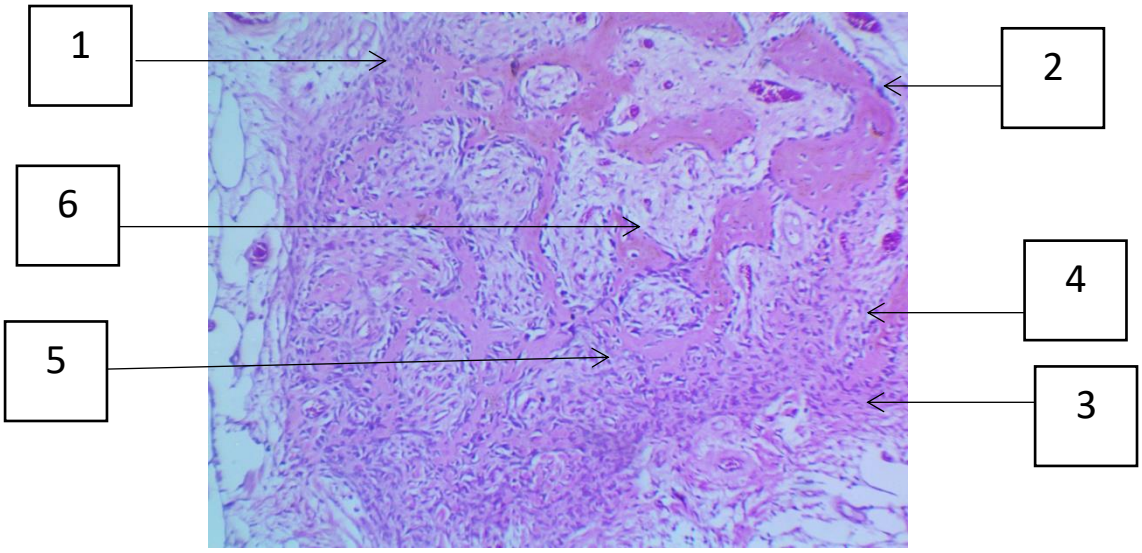


Fig 33: photomicrograph for HA group in diabetic dogs at 5 week scarification period showing:. inflammatory cells(1) , wdilated blood BVs(2) ,well organized granulation tissue(3) , thin condensed collagen fibrils(4), thin bony trabeculae(5), osteoblast(6) . (H&E.100x)

C- PRF group:

Healthy dog

Histological examination of section stained with H&E(at 5 weeks) revealed that, the presence of well organized granulation tissues which characterized by multiple dilated blood capillaries . There was osteoblast with early formation of islands of thick bone trabeculae with variable bone marrow spaces.

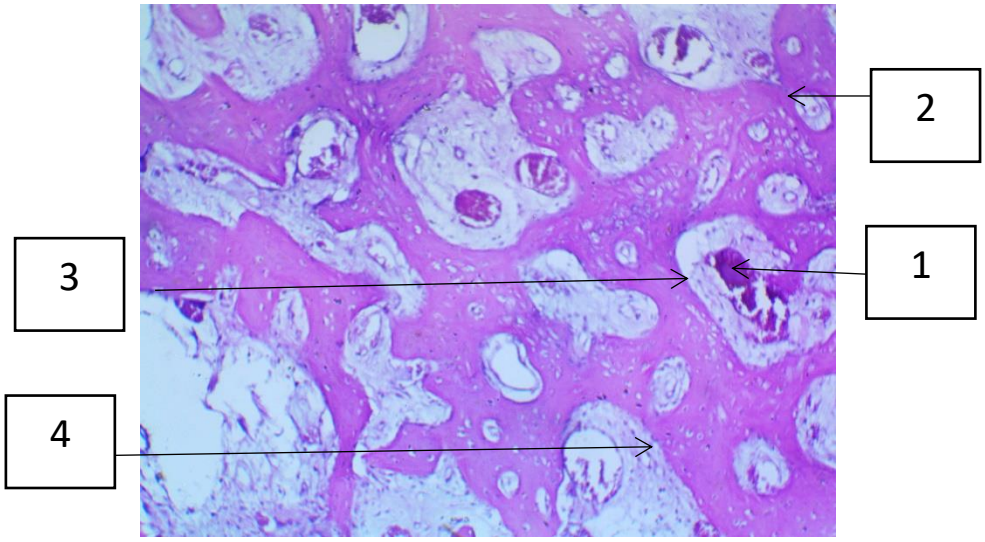


Fig 34: photomicrograph for PRF group in healthy dogs at 5 week scarification period showing:-blood vessels(1), (2),bony trabeculae(2), bone marrow spaces(4), woven bone(4)

Diabetic dogs:

Histological examination of section stained with H&E(at 5 weeks) revealed that, the presence of blood clot infiltrated with inflammatory cell infiltration, well organized granulation tissues which characterized by multiple small blood capillaries regarding low angiogenic activity compared to healthy one. There was recruitment of osteoblast with early formation of islands of very thin bone trabeculae .

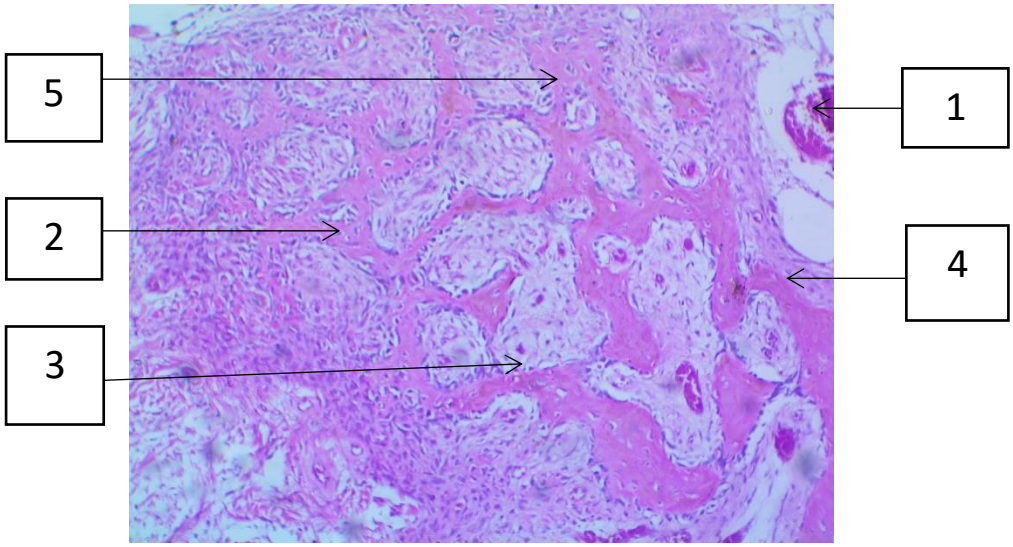


Fig 35: photomicrograph for PRF group in diabetic dogs at 5 week scarification period showing: remnant of blood clot**(1)** ,well organized granulation tissue**(2)**,bone trabeculae **(3)**,very wide bone marrow spaces**(4)**,osteoblast**(5)**.

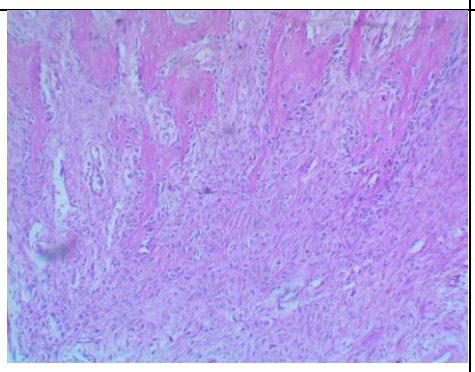
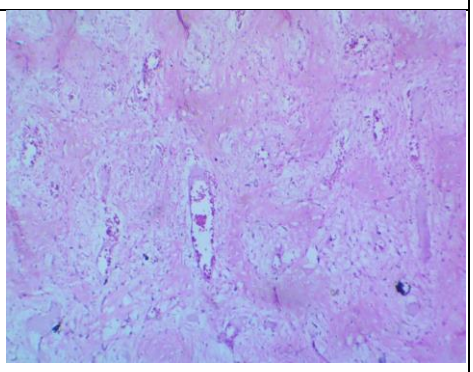
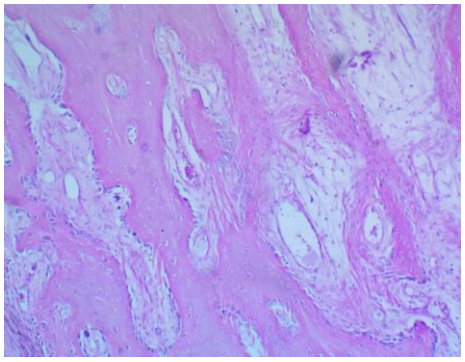
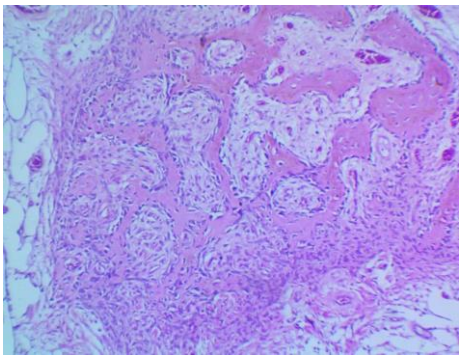
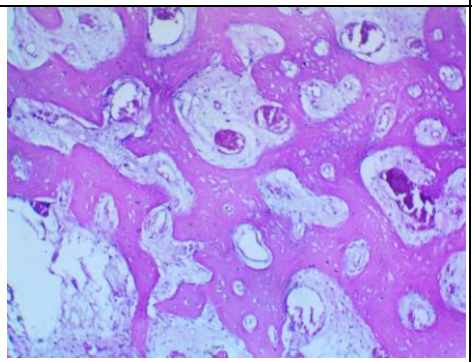
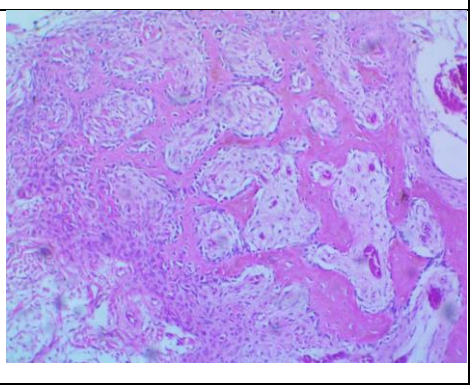
Five weeks	Healthy	Diabetic
HA		
Blood clot		
PRF		

Fig 36 photomicrograph at five weeks sacrification period in healthy and diabetic dogs showing: a) HA in healthy dogs, b) HA in diabetic dogs, c) blood clot in healthy dogs, d) blood clot in diabetic dogs, e) PRF in healthy dogs, f) PRF in diabetic dogs.

3 - Nine weeks scarification period:

A- HA group:

Healthy dogs:-

Histological examination of section stained with H&E(at 9 weeks) revealed that, the presence of well organized granulation tissues. There was osteoblast with dramatic features reflecting formation bone trabeculae with bone marrow spaces. The spread of bony trabeculae appears clearly from the cenrer (bone marrow spaces) to be anastomoed with each other to form woven bone. Early formation of circumfrential lamellae appears at some areas .

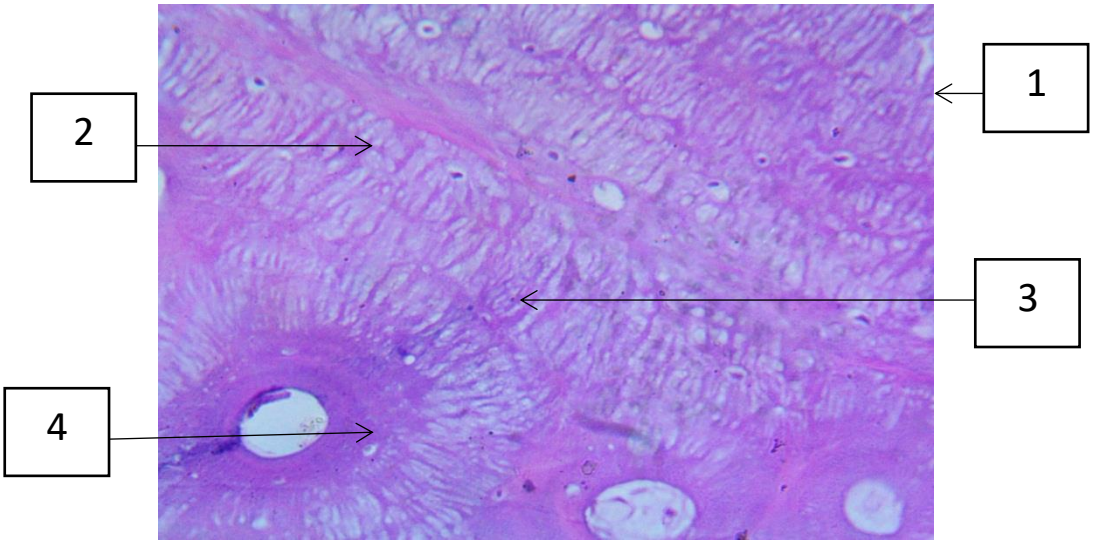


Fig 37 photomicrograph for HA group in healthy dogs at 9week scarification period showing: well organized granulation tissue(1),bony trabeculae(2), interstitial lamellae(3),bone marrow spaces(4).(H&E.100x)

Diabetic dogs: -

Histological examination of section stained with H&E(at 9 weeks) revealed that, the presence of remanent of blood clot with inflammatory cell infiltration, well organized granulation tissues which characterized by multiple blood capillaries regarding . There was recruitment of osteoblast with early formation of bone trabeculae with variable thicknes reflecting formation woven bone formation.

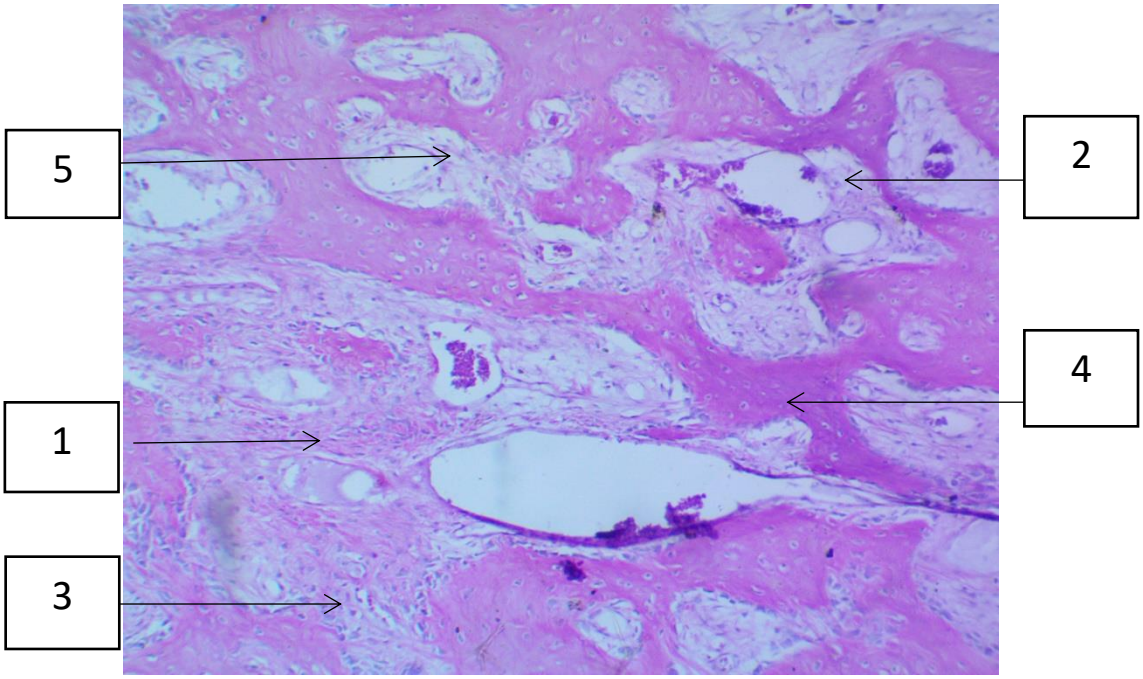


Fig 38: photomicrograph for HA group in diabetic dogs at 9week scarification period showing:-well organized granulation tissue(1), blood cappilaries (2),osteoblast(3), bony trabeculae(4) and bone marrow spaces (5).

B- Blood clot group:

Healthy dogs: -

Histological examination of section stained with H&E(at 9 weeks) revealed that, well organized granulation tissues which characterized by multiple dilated blood capillaries . There was recruitment of osteoblast with early formation of woven bone with variable thickness of bony trabeculae as well as bone marrow spaces and the trabeculation not completely anastomosed with each other.

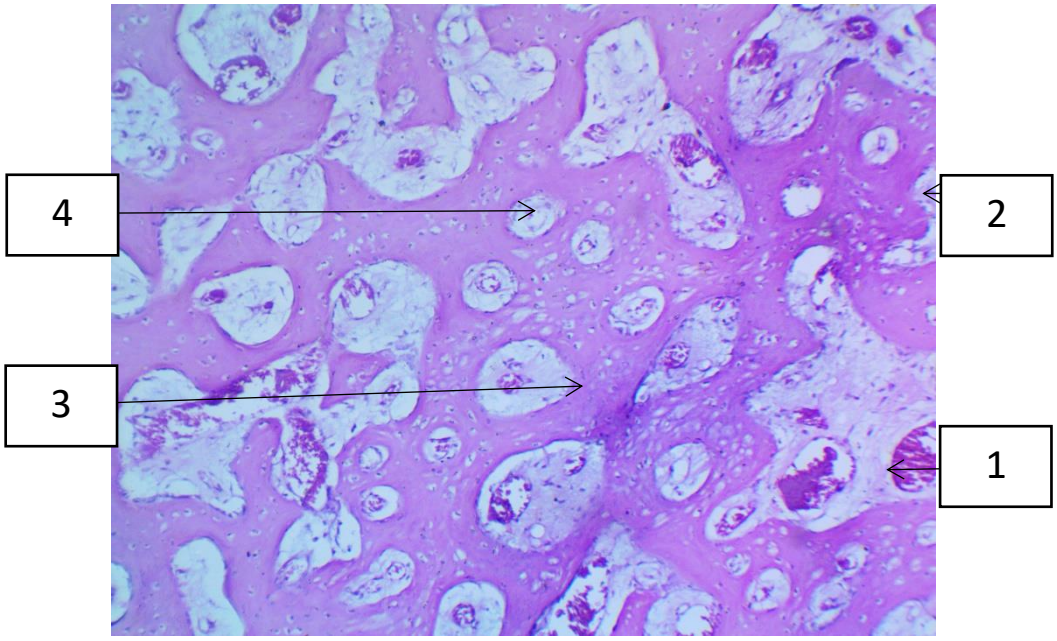


Fig 39 photomicrograph for Blood clot group in healthy dogs at 9week scarification period showing: blood capillaries(1), bone trabeculae with variable thickness (2) , bone marrow spaces(3), woven bone(4). (H&E.100x)

Diabetic dogs:-

Histological examination of section stained with H&E(at 9 weeks) revealed that, remanent of blood clot infiltrated with fibroblast, well organized granulation tissues which characterized by multiple blood capillaries . Osteoblast with early formation of woven bone with variable thickness of bony trabeculae and very wide bone marrospaces

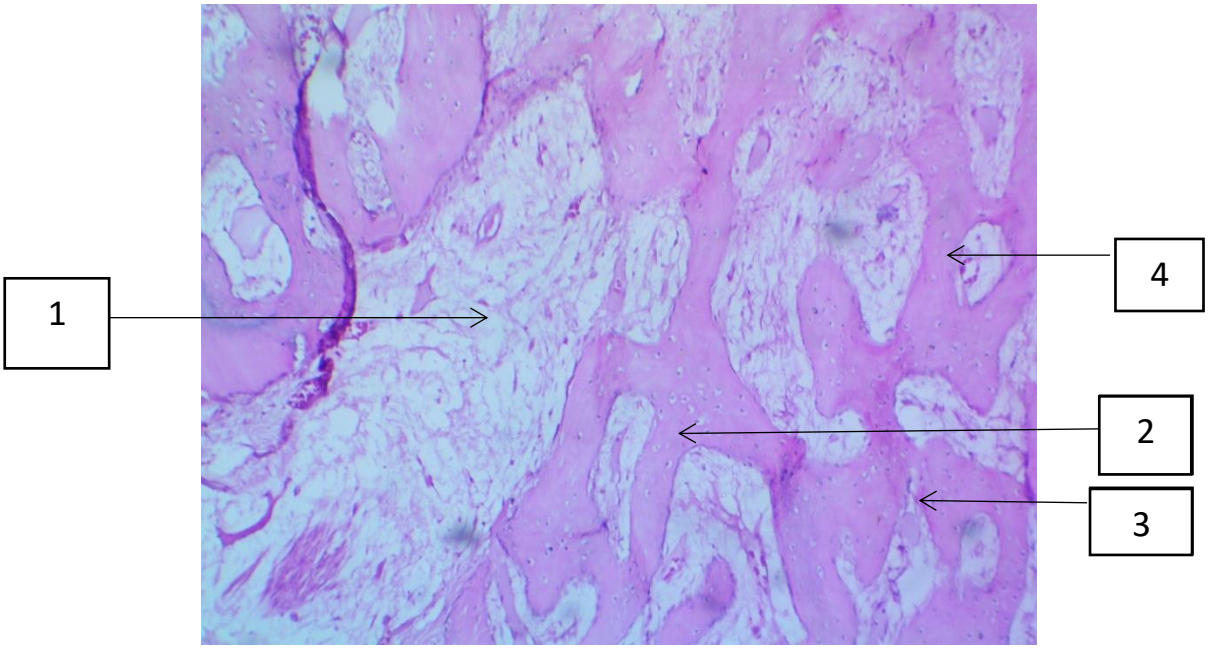


Fig 40: photomicrograph for Blood clot group in diabetic dogs at 9 week scarification period showing: blood vessels(1),bone trabeculae(2), bone marrow spaces(3), woven bone(4)

C- PRF group:

Healthy dogs: -

Histological examination of section stained with H&E(at 9 weeks) revealed that, the presence of multiple blood capillaries formation of islands of very thick bone trabeculae with variable bone marrow spaces. woven bone formation ,at some areas there was the same dramatic appearance of trabculation with spread of bony trabeculae from the center .most ares of the slides reflects formation of lamellar , at this stage woven bone (non- lamellar bone) still present with low grades however the most dominant features was replacement of no-lamellar(woven bon) by lamellar one .

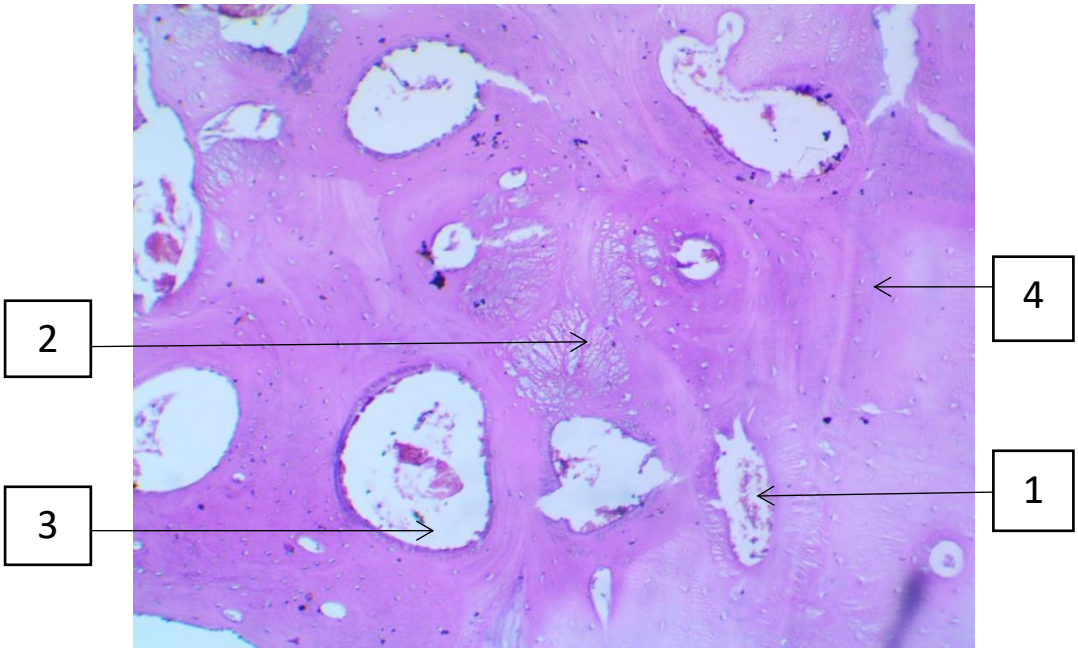


Fig 41: photomicrograph for PRF group in healthy dogs at 9 week scarification period showing: blood vessels(1), woven bone(2), bone marrow spaces(3) and lamellar bone(4). (H&E.100x)

Diabetic dogs:-

Histological examination of section stained with H&E(at 9 weeks) revealed that, the presence well organized granulation tissues which characterized by multiple blood dilated blood capillaries . woven bone with variable thickness of bony trabeculae and very wide bone marrow spaces

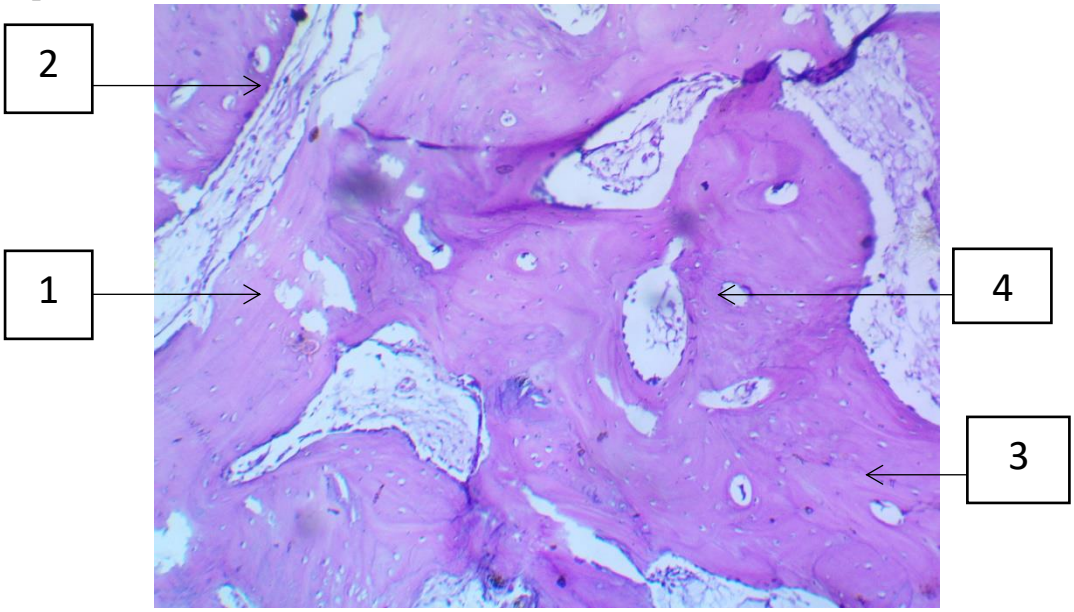


Fig 42: photomicrograph for PRF group in diabetic dogs at 9 week scarification period showing: well organized granulation tissues(1) blood vessels(2), woven bone(3), bone marrow spaces(4) . (H&E.100x)

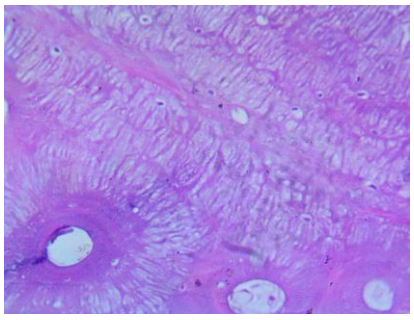
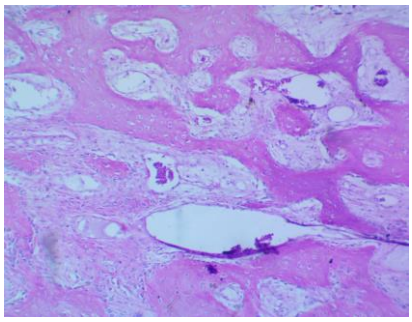
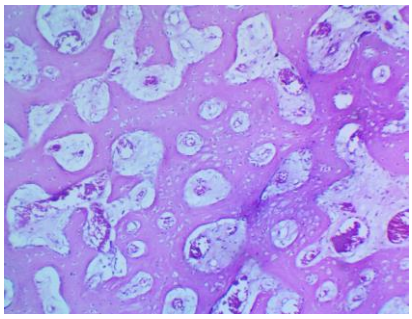
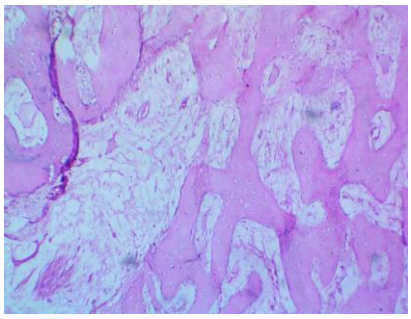
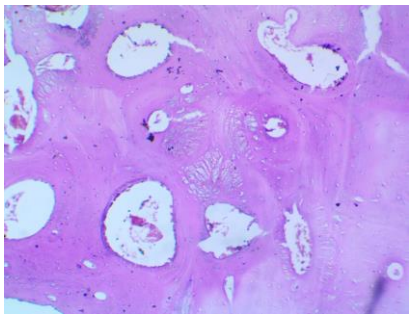
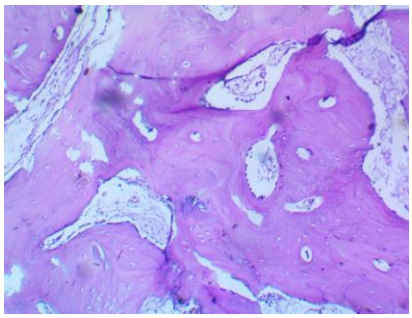
Nine weeks	Healthy	Diabetic
HA		
Blood clot		
PRF		

Fig 43: photomicrograph at nine weeks sacrification period in healthy and diabetic dogs showing: a) HA in healthy dogs, b) HA in diabetic dogs, c) blood clot in healthy dogs, d) blood clot in diabetic dogs, e) PRF in healthy dogs, f) PRF in diabetic dogs.

3 - Thirteen weeks scarification period:

A- HA group:

Healthy dogs: -

Histological examination of section stained with H&E(at 13 weeks) revealed that, dilated blood capillaries, There was osteoblast with dramatic features reflecting formation bone trabeculae with bone marrow spaces. The spread of bony trabeculae appears clearly from the cenrer (bone marrow spaces) to be anastomoed with each other to form woven bone with higher rat comparing to the same group in the previous period. Early formation of circumfrential lamellae appears at some areas .

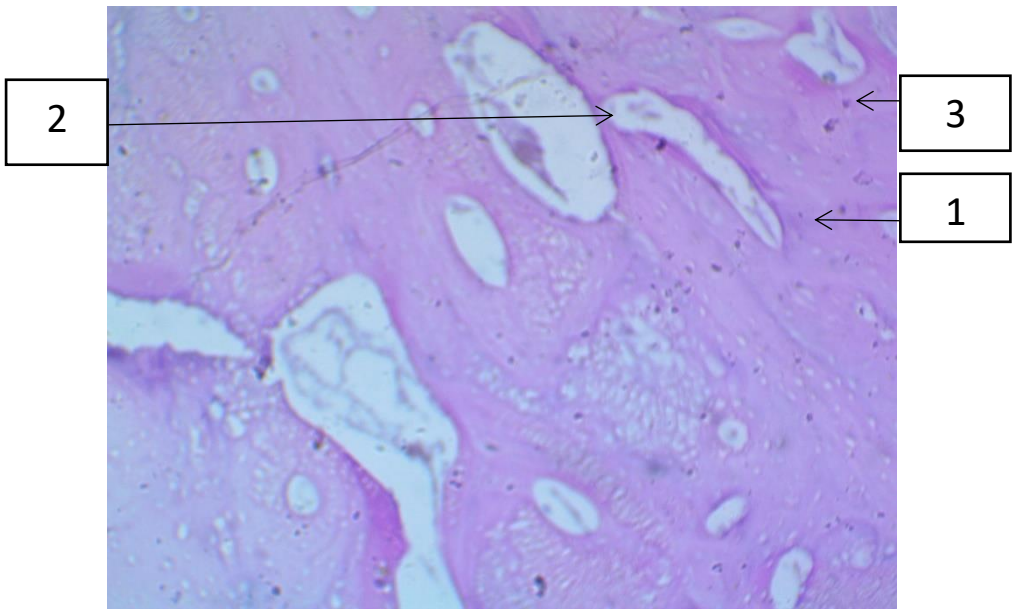


Fig 44 photomicrograph for HA group in healthy dogs at 13bweek scarification period showing:dilated blood capillaries(1). Bone marrow spaces(2), thick bony trabeculae(3)

Diabetic dogs: -

Histological examination of section stained with H&E(at 13 weeks) revealed that, the presence of well organized granulation tissues. Dilated blood vessels There was osteoblast, bone trabeculae with bone marrow spaces with variable thickness,. The spread of bony trabeculae appears clearly from the cenrer (bone marrow spaces) to be anastomoed with each other to form woven bone. Early formation of haverian system.

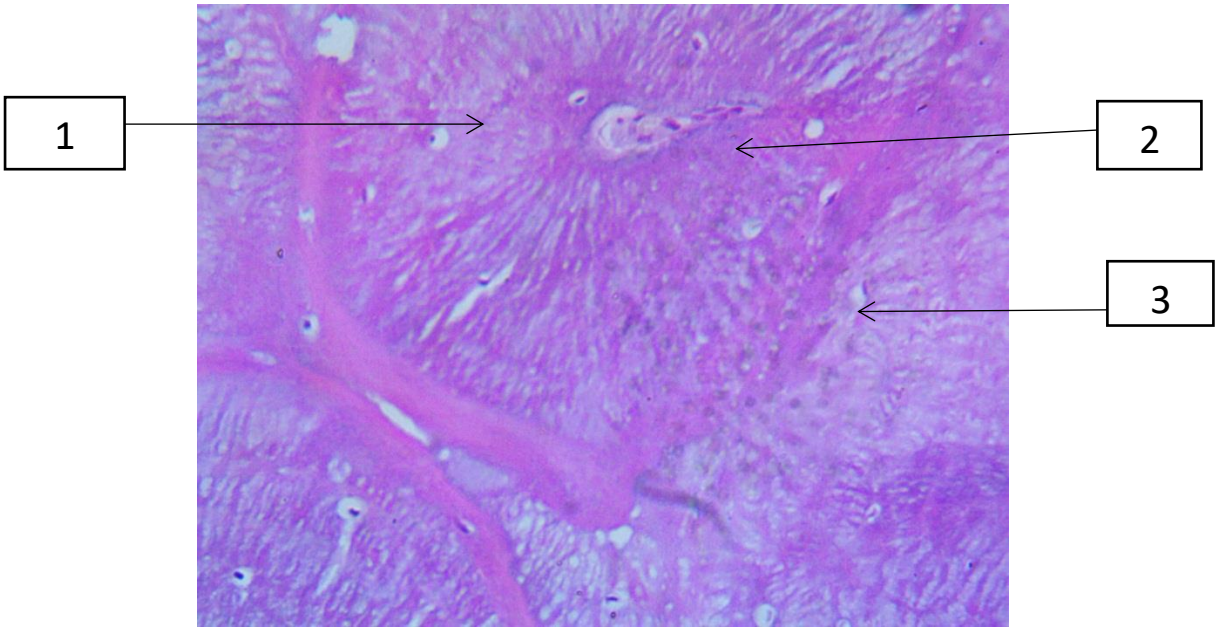


Fig 45: photomicrograph for HA group in diabetic dogs at 13 week scarification period showing: well organized granulation tissue(1), B,Vs(2), spread of bony trabeculae(3)

B-Blood clot group:

Healthy dogs: -

Histological examination of section stained with H&E (at 13 weeks) revealed that, blood capillaries, There was osteoblast, very thick anastomosing bony trabeculae with very narrow bone marrow spaces, the most dominant features of this period replacement of woven bone (non - lamellar) by lamellar one.

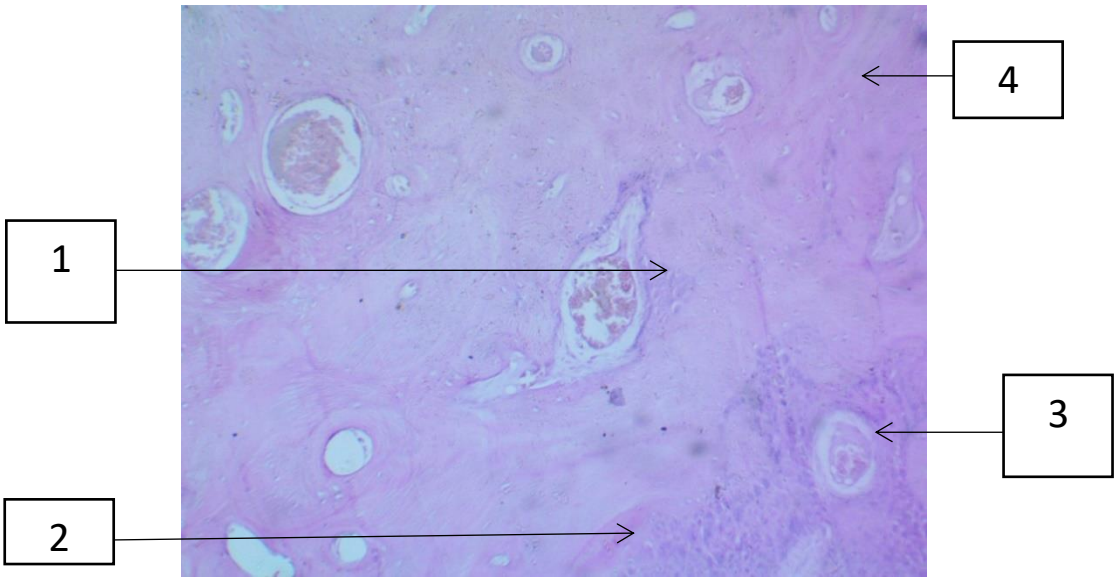


Fig 46: photomicrograph for Blood clot group in healthy dogs at 13 week scarification period showing:- osteoblast(1), B.Vs(2),bone marrow spaces(3), lamellar bone(4)

Diabetic dogs:-

Histological examination of section stained with H&E(at 13 weeks) revealed that, the presence of well organized granulation tissues. blood vessels There was osteoblast, bone trabeculae with bone marrow spaces with variable thickness,. The spread of bony trabeculae appears clearly from the cenrer (bone marrow spaces) to be anastomoed with each other to form woven bone.

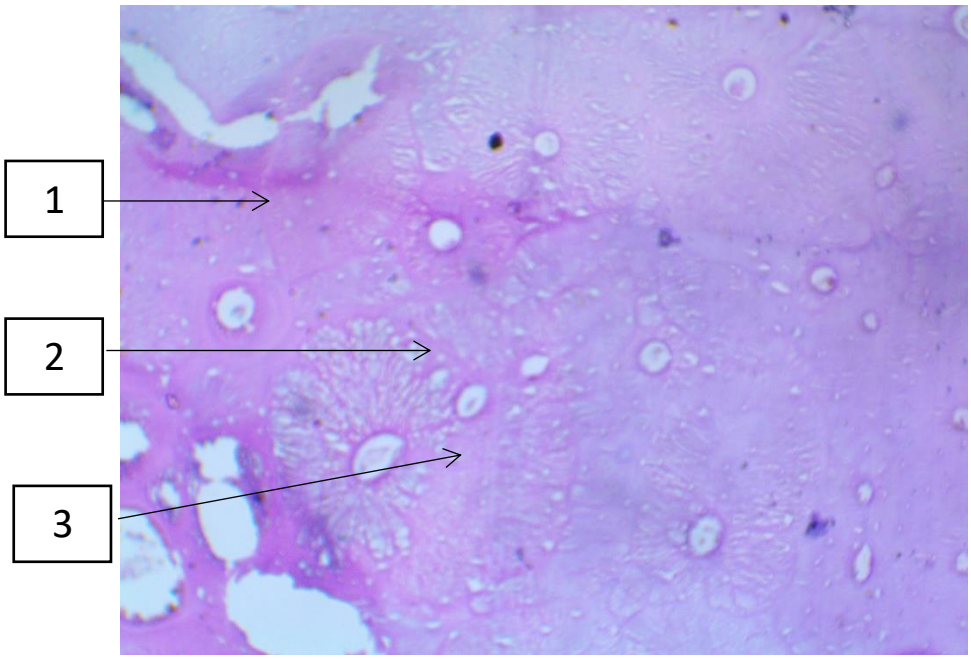


Fig 47:- photomicrograph for Blood clot group in diabetic dogs at 13 week scarification period showing: woven bone (1).bony trabeculae(2), bone marrow spaces(3)

C- PRF group:

Healthy dogs:-

Histological examination of section stained with H&E(at 13 weeks) revealed that, the presence of multiple blood capillaries however replacement of no-lamellar(woven bon) by lamellar one appears clearyly at this section ,haversian system, circum frensial lamellae also ppears .

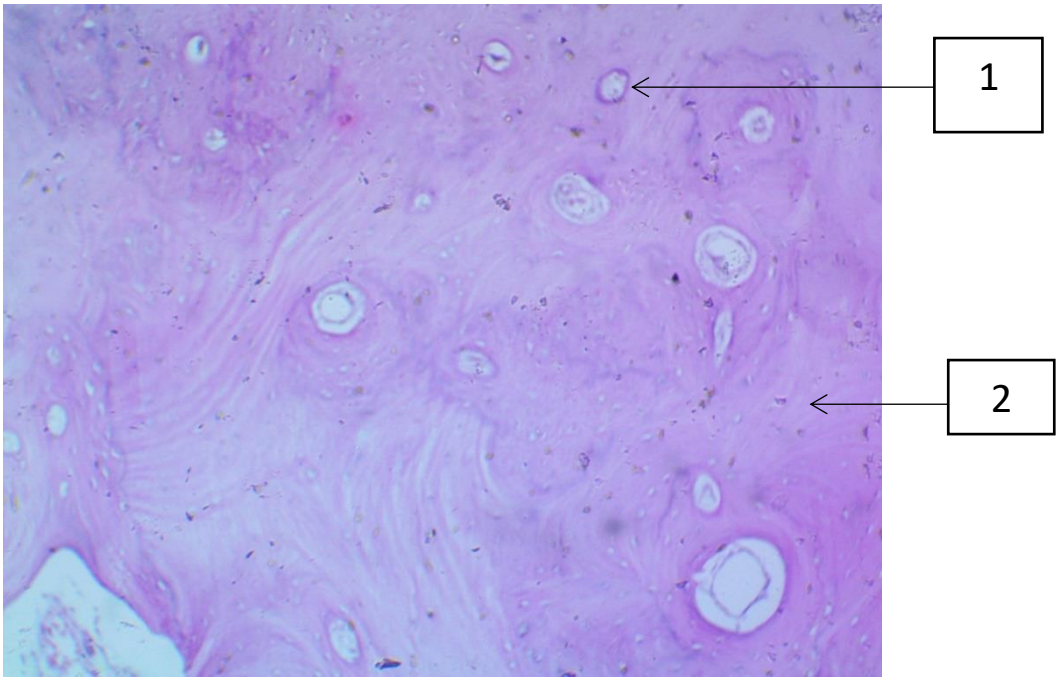


Fig 48: photomicrograph for PRF group in healthy dogs at 13 week scarification period showing: blood vessels(1), lamellar bone(2) (H&E.100x).

Diabetic dogs: -

Histological examination of section stained with H&E(at 13 weeks) revealed that, the presence of multiple dilated blood capillaries very thick bone trabeculae with variable bone marrow spaces. woven bone formation , however it was replaced by lamellar bone at some areas .

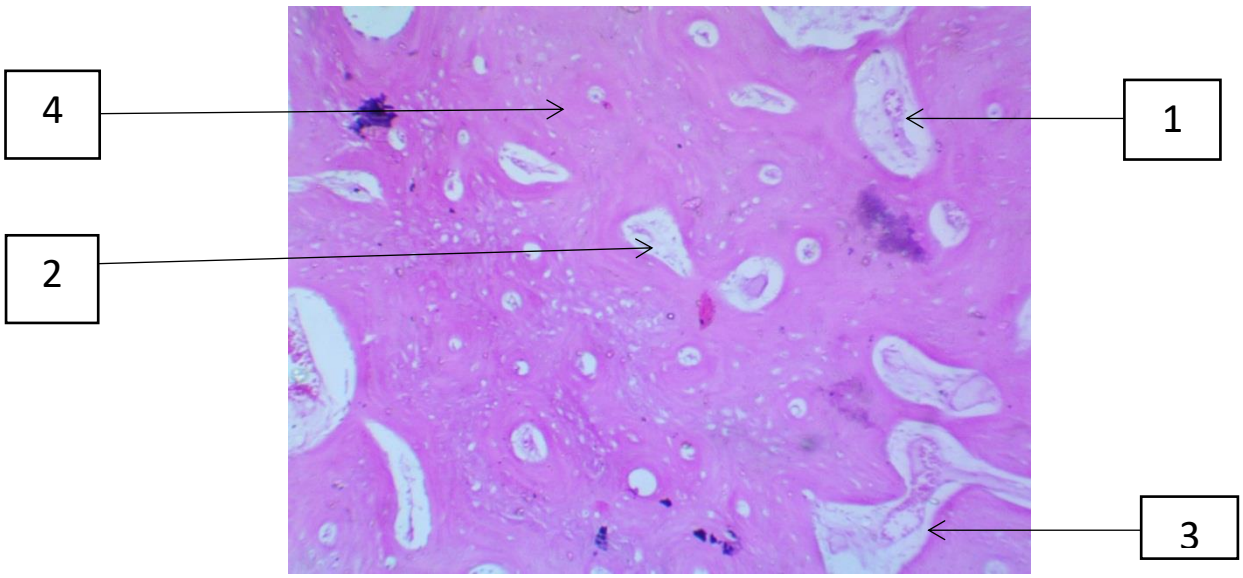


Fig 49:photomicrograph for PRF group in diabetic dogs at 13 week scarification period showing:- B.Vs(1), bone marrow spaces(2), woven bone(3) ,and lamellar bon(4)

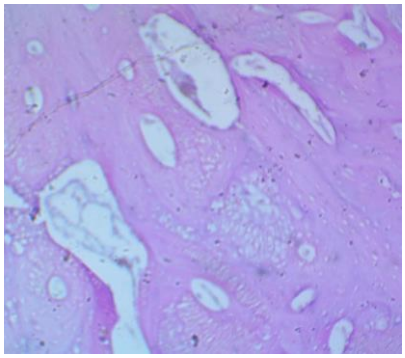
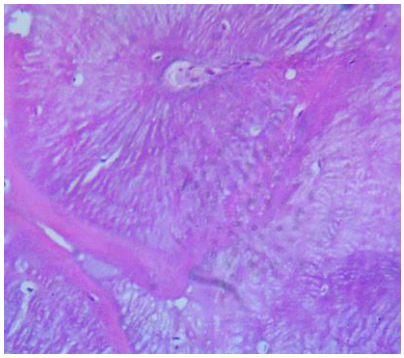
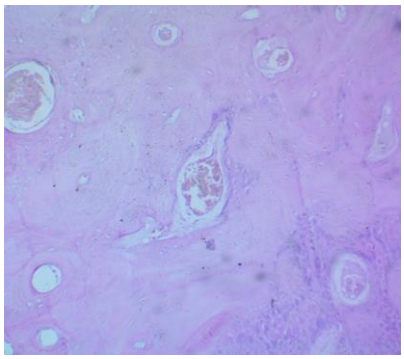
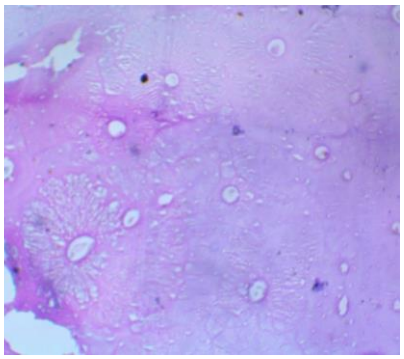
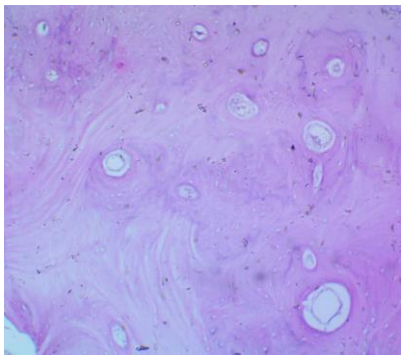
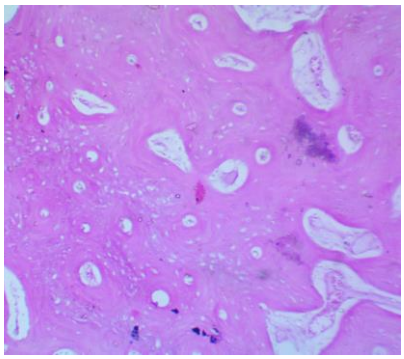
Thirteen weeks	Healthy	Diabetic
HA		
Blood clot		
PRF		

Fig 50: photohistogram at 13 weeks sacrification period in healthy and diabetic dogs showing: a) HA in healthy dogs, b) HA in diabetic dogs, c) blood clot in healthy dogs, d) blood clot in diabetic dogs, e) PRF in healthy dogs, f) PRF in diabetic dogs.

Discussion

The healing of periapical lesions after root canal treatment (RCT) is not the result of the curative action of the treatment. The process of healing begins with inflammation, and is resolved by the clearance of the immunogen that induces the immune response. Then, the periapical tissue itself carries out the healing of the periapical lesion, by repair or by a combination of repair and regeneration, depending on the host's reparative response working properly. The ultimate objective of RCT is to achieve wound healing by removing the source of bacterial antigens and toxins, allowing chronic inflammatory tissue to become reparative tissue. Some systemic conditions such as diabetes, cardiovascular diseases, osteoporosis, HIV infection, inflammatory bowel disease, and others, can influence or interfere in the repair of periapical tissues and increase the susceptibility of the host to infection or impair the tissue reparative response, maintaining the inflammatory process and periapical bone resorption after RCT. This can cause the failure of RCT and even the need for extraction of the affected tooth[(172).]

Diabetes mellitus is considered a group of complex metabolic disorders caused due to deficiency in insulin secretion as a result of dysfunction of pancreatic β-cell and/ or insulin resistance in the liver and muscle. The dental Pulp of patients with Diabetes mellitus may tend to limit collateral circulation, impaired immune response, increased risk of acquiring pulpal diseases, or necrosis of the pulp. hyperglycemia may also be a stimulus for bone resorption, which inhibits the differentiation of osteoblasts and reduces bone recovery [(173,174)]

Periradicular lesions of pulpal origin have been noticed in 74-97% of a diabetic patient who has teeth with symptomatic pulpitis[(175,176,177)].

Diabetes may also influence the development and the course of periradicular diseases. In diabetic patients, the initial periradicular lesion may increase in size, even with proper endodontic treatment [178]. In such cases, periradicular surgery is indicated to remove the periapical lesion and allow the regeneration of new bone which can be facilitated by placing a bone graft into the periapical defect. Different types of bone grafts are available for dental surgical procedures. These include autografts, allografts, xenografts, and alloplasts. The ideal bone replacement material should be clinically and biologically inert, noncarcinogenic, easily maneuverable to suit the osseous defect, and should be dimensionally stable. It should serve as a scaffold for bone formation and slowly resorb to permit replacement by new bone[179]

The purpose of this study was to radiographically and histologically evaluate the effect of platelet-rich fibrin and hyaluronic acid on bone regeneration after periradicular surgery in healthy and diabetic dogs

.

The selection of the dogs as an animal model in the present study is based on the anatomical, physiological, and bio-mechanical similarity to human beings with better experimental manipulation for various diagnostic and functional tests than rodents especially in studying Diabetes Mellitus[180,157].

The age of the dogs ranged between 1 and 2 years as premolar teeth completely erupted and the animals can tolerates general anesthesia and surgicalprocedures.

Out of seventeen dogs, sixteen dogs were selected with eight dogs per group based on 95% confidence power. After clinical and radiographic examination one dog was excluded due to congenital missing of second premolars Randomization of the dogs was done using a randomizer software to avoid bias followed by induction of diabetes in diabetic group.

There are several methods for diabetes induction in experimental animals such as surgical, genetic, and chemical methods. The surgical and genetic methods of diabetes induction are associated with a high percentage of animal morbidity and mortality. The chemical induction of diabetes appears to be the most popularly used procedure for inducing diabetes mellitus in experimental animals. The foremost drug-induced diabetic model is the alloxan diabetes that is capable of inducing type I diabetes mellitus in experimental animals through the selective necrosis of the βcells of pancreatic islets as it preferentially accumulates in the beta cells as glucose analogues. Alloxan-induced diabetes model appears to be the most reliable and easily reproducible method of inducing diabetes mellitus in experimental animals [(181)].

Compassion with dogs was taken into consideration so efforts were made to minimize the number of times the doges were exposed to general anesthesia as reported, exposure to general anesthesia may affect the general health of the dogs, so root canal treatment and periradicular surgery were made in single appointment[(182)].

PRF was prepared immediately before the beginning of the surgery allowing the PRF clot to remain in the tube for a period longer than the recommended time, at least 60 minutes may result in a more complete physiological reaction taking place leading to the formation of PRF that had significantly higher concentrations of platelets compared with fresh prepared PRF [(183)] .

PRF is a biomaterial that contains living cells[184] and In order to sustain cell viability over time, PRF was stored in the same tubes in which it was formed and still sealed until using it to prevent exposure to other environmental factors that may contaminate it. The remaining serum acts as a natural isotonic solution that sustains cell survival [171].

Disinfection of the operative field followed by rubber dam application was of paramount importance to allow for an aseptic field while working as It is considered the standard of care in root canal treatment because of the advantages that it offers concerning infection Blood clot, dogs protection, and treatment efficacy. [185]

Root canal treatment for mesial and distal roots were done and Sodium hypochlorite irrigant solution 5.25% was used due to its antimicrobial and tissue-dissolving capabilities[186] The distal root of the second and fourth premolars were selected for surgery rather than other roots as there is a large distance of bone and soft tissue in between and opposite to the third premolar. Separate two rectangular flaps were elevated opposite to each root to avoid seepage of experimental materials between experimental cavities. The rectangular flap was selected because it is extremely easy to perform, replace, and suture, resulting in low morbidity. This flap has great mobility and permits it to reach even very long roots. As well as every complete full-thickness flap, it exposes the entire buccal cortical with better exposure to the surgical field[187]

A CNC (Computer Numerically control) metallic template was manufactured to standardize the bone cavity size and improve the accuracy of cutting. Its metallic nature allow to resist cutting by bur when it contacts the template. [188]. The metallic template was replaced with a new one when the old one was affected by cutting.

A 3mm of the root apices was resected to reduce 98% of the apical ramifications and 93% of the lateral canals [189]. As these percentages are very similar at 4 mm from the apex, Kim and Kratchman[190] recommended root-end amputation of 3 mm, since this leaves an average of 7 to 9 mm of the root, providing sufficient strength and stability. A root-end resection of less than 3 mm did not remove all of the lateral canals and apical ramifications, therefore, increasing the risk of reinfection and eventual failure.

MTA was used to seal the roots with the periradicular surgery as it has been shown the highest apical healing (90.4%) in comparison to other root-end filling materials[191,,192]. it can induce bone, dentin, and cementum formation in vivo. [193,194,195]

Following the procedure of filling the bony defect, the flap was repositioned and sutured with 3-0 resorbable suture vicryl suturing material as it has been proven clinically excellent because it does not allow adherence of plaque and is well suited for handling. In addition, it shows no intensive local reaction, contributes to faster healing of wounds with a lower incidence of dehiscence, doesn't need removal, and remains in place for adequate time for healing. [196]

Measuring the bone mineral content and bone mineral density using the DEXA method because it is a simple method, suitable for all ages, not dependent on other measurements, and the associated x-ray dose is very low [197] in addition Excellent accuracy and precision of DXA[198].

Histological examination was done to explain the healing power of different materials at different times as it is directly related to the newly formed bone.

The effect of time on the bone mineral content in both healthy and diabetic dogs revealed that the highest mineral content was found after a thirteen-week sacrification period followed by nine weeks and five-week while the lowest mineral content was found after a one-week sacrification period.

This may be attributed to that the healing after periarticular surgery occurs by mesenchymal cells that differentiate into osteoblast, This cell will synthesize bone matrix in the periphery and the mesenchymal cells continue to differentiate into osteoblasts leading to woven bone formation that will be reshaped and replaced by mature lamellar bone.[199]

A potential explanation for the extended time required for apical lesions to heal may be the persistence of an activated state of macrophages and lymphocytes within the lesion. Activated macrophages and activated lymphocytes are the primary sources of IL-1β and TNFβ cytokines, which represent the main osteoclast-stimulating activity in the apical lesion [200] Such an activated state may outlive its biological purpose as an essential element of the host's defensive response in the area of the lesion. As long as such activation persists and these cytokines are produced, the osteoclastic potential of the lesion persists, keeping the vast surrounding osteogenic potential at bay. When the osteoclastic activity subsides, the lesion finally heals[201 ,202]

Harrison and Jurosky[203] described the osseous response to excisional wounding at postsurgical intervals ranging from 1 to 28 days. The results revealed that excisional defects were initially filled with a coagulum which was subsequently replaced by granulation tissue emanating from the endosteal tissues. Cortical and trabecular bone forming the wound edges was devitalized, as evidenced by an absence of osteocytes in the peripheral lacunae. At 14 days post-surgery, woven bone trabeculae occupied most of the defect, with the more superficial trabeculae in direct contact with a thick band of dense fibrous connective tissue separating the osseous defect from overlying mucosal tissues. Within the defect, new bone was deposited on devitalized bone without evidence of preceding osteoclastic activity. At 28 days, the woven bone trabeculae were more mature and functioning periosteum was now active in the repair of the cortical plate.

Concerning the effect of material on both healthy and diabetic dogs, There was a significant difference between PRF and Blood clot and between PRF and hyaluronic acid without a significant difference between the Blood clot and hyaluronic acid and this results may be due to the effect of PRF which gradually increases the osseous healing at every follow-up period[204]. The results of this study were in agreement with other studies and showed that PRF acts as an appropriate scaffold with a strong fibrin structure that optimally supports the transplanted mesenchymal cells and allows for a gradual release of growth factors over a long period ranging from 7 to 28 days[(205),(206),207 ,208,209]. Also, it was found that PRF has a role in phosphorylated extracellular signal-regulated protein kinase expression and suppresses osteoclastogenesis by promoting the secretion of osteoprotegerin in osteoblasts cultures[(210)]. Besides that, PRF may hasten natural healing in immuno-compromised patients, and those with a history of radiotherapy because it can stimulate natural defense mechanisms[(211)]. Furthermore, PRF is rich in vascular endothelial growth factor VEGF which has been found to have its topical application and enhances neovascularization at the site of injury with a clinically significant effect. The mechanism for this effect is through stimulation of local angiogenesis, enhanced expression of growth factors including PDGF and FGF-2, and a systemic mobilization of bone marrow-derived stem cells [212)].

The hyaluronic acid and Blood clot groups showed less bone formation among the different periods This effect may have different explanations.

The effects of hyaluronic acid are transmitted by different receptors. Entwistle et al [213]. documented that the effects of hyaluronan are transmitted by CD 44- (cell surface glycoprotein) which involved in multiple cellular functions, such as cell proliferation, differentiation [214] and bone metabolism[215], RHAMM (receptor for HA-mediated motility) is a critical regulator of cell motility and cellular responses to growth factors[216], and ICAM-1-receptors (intracellular adhesion molecule-1) It

may be that the hyaluronic acid used in our study stimulated the RANKL expression(Receptor activator of nuclear factor kappa-B ligand) since The stimulation of RANKL expression itself can lead to decreased cortical thickness and a bigger intramedullary cavity under study conditions. as shown by Cao et al.[203] . THE results are in agreement with data obtained from a study performed by Oakes et al. [217] who found no signs of radiographic healing, enchondral ossification, and only a minimal periosteal ossification of defects treated solely with hyaluronic acid in a rat femoral defect model.

another pilot multicenter placebo-Blood clotled randomized clinical trial showed that Gengigel ProfVR /Gengi ProVR (0.8% HA gel) was safe but did not have an improvement in surgical wound healing.[218]

Also, A dog model of apical lesions following periradicular surgery evaluated the percentage of new bone tissue and bone trabeculae thickness and the results showed no significant differences between the groups treated by b-tricalcium phosphate (b-TCP) alone or HA1b-TCP, respectively. These results indicated that adding HA to b-TCP did not improve bone tissue healing of induced apical lesions in a dog model.[219] From the results of these studies, it can be concluded that HA could be used in conjunction with bone filler materials for the healing of bone defects.

Another explanation for the results may be that hyaluronic acid did not have the optimal molecular weight because hyaluronic acid used in our study was high-molecular-weight and high-molecular-weight hyaluronic acid (HMW-HA) as reported in many studies inhibits cell proliferation, migration of vascular endothelial cell, and angiogenesis, whereas low-molecular-weight hyaluronic acid (LMW-HA) with completely different physiological functions promotes the adhesion and proliferation of endothelial cells[220,221,222,223] , Also Pilloni and Bernard examined the effects of various molecular weights on osteogenesis in vitro

in a bone marrow ablation model in rats and revealed that low molecular weight hyaluronic acid accelerated[224].

Regarding the effect of health status, the highest bone mineral content, bone mineral density, and newly formed woven bone was found with the healthy group as diabetes may affect wound healing by locally increasing Pro-inflammatory mediators including TNF-α, IL-1β, IL-6, and IL-18 are which thought to contribute to diabetic complications [225,226]. Increased levels of TNF may decrease the ability of diabetics to downregulate other inflammatory genes and increase apoptosis, which has been shown to reduce bone coupling in diabetic animals[144]

Uncontrolled diabetes increases and prolongs inflammation, which may lead to enhanced osteoclastogenesis. Hyperglycemia may affect bone through enhanced expression of pro-inflammatory cytokines such as TNFα, which reduces osteoblast differentiation, and osteoblast activity and increases osteoblast apoptosis [227,13].

Diabetes decreases hemostasis, inflammation, and angiogenesis. Wounds in diabetic patients showed altered blood flow, impaired neutrophil anti-microbial activity, and a dysfunctional inflammatory state associated with abnormal chemokine expression. Also, it was found that the number of growth factors essential for wound healing, including FGF-2 and PDGF-B, have also been found to reduced in experimental diabetic wounds[228,229,230,132]. Insulin binds to receptors on osteoblasts and stimulates anabolic effects [231]. It is possible that the reduced insulin levels or reduced insulin signaling in osteoblasts negatively affects bone and contributes to reduced bone formation caused by diabetes [155]. The results of this study rejected the null hypothesis as the tested groups showed different bone regeneration capabilities.

Conclusion

Within the parameter of this study and depending on radiographic and histologic the finding:

1- Platelet rich fibrin is valuable in bone healing after periradicular surgery.
2- Platelet rich fibrin improves wound healing in diabetic animals undergoes periradicular surgery
3- Hyaluronic acid has similar effect to blood clot on bone healing after periradicular surgery.

Recommendation

1- Further extended follow up periods are needed to monitor the bone healing power of platelet rich fibrin and hyaluronic acid
2- Further research should be done to evaluate bone healing using different evaluation mechanisms.
3- Further research should be done to evaluate the effect of platelet rich fibrin on individuals with other systemic diseases.
4- Further research should be done to improve the healing power hyaluronic acid after periradicular surgery.

Summery

Surgical management of periradicular lesions results in development of bony defects of variable sizes around the apex of the involved teeth that needs bone substitute to enhance bone regeneration. Recent bone healing studies aim to accelerate bone regeneration to improve treatment outcome by using bone substitute of different types to fill the bony defect and induce healing, a process that include several drawbacks ranging from slight morbidity to disease transmission

Recently PRF which is a healing biomaterial with a great potential for bone and soft tissue regeneration, without inflammatory reactions promoting hemostasis, bone growth, and maturation.

In the present study PRF was examined as a bone substitute and compared to hyaluronic acid and blood clot in healthy and diabetic dogs. This study was approved by the Ethical Committee at Faculty of Dentistry, Al Azhar University based on the guidelines and regulations. The procedures were carried out in the Department of Veterinary Surgery, General veterinary hospital in Alabbasyya, Cairo, Egypt.

Based on power study, 16 dogs were used for this study in which 3 bony defects were created in each jaw in relation to the distal roots of the second and fourth premolar teeth. sacrification of the dogs was done at 1,5,9 and 13weeks. The dog's mandibles were harvested and scanned with DEXA to measure bone mineral content and bone mineral density at each defect site then the specimens were send for histological examination.

Statistical analysis was performed with IBM® SPSS® Statistics Version. Data were represented by total number and percentage for each method used in the study. Significance was set when P value ≤ 0.05 with confidence level 95 %.

The result showed that at all stages of follow up PRF group was superior to the other groups in both healthy and diabetic groups and there was no difference between hyaluronic acid and blood clot group.

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الملخص العربى

يعتبر الحل الجراحي للآفات حول جذورالاسنان الناتج من تعفن اللب او المعالجة الخاطئة حلا مثاليا الا انة ينتج عنة ظهور عيوب عظمية بأحجام مختلفة حول قمة الأسنان المصابة والتي تحتاج إلى بديل للعظام لتحسين نتائج العلاج لملء الخلل العظمي والحث على الشفاء .يتم تحضير البدائلا العظمية باخذ رقعة عظمية من نفس المريض من مكان اخر او باستخدام بدائل العظام بأنواع مختلفة طبيعة او مصنعة وفى كل الاحوال فان هذة العملية تتضمن عدة عيوب تتراوح من الاعتلال الطفيف الى نقل العدوى.

على الجانب الاخرتهدف الدراسات في الآونة الأخيرة الى استخدام الصفائح الدموية الغنية بالفيبرين وهو مادة حيوية علاجية ذات إمكانات كبيرة لتجديد العظام والأنسجة الرخوة ، بدون تفاعلات التهابية بمفردها او مع البدائل العظمية ، مما يعزز الإرقاء ونمو العظام والنضج.

فى الدراسة الحالية تم اختبار استخدام الصفائح الدموية الغنية بالفيبرين لملء الخلل العظمي المفتعل ومقارنته بحمض الهيالورونيك و الجلطة الدموية في الكلاب الصحيحة والمصابة بداء السكرى المفتعل.

تمت الموافقة على هذه الدراسة من قبل الجنة العلمية بكلية طب الأسنان بجامعة الأزهر بناءً على الضوابط واللوائح وتم تنفيذ الإجراءات في قسم الجراحة البيطرية ، المستشفى البيطري العام في العباسية ، القاهرة ، مصر.

بناءً على دراسة القوة ، تم استخدام 16 كلبًا لهذه الدراسة (8 كلاب اصحاء و8 مصابين بداء السكرى المفتعل)و محاكاة للواقع وتحت تأثير التخدير الكلى تم إنشاء 3 عيوب عظمية في كل فك اسفل الجذور البعيدة للأسنان الضاحك الثاني والرابع بابعاد ثابتة 7×7×7 ملليميترحيث تم ملء كل فراغ بواحدة من المواد المستخدمة فى الدراسة. تم التضحية بالكلاب عند 1 ,5 ,9 , 13 أسابيع وتم استئصال الفك السفلي للكلب ومسحها باستخدام جهاز ال DEXA لقياس الكثافة العظمية عند كل موقع اختباروبعدها تم إرسال العينات للفحص النسيجي.

تم إجراء التحليل الإحصائي باستخدام إصدار إحصائيات .IBM® SPSS® حيث تم تمثيل البيانات بالعدد الكلي والنسبة المئوية لكل طريقة مستخدمة في الدراسة. تم تعيين الدلالة عندما تكون القيمة P 0.05 بمستوى ثقة 95٪.

أظهرت النتائج أنه في جميع مراحل المتابعة تقدم مجموعة الصفائح الدموية الغنية بالفيبرين على المجموعات الأخرى .

وفى ضؤما سبق يتضح انة يمكن استخدام الصفائح الدموية الغنية بالفيبرين لملء العيوب العظمية التى تتنتج اثناء الجراحات بغض النظر عن حجم العيب العظمي حول قمة الأسنان .