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A comparative study on effects of using bone xenograft with each of hydroxyapatite Nano particles and hyaluronic acid on the healing of induced tibial defect in dogs

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A comparative study on effects of using bone xenograft with each of hydroxyapatite Nano particles and hyaluronic acid on the healing of induced tibial defect in dogs

A Dissertation submitted

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بسم الله الرحمن الرحيم

صدق الله العظيم

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Supervisor Certification

I certify that this dissertation entitled **"A comparative study on effects of using bone xenograft with each of hydroxyapatite Nano particles and hyaluronic acid on the healing of induced tibial defect in dogs"** was prepared under my supervision at the College of Veterinary Medicine / University of Mosul, as a partial fulfillment of the requirements for the degree of Ph.D. in Veterinary Medicine/Veterinary surgery.

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Linguistician Certification

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Fouad

Abstract

This project was targeted to explore the character of bioactive materials (hydroxyapatite Nano gel and hyaluronic acid) on improvement the healing process of bone xenograft of the reconstructed tibial defects in dogs.Thirty six adult healthy stray dogs of both sexes were included in the present study, their weights and ages were (21±0.3kg), (2.1±0.9 years) respectively.These experimental animals were randomly allocated into 4 equal groups, nine for each including control, xenograft, hyaluronic acid and hydroxyapatite Nano gel groups.The induction of bone defect were carried under general anaethesia and aseptic technique. A(3-5cm) longitudinal skin incision was done at the proxomedial aspect of the right tibia to reach it, then in all animals a (2.5× 0.7cm) rectangle bone defect was induced at the proxomedial aspect of the right tibial bone using electrical saw.

In control group, the rectangle bone piece was re-implant at the same induced defect and fixed firmly with cerclage stainless steel wire suture material then, the muscles and skin were closed with routine methods. In the xenograft group, the same procedure was done as with control group except using of (2.4× 0.6cm) deproteinized lamb rib xenograft for repairing the defect.While for 3rd and 4th groups a 1ml of hyaluronic acid and hydroxyapatite Nano gel were added into xenograft as filling materials to reinforce the repaired defect.

All experimental animals were inspected daily until the end of 60 days to record the clinical animal status and health condtion as well as, a radiological, macroscopical, histopathological, histochemstry, biochemical and serological investigations on 0,7,14,30,60 days post surgery were carried on according to the requirement parameters.

The clinical finding indicated no significant signs with mild degree of lameness during the first few days that became more intence at day 7 post surgery in control and xenograft groups, then the signs subsided gradually until 60 days post surgery and animal returned to the normal weight bearing in all groups.

While the biochemical and serological investigations exhibited an increased in alkaline phosphatase at 7 and 14 days post surgery in all groups. Highest rates at 7days were recorded in hyaluronic acid and hydroxyapatite Nano gel groups, then gradually decreased until 60 days post surgery in both these treatment groups .

On the other hand, the results of serum insulin like growth factor exhibited an increased levels at 7 days in all groups. On day 14 post surgery the level of insulin like growth factor gradually decreased in all groups and subsided in all groups especially in hyaluronic acid after 60 days post surgery.

The results of radiographic investigations indicated that the fastest healing process was obtained in both hyaluronic acid gel and hydroxyapatite Nano gel groups that was appeared as good periosteal reaction with new bone formation around the repaired defect at 60 days post surgery as compare to the control and xenograft groups that demonstrated a faint lucent line around the fixed bone segment.

The macroscopical examination for both hyaluronic acid and hydroxyapatite Nano gel groups showed complete healing process and best new bone formation with no inflammatory signs that made the bone appeared healthy especially in hyaluronic acid and hydroxyapatite Nano gel groups besides, there was mature fibrous connective tissue filling the space between the implant and edge of the defect at thirty and sixty days post surgery as compare with control and xenograft groups.

Furthermore, the histopathological examination exhibited mature compact bone lamellae formation with in the hyaluronic acid group, where as for the hydroxyapatite group, it showed mature compact bone formation with formation of haversian canal and numbers of osteocytes at sixty days post surgery.The histobiochemical finding of this study exhibited that the group of hydroxyapatite Nano gel was reveled increase in neutral mucopolysaccharides (NMP) reaction as compared with others groups.

In nut shell, the application of both hyaluronic acid and hydroxyapatite Nano gel as a filling bioactive materials were a superior than the control and xenograft group and had a beneficial effect on biocompatible properties between the host and xenoimplant that improved bone healing process in dogs.

Contents

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Abbreviation and Symbols

Abbreviation	**Name**
IGF-I,IGF-II	Insulin like growth factor I,II
H&E	Hematoxylin and eosin
HAp	Hydroxyapatite
HA	Hyaluronic acid
ALP	Alkaline phosphatase
BW	body weight
Kg	Kilogram
Ma	Milliampere
MSCs	Mesenchymal stem cells
ECM	Extracellular matrix
BMPs	Bone morphogenetic protein
TGF	Transforming growth factor
IL	Interleukin
PDGF	Platelet derived growth factor
PG-E2	prostaglandin E2
TNF-α	Tumor necrosis factor alpha
IFN-ϒ	interferon γ
FGF	Fibroblast growth factor
VEGF	Vascular endothelial growth factor
OP-1	Osteogenic protien -1
HAC	Hydroxyapatite ceramic
DBM	Deminerlized bone matrix
HIV	Human immunodeficiency virus
CaP	Calcium phosphate
TCP	Tricalcium phosphate
CPC	Calcium phosphate cement
BCP	Biphasic calcium phosphate
CaS	Calcium sulfate
IP-CHA	Interconnected-porous calcium hydroxyapatite cermic
rhBMP-2	Recombinant bone morphogenetic protein-2

IR	Insulin receptor
GH	Growth hormone
b-FGF	Basic fibroblast growth factor
PTH	Parathyroid hormone
PRP	Platelet-rich plasma
AGF	autologous growth factor concentrate
(P2O5)	phosphorus pentoxide
(Na2O)	calcium, sodium oxide
(SiO2)	silicon-dioxide
CaO	calcium oxide
MgO	magnesium oxide
K2O	potassium oxide
B2O	boric oxide
PMMA	Polymethyl methacrylate bone cement
ACUC	Animal Care and Use Committee
ELISA	Enzyme linked immunosorbent assay
NBF	neutral buffer formalin
Two-way ANOVA	Analysis of Variation-ANOVA two way
NMP	neutral mucopolysaccharides
NaOH	sodium hydroxide
PRF	Platelet-rich fibrin
FFD	Focus film distance

Chapter One

Introduction

Tibial bone defect in dog represents the highes rate of long bone injures and defects and the neighboring tissue as well as, most costly and problematic challenge in clinical practice (Christou *et al*., 2014).The successful ways to restore the functions of bone tissue defect achieved by reconstitution the lost bony tissue with grafting by different bioactive bone substitute materials (Bayani *et al*., 2017).

Bone grafting is one of the surgical procedures used to supplement bone restoration in orthopedic surgery(Dimitriou *et al*., 2011).The term bone grafting refers to the procedure in which bone transported from a donor to recipient (Sinibaldi and Roy,1998;Haben,2020) and indicated for violent tumor resection, mandibular and calvarial reconstruction, refractory nonunion and complex defect (muscle, skin and bone) and to substitute of comminuted bone fragments, to grow bones, to precise delayed unions, malunions, for primary construction of osteomyelitis and bone (Brinker *et al*,1990).In general bone implant used as background to deliver constancy, cure of pseudoarthrosis and to steady spinal sections and in addition of bone frameworks in whole joint substitution (Friedlaender,1987).

Using of bone grafting for replace the lost bone that underwent trauma or congenital deformities lead to bone defeat and to substitute the damage a bone we want the bone transference and this graft is used as well as,to restore a segment of bone defect or to enhance the nonunion fracture. A bone implant was obtained from the bones of tibia, iliac crest, humerus, sternum and ribs.A perfect implant material must be inert naturally, osteogenic source, act as a scaffold, obtainable, easily flexible in

positions of shape, length, size and substituted by the original bone (Joshi *et al*., 2010).

Large critical bone tissue defect defined as the smallest measure size of bone tissue shattering that does not self-healing if lifted without surgical intervention during limited period(Marei *et al*.,2018).Its consider as challenge in orthopedic field because of the difficulty of reestablishing the esthetic and mechanical structures of the bone in case of bone neoplasm exclusion, infection ,fracture associated with substantial soft tissue injuries (Huang *et al*., 2016).In these cases the size of lost tissue need restoring with sufficient material of bone tissues or substitutes (Giannoudis *et al*., 2007).

The autologous,allogeneic,xenograft and artificial bones are used as graft materials for creation of bony tissues (Dimitriou *et al*.,2011; Merli *et al*.,2015). Bone implanting techniques are gradually transformed from synthetic to natural bone implant substitutes and biological elements(Wang andYeung,2017). Many types of grafted materials have been used successfully in veterinary field as cancellous, cortical, corticocancellous or synthetic materials (Johnson,1991;Akram *et al*., 2014) or Nanoscale silk-hydroxyapatite hydrogels as bioactive materials (Ding *et al*.,2017).

The bone implantation is considered proper tissue that used in surgical interventions when there are diseases, trauma or osteomyelitis to replace the shuttered bone tissue (Duan and Lopez, 2019). Xenograft can be used in treatment of critical size of bone defect,ovine deproteinized preserved rib bone implant to fill the empty space in tibial defect in rabbit model with beneficial and success outcome (Nazht *et al*.,2018).

Hydroxyapatite (HAp) implant which have a chemical formulation Ca10(PO4)6(OH)2 is an important bioactive materials used for repairing segmental bone tissue defects and bone augmentation and it is most

widely used in the orthopedic surgery because it has better bioactive, biocompatibility and osteoconductive properties as well as these properties permit their implantation into the bone site without an immune response, this material is considered an ideal biological graft material(Kurien, *et al*.,2013).It is used as filling materials for bone defects and creation of the residual ridge. Though, other gelatinous bone-filling materials and paste can simply be used to seal bone defects and have good operability, later they permits for soft-tissue growth to be performed concurrently(Alves Cardoso *et al*.,2014;Ding *et al*., 2017).This agent is able to bind directly to bone tissue, promoting osteogenesis and new bone formation along its surface (Myers,1991) in addition, the chemical composition is similar to the elements of bone tissue structure, this substance may be synthesized from synthetic materials or natural material as egg shell (Azis *et al*., 2015).Hydroxyapatite activates of osteoblast action and lead to enhancing the bone restoration and local growth factors (Bayani *et al*., 2017).

Hyaluronic acid (HA) is recognized as hyaluronate or hyaluronan (C14H21NO11) is a major component of extracellular matrix and high molecular weight polysaccharide (glycosaminoglycan) nearly in all natural tissues (connective tissue, synovial fluids)(Bansal *et al*., 2010).The use HA in grafting process of tibial bone defect gives best outcome (Abid and Mukhtar,2019). It plays a critical role in the extracellular mineralized and non-mineralized materials functions, involving cell migration, tissue hydrodynamics, space filling, lubrication, shock absorption, and protein exclusion, proliferation and differentiation (Sasaki and Watanabe 1995).Therefore, administration of exogenous HA in the treatment of inflammatory stages is exposed in several medical sciences for example ophthalmology, dermatology and orthopedics (Necas *et al*.,2008).

Due to the rareness of studies in using ovine lamb rib xenograft for reconstruction of tibial bone defect and the bioactive materials like hydroxyapatite and hyaluronic acid as a filling materials, the present study was suggested in order to:

A. Use of ovine lamb rib bone xenograft for reconstruction of large tibial bone defect in dogs.
B. Evaluate the effects of using bioactive hydroxyapatite Nano gel on the healing of the bone xenograft of large tibial bone defect in dogs.
C. Evaluate the effects of hyaluronic acid on healing of the bone xenograft of large tibial bone defect in dogs.
D. Compare between these bioactive materials in improvement of healing process using the following techniques
    1. Clinical evaluations.
    2. Macroscopic investigation.
    3. X-rays analysis.
    4. Histopathological examination.
    5. Histobiochemistry stains.
    6. Serological and biochemical tests.

Chapter Two
Review of Literature

2.1.The Bone

Bone is a very compound heterogeneous body structure having versatile composites from Nano to macro-scale diameter with unique mechanical properties (Rho *et al.*, 1998). It represents the largest structure of the body composing of connective tissue with highly mineralized, massively vascularized dynamic living tissue. It is consisted of metabolically living mixtures that are arranged in a firm frame. The main roles of the bone are to shield the body vital tissues and organs and to supply the framework for mechanical support of the skeletomuscular organ, besides, it has a unique ability to regenerate itself after exposing to any injury or external strength without scar tissue formation (Taylor *et al.*,2007 ; Shapino, 2008).The bone is consist of several important crystals, like phosphate and calcium that have an essential part in the regulation of extracellular fluid ion concentrations (Mistry and Mikos, 2005; Loi *et al.*, 2016), it also considers as a reservoir for many growth factors and cytokines (Taichman, 2005). As well as, bone considered as an important factory for synthesis of red and white blood cells and storage of the fat in the normal mature animals (Evans and De Lahunta, 2013).

2.2.Structure of bone

The bony tissue is composed of 69% of inorganic materials which are mainly calcium phosphate (mostly HAp), that provides bone with, firm, rigidity, strength and solidity; it constitutes approximately 99% of the body's calcium,85% of the phosphorus, and 65% of the sodium and magnesium. While, the ratio of organic substance (collagen and non-collagen) constitutes 21% of the bone structure, besides a 9% of water and 1% of numerous further constituents (Kini and Nandeesh,2012).

Bone marrow, one of the essential bone structure acts as source of stem cells (MSCs), which consider as multipotent cells that have ability to distinguish into different tissues like muscle, cartilage, bone, tendon, fatty and cutaneous matter. Within the medullary canal there are also several types of hematopoietic cells that create the white and red blood cells to achieve many vital functions including transportation of gases and providing immune body response (VanPutte *et al*., 2013).

2.3. The bone components and extracellular matrix

Bone is an organic structure having both an extracellular matrix (ECM) and living cells.The living cells involve osteoclasts, osteocytes, and osteoblasts while the extracellular matrix consist of many elements such as glycoproteins, proteoglycans and collagen fibers type (I) which make up approximately 90% of the organic components and numerous non-collagenous proteins, like bone osteocalcin, sialoprotein and osteopontin (Roach,1994).The extracellular matrix vital function is in bone mineralization that linking the calcium hydroxyapatite compound to the collagen fibers (Kini and Nandeesh, 2012).

2.4. The bone cells

2.4.1. Osteoblasts

They are oval and rounded in shape. These cells are highly differentiated cells that originate from less differentiated precursor cells recognized as osteoprogenitor or mesenchymal stem cells.They produce osteoid, the extracellular bone matrix and their mineralization's as well as they are responsible for hormones production like prostaglandins that do on bone tissue itself and create of ALP, the enzyme that has a role in mineralization of bone tissue. In addition, they greate the biological particles, like collagen type I, glycosaminoglycans, BMPs or

transforming growth factors and regulate the collagen mineralization. Afterward satisfying their secretory action, osteoblasts endure both apoptosis about 80% or last distinction into osteocytes about 20% (Ginebra *et al*., 2018). On the other hand, some osteoblasts are remaining on the endosteal or periosteal surfaces as lining cells when bone forming activity reaches its end. Finally, they play an important role in the stimulation the resorption of bone tissue by osteoclasts (Kalfas, 2001).

2.4.2. Bone-lining cells

They are inactive thin type of osteoblasts that have an elongated shaped, flat surface, squamous. These cells still inactive at the surface of bone once the bone construction or bone remodeling is completed. However, these cells could reactivate the chemical and mechanical events of bone healing. Most bone surfaces are covered by these cells named as osteoprogenitor cells, inactive osteoblasts, surface osteocytes and flattened mesenchymal cells. They play important role in bone resorption by producing collagenases that destruct of osteoid and permitting osteoclasts to start resorption of bone tissue on the bone extracellular matrix directly (Bellido *et al*., 2019).

2.4.3. Osteocytes

They are the most abundant and important type of bony cells.They are dead osteoblasts that are implanted in the mineralized matrix of bone.The cells connected with each other by cytoplasmic procedures through connecting networks (canaliculi) in the matrix.Osteocyte are very vital for preserving the bone tissue, contribute in extracellular interactions and are included in the mechanotransduction procedure; besides they secrete enzymes which act a crucial role in bone matrix maintenance and mineral content.These cells originate from osteoblasts that often entrapped throughout the extracellular matrix (Nakashima,2013).These cells are

also included in the controlling of the extracellular concentration of phosphorus and calcium, besides have adaptive remodeling actions through cell-to-cell connections in response to the local environment (Kalfas, 2001).

2.4.4. Osteoclasts

These enormous multinucleated cells, which have a macrophage/monocyte linage, are derived from hematopoietic cell lines. It is thought that chemokines and chemotaxis events on osteoprogenitor cells cause the fusion-differentiation of monocytes into osteoclasts and subsequently drive osteoclastic action. Monocyte- macrophage lineage cells play a significant role in the initiation repair methods of bone tissue. Osteoclasts play vital role in degradation and resorption of bone matrix and mineralization during healing, remodeling and growing process.They have the capacity to break down both the inorganic and organic stages of bone tissue by digesting the organic components and releasing acidic types, such as protons, from the inorganic phase.The differentiation of these cells occurs during the microenvironment of bone tissue, anywhere relations between osteoblasts and monocyte precursors permits the cells to differentiate into osteoclasts (Hadjidakis and Androulakis, 2006; Goldberg *et al*., 2012). In group, these cells named “cutting cones” when they connect to bone simple surfaces and liberating hydrolytic enzymes to dissolve all inorganic and organic substances of bone tissue and any crystalied cartilage.The outcome of such procedure lead to creation of shallow erosive pits at the surface of bone that named how ship lacunae. Following resorption, after osteoblasts take possession of create the bone matrix, they submit to cell death or apoptosis (Kalfas,2001).

2.5. Inorganic extracellular matrix

The main inorganic compound of the solid bony tissue is hydroxyapatite (HAp) Ca5(Po4)3OH, the deposition of HAp occurs during the bone biomineralization interaction process that occurs between minerals and matrix, as amino acid in non-collagenous proteins determine HAp producing.The collagen formed through mineralization of tissue and this due to significant chemical and physical similarity of HAp mineral constitute of bone (Ramesh *et al*.,2018).

2.6. Bone macroscopic organization

Usually, the bone is consist of 80% cortical bone and 20% trabecular bone (Sikavitsas *et al*.,2001).

2.6.1. Cancellous bone

The cancellous bone is consisting of high-porosity trabecular bone of honeycomb branches with a porosity of about 50% to 90% that make its inside like a sponge (Salgado *et al*., 2004).It can be found in the vertebrae, ribs, iliac crest, and the interior part of the cortical bone of long bone extremities.Cancellous bone is made up of a 3D network of tiny rods called trabeculae that are directed in the main directions of mechanical strain.This structure resembles a mesh and has a low density of cancellous bone compared to high density for cortical of many bones (Ansary,2003).About 25–30% of the volume of the cancellous bone is made up of the 200– μm -long trabecular rods and plates.Trabecular number, thickness, and separation are the three basic properties of the cancellous net, and the remaining area is known as the marrow cavity of bone tissue (Barak *et al*.,2013).The red marrow, which is an organ that produces blood cells, is located in cancellous bone (Igashira-Kamiyama *et al*.,2013).

2.6.2. Cortical bone

It forms the external surface of all skeleton and it is especially found in the iliac crest (Castillo *et al*.,2012), skull (Torimitsu *et al*., 2014), peripheral surroundings of cancellous bone, shafts of long bone and the vertebral endplates (Evans and De Lahunta,2013).The cortical bone found as a cylindrical shaped osteon, which consists of layers named lamella that arranged longitudinally and oriented vascular channels this system called haversian system.Whereas Volkmann canals oriented horizontally. The mechanical power of cortical bone depends upon the fitted filling of the osteons (Mistry and Mikos,2005).

2.7. Gross anatomy of the bone

Bone categorized into several types such as flat bones, long bones, irregular bones, short bones and sesamoid bones.The anatomical feature of the bone divided into three several parts including diaphysis, proximal and distal epiphysis (Fig. 1). Diaphysis is the mid shift of bone that lies between two parts of epiphysis the proximal and distal part, inside the diaphysis there are cavities named medullary cavity that are occupied with plentiful amount of red and yellow marrow consisting the adipose tissue and blood creating cell. The metaphysis part is the connection point between epiphysis and diaphysis, during the bone growing, the epiphyseal plate plays a role in bone growing, development and maturity.

Histologically,the external surface of the bone tissue is generally enclosed by dense connective tissues named periosteum that mixtures gradually with ligaments and tendons at their connections.The periosteum is crucial in bone reconstruction and regeneration. It has nerves, osteoblast and osteoclast besides, it contains blood vessels to provide nourishing. Requirement and protection to the bone, it separates the bone from surrounding tissues and protect it. During fracture repair, it plays a

vital role in appositional regenerate of fractured site due to its osteogenic activity (Kini and Nandeesh, 2012; Pastides *et al*.,2012).Meanwhile, the endosteum is a thinner connective outer surface of medullary cavity that provides osteoblasts to help in repairing the trauma and defective bone (VanPutte *et al*.,2013).

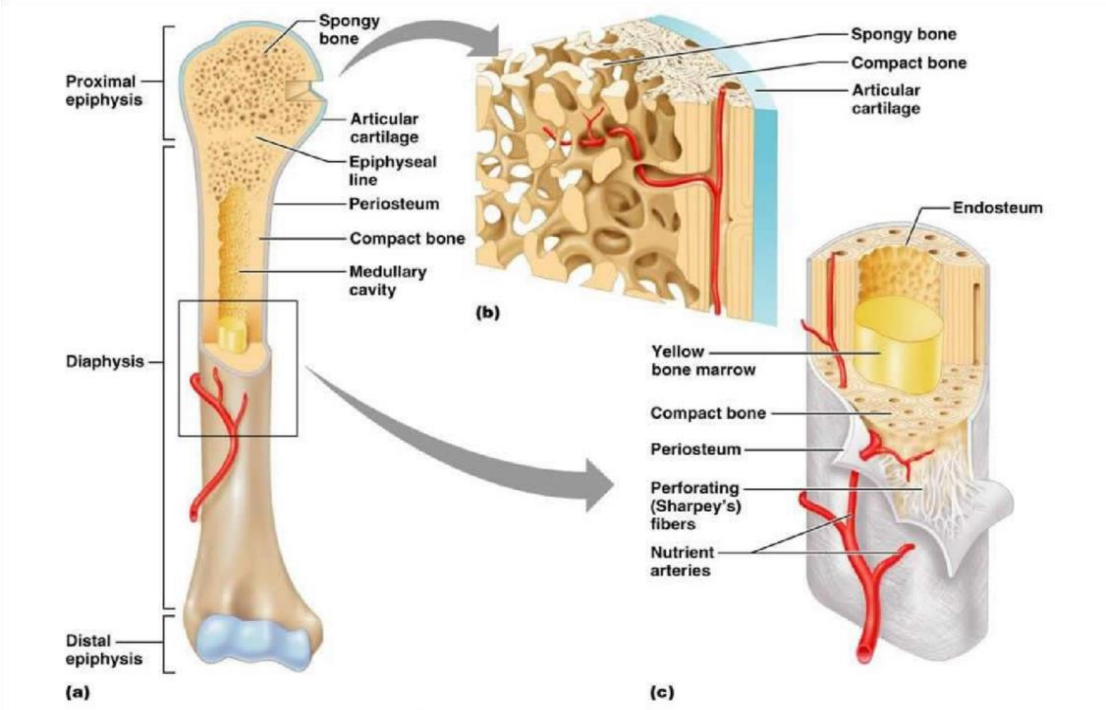


Figure 1:Structure of long bone (VanPutte *et al*., 2013).

2.7.1. Anatomy of canine tibia

The tibia bone represents one of most important bone in skeletal structure of dog as it is considered as a compact solid bone. It lies at the medial border of hind limb.The tibial bone attached with many articulations, proximately with femur, distally with the tarsus bone that attached firmly at the lateral side with the fibula's partner bone, both proximally and distally.The proximal half of the tibia has a triangular cross shape and is heavier than the distal, almost cylindrical, half (Evans and De Lahnta,2013).

The medial and lateral menisci divide the medial and lateral tibial condyles from the medial and lateral femoral condyles.The tibial

tuberosity standout from the cranial aspect of this extremity and it is continued by a gradually subsiding crests the broad, quadrangular, proximocranial structure that serves as the attachment point for a number of strong muscles, including the sartorius, biceps femoris, and quadriceps femoris.The tibial tuberosity, formerly known as the tibial crest, is where the gracilis, semitendinosus, and a portion of the sartorius and biceps femoris muscles insert.The proximal half of the body has three sides, whereas the distal part is approximately cylinder-shaped. In the proximal part of the tibia, three surfaces and three borders can be seen.These are the lateral, medial, and cranial borders as well as the caudal, medial, and lateral aspects. In the distal part of the tibia, a small, flat surface opposite the nearby, thoroughly beneath fibula replaces the lateral border interosseous border. Due to the fact that the cranial border of the tibia contributed to its formation, the medial aspect is broad, flat, and subcutaneous (Dyce *et al*., 2010;Evans and De Lahunta,2013).

2.7.2. Anatomy of the canine fibula

It is a long thin laterally compressed bone that placed in the lateral part of the crus. It articulates with the caudolateral part of the lateral condyle of the tibia proximally and with the tibia and talus distally. It serves mainly as a muscle attachment. It is separated into a neck, proximal head and body distally as a lateral malleolus (Fig.2) (Evans and De Lhunta,2013).

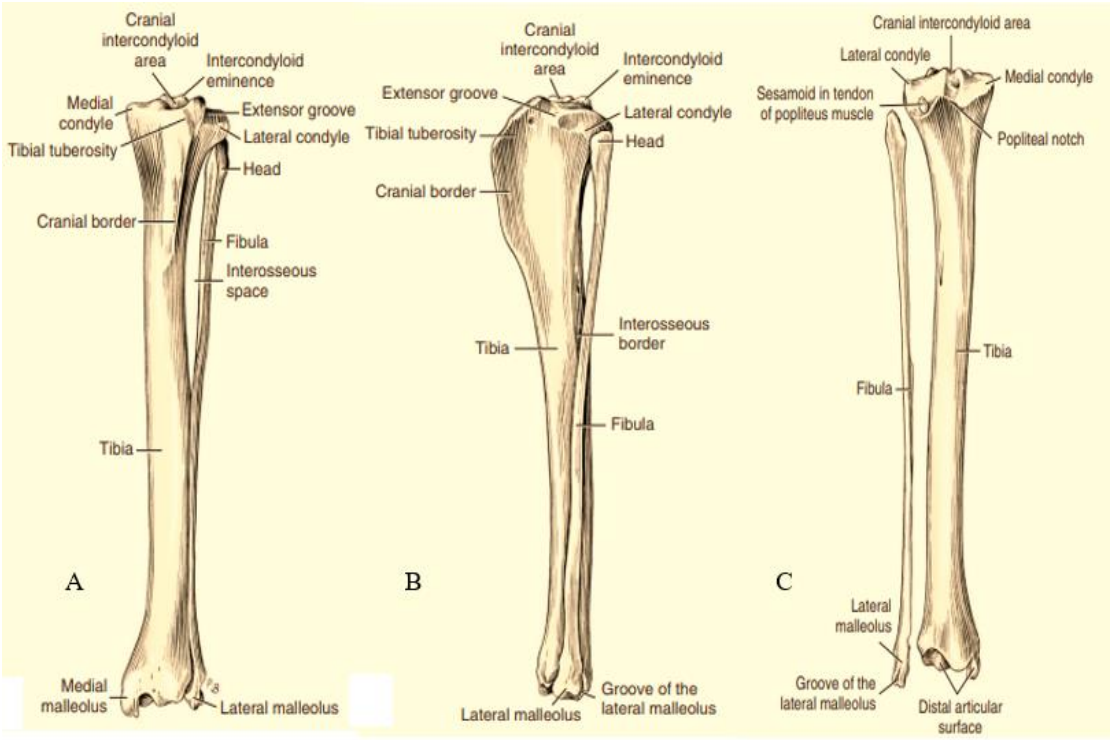


Figure2:Anatomy of tibia and fibula (A) Articulated left tibia and fibula,cranial view (B) Articulated left tibia and fibula,lateral view (C) Disarticulated left tibia and fibula, caudal view (Evans and DeLahunta, 2013).

2.8. Blood supply of bones

The vascularization of the hind limb runs from the external iliac arteries (right and left) which originated directly from the aorta, the external iliac artery branched to the femoral, cranial tibial and saphenous arteries, dorsal pedal artery, perforating metatarsal artery, plantar metatarsal arteries and plantar proper digital arteries (Dyce *et al*.,2010).

Bone receives up to 10% of cardiac output, and because of this blood supply,it may remodel and repair itself at a far higher level than cartilage, which lacks blood vessels.The Nutrient arteries deliver blood to the endosteal cavity of the bone, where it passes through the marrow sinusoids and out through a network of countless tiny capillaries that ramify through the bone cortex (Marenzana and Arnett,2013).

The nutrient (medullary) artery and vein passing through the nutrient foramen and canal of a bone and reaching the marrow cavity, divide into proximal and distal branches that subdivide repeatedly and supply the bone marrow and the adjacent cortical bone.The periosteal arteries and veins are supplying the extremities of long bones.They enter minute canals that lead in from the surface, and ramify proximally and distally in the microscopic tubes that tunnel the compact and spongy bone.The arterioles of the nutrient artery anastomose with those of the periosteal arteries deep within the compact bone. Lymph vessels are present in the periosteum as perivascular sheaths and probably also as unaccompanied vessels within the bone marrow (Evans and De Lahunta,2013).The metaphyseal artery and vein begin adjacent to the epiphyseal line in the metaphyseal section of the diaphysis.While the epiphyseal artery and vein supply the extremity of the bone with its covering of articular cartilage (McCarthy,2006).

2.9. Nerve supply of bones

The innervations of the tibial bone originated from the sensory nerves, so the innervation plays an important role in regeneration and remodeling of bone matrix (Sims and Walsh,2010) as well as, the central and sympathetic innervation of peripheral nervous fibers play same role in healing process of bone.The ischiatic nerve, common peroneal, superficial peroneal nerve, deep peroneal and tibial nerves all contribute in the innervation of hind limb especially the tibial bone (McCarthy, 2006).

2.10. Bone remodeling and healing

During the whole life span of bone,it is undergoing continuous remodeling due to its living ability (Rucci,2008). Bone remodeling includes the demineralization process by the act of osteoclasts followed then by the action of osteoblasts for building of bone matrix that lastly become mineralized.The remodeling mechanism occurs during a process called osteogenesis that having three consecutive phases: resorption, during which osteoclasts digest older bony tissue; reversal, when mononuclear cells seen on the bone surface; and formation, when there is a lay down of new bone by osteoblasts until the resorbed bone is entirely restored (Hadjidakis and Androulakis,2006) (Fig. 3).

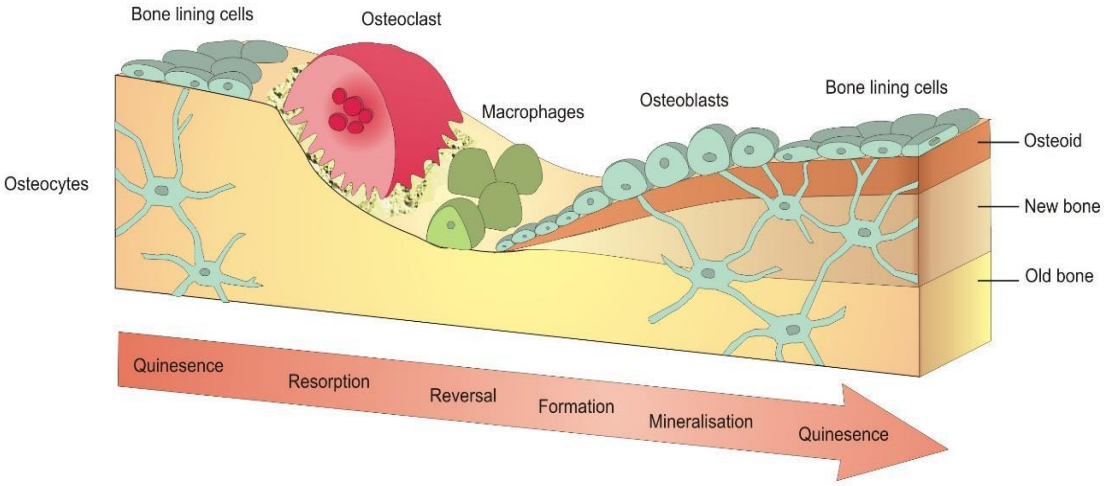


Figure 3:Bone remodeling process (Rucci,2008).

2.10.1. Bone healing

When there is losing of the bone strength and disruption of its mechanical continuity that accompanied with many pathological events between its broken ends, this condition is known as fracture or defect that is generally heals through a regenerative biological process without scarring. The key to complete the healing process of bone tissue fracture is the existence or re-establishment of some degree of constancy among

the broken bone ends that helps the creation and preservation of the osteoconductive feature of the implants above which the bone creating cells contribute in resting bone(Kalfas, 2001;Shenoy and Pillai, 2017). Bone healing process includes many cascades of triggered events that follows bone defect to return the original mechanical and physical tissue features as it was before that defect. It is well known that process of bone healing is influenced by a number of local and systemic elements (Shenoy and Pillai,2017).This process can be considered as a form of tissue regeneration. Though, regardless of this regenerative capability of skeletal system, this biological process occasionally fails and bone defect may show a delay in healing or even develop pseudoarthrosis or nonunion fate (Marsell and Einhorn,2011).

According to this theory, bone healing recapitulates the ontological processes that occur through the development of the skeletal system in the embryo, allowing the injured organ to be completely repaired to its pre-injury structure, construction, and function (Einhorn and Gerstenfeld,2015).The degree of tissue loss is one of many parameters that influence healing that can be used to classification of bone healing process(Marsell and Einhorn,2010).Similar to this, there are two categories of bone healing: primary and secondary bone healing.

2.10.1.1. Primary bone healing

It is known as intramembranous ossification with direct union of bone that normally happens with a very small gap less than 0.1mm (order of 10-100 μm) and a strain under 2%. It often needs a low rigid fixation in place like compression plate for maintaining absolute stability at defect area for much longer time even years (Ghimire *et al*.,2021).In this process, the osteoblasts formed during differentiation of mesenchymal stem cell are responsible for direct bony tissue creation (Einhorn and

Gerstenfeld, 2015)while osteoclast that advances directly from the defect site are resorbing dead bony at the necrotic ends leading to produce longitudinal cavities.Behind the cutting head, the creation of new bone and the reestablishment of the Haversian system are formed by the action of osteoblasts that bridge the gap defect with new bone formation that lake any cartilaginous or connective tissue (Loi *et al*.,2016). The primary bone healing that can happened any where is a slow process that can lasts for several months or years until healing is complete (Rafiee, 2009).While when there is a somewhat larger gap separating the bone fragments, a similar mechanical stable condition is occurred and the gap healing is occupied by direct deposition of intramembranous woven bone and the Haversian system of the matured bone is rebuilt via the osteoclast-mediated remodeling development (Perren,2002). Many authors like (Marsell and Einhorn, 2011) said that intramembranous ossification happens concurrently in callus neighboring to the distal and periosteal end of cortex,away from the wounded region(below relatively low strain).

2,10.1.2. Secondary bone healing

When the fractured borders are fewer than twice the diameter of the damaged bone with motion on the fracture site, a secondary bone healing is usually occurs that involves many stages including an inflammatory response, hematoma formation, repair phase (soft callus and hard callus), and finally remodeling (Ercin *et al*., 2017). It is known as indirect healing of bone defect that undergo endochondral ossification due to the presence of a large gap defect with higher micro movements.It can be completed through enhancing the fixation system elasticity like locking plates(Perren, 2002). Between injured ends and external to periosteal callus and relatively below huge strain an endochondral ossification

process is happening that involve the formation of cartilage tissue then calcified, then resorbed and finally substituted by bone (Marsell and Einhorn, 2011; Holstein *et al*., 2013).Within fracture of bone ,anabolism is firstly stimulated in the manner of bone volume increasing by recruiting the differentiation of MSCs and then retarded with apoptosis of chondrocyte (Gerstenfeld *et al*., 2003). In secondary bone healing process and within the injured site both intramembranous and endochondral ossification can occur depending on vascularity and strain level(Gerstenfeld *et al*., 2003;Ghiasi *et al*.,2017; Baker *et al.*,2018).

Bone healing process is a complex procedure that has three different but stages of overlapping: the early inflammatory stage; the repairing stage;and finally, the late remodeling stage (Baker *et al*.,2018).

2.10.1.2.1. The inflammatory stage

Hemorrhage from the damaged bone edges and blood vessels during the first few hours and days following fracture leads to formation of hematoma at injured area. This interference with blood supply will cause reduction in oxygen tension and nutrient delivery and both of them in turn lead to hypoxemic events progress within the bony tissues. Following that, the osteocytes nutrients and gases are deprives, causing necrosis of bone at the site of injury.The distribution of necrosis varies depending on the intensity of injury, quantity of blood supply and nutrition (Claes *et al*., 2012;Marongiu *et al*.,2020). During this early phase, there is localized vasodilatations with leucocytes and plasma exudation, besides the local accumulation of histiocytes and mast cells with the clearing up of the debris commences(Mckibbin,1978).The prostaglandin is the key mediator for infiltration of several inflammatory cells including lymphocytes , monocytes ,macrophages, and polymorpho-nuclear cells as well as fibroblasts. Within hematoma, there is plenty of platelets and

macrophages that act to stimulates the release of a series of mediators and signaling materials including cytokines and growth factors which they have an essential role in the inflammatory response by drafting inflammatory cells. Macrophage and platelets in the blood clot, proliferating osteogenic tissue and even from the dead bone will release different growth factors like platelet-derived growth factors, TGF-B and bone morphogenic protein through lysis and acidification of the matrix. These growth factors play an important role in stimulating the proliferation of the repaired tissue (woven bone). Finally, the result will lead to new tissue formation known as granulation tissue, new vascular tissue and migration of MSCs and exposing to cancellous bone.While the muscle provided nutrition and oxygen to the injured area (kalfas, 2001; Weisbrode, 2007; Claes *et al*., 2012). The hematoma is also intruded with immune cells; an inflammatory response provokes with pro-inflammatory cytokines IL-1, IL-6, TNF-α, and IFN-ϒ. Inflammatory response control is critical for further process.Following inflammatory response resolution, the mesenchymal stem cells gather at the injured area then differentiate into chondrocytes and osteoblasts. Under controlling of macrophages, cytokines, T- and B-lymphocytes and growth factors, soft callus is formed with endochondral formation followed by hard callus (Ercin *et al*.,2017). At this stage of defect healing, the important controlling cytokines are prostaglandin E2 (PG-E2), the transforming growth factor beta group of proteins (TGF) interleukin-1, interleukin- 6 (IL-1 and IL-6) and platelet derived growth factor (PDGF) (Ridiandries *et al*.,2018).

2.10.1.2.2. The repair stages

Bone repairing is a biological process controlled by various cell mediators and growth factors that deliver localized signals at damaged area, allowing progenitors and inflammatory cells to migrate and

generate healing processes that starts the inflammatory phase overlaps within a few days (Hasan *et al*.,2014).The cellular layer of the periosteum, which has two layers an exterior fibrous layer and an inner cellular layer and cells that have moved to the damaged area along blood vessels are the sources of pluripotent mesenchymal cells that become fibroblasts, chondroblasts and osteoblasts.(Buckwalter and Grodzinsky,1999). During the inflammatory phase, the environment is acidic and hypoxemic that turns gradually to neutral then become alkaline. Alkaline phosphatase and bone mineralization and the reparative bone can be molded either directly without an intermediate cartilage formation known as intramembranous ossification from the nearest precursor cells close or through chondrogenesis of the periphery with gradual endochondral calcification that led to degradation of the cartilage matrix and mineralization. Like to the arrangement of events at the development plate, this mobility of the faulty site then permits proliferation of the vascular bed, higher limit oxygen tension, osteoblasts invasion and primary spongiosa laying down (woven bone) (Lieberman *et al*.,2002).The lay down of stroma by fibroblasts support vascular ingrowth, with new blood vessels formation, also the collagen matrix is laid down whereas osteoid is secreted and then mineralized that leads to the creation of a soft callus round the repaired area. During the first month to 6 weeks of the process of healing, the callus is very weak and fragile, in terms of resistance to movement that usually requires suitable protection like bracing or internal fixation. Finally, the callus ossifies, creating a bridge of woven bone among the defect ends.However, if correct immobilization is not used, ossification of the callus may delay or not occur, and an unstable fibrous union may establish instead (Marsell and Einhorn,2011).

2.10.1.2.3. Remodeling stage

It is the phase in which the bone tissue repaired its mechanical strength, dynamic properties and original shape.This stage of bone healing occurs slowly over many months every years that is eased through the structural pressure located on the bone tissue (Hadjidakis and Androulakis, 2006).To achieve integration and remodeling of a bone implant, the MSCs should have a vascular access to the implant in order to distinguish into osteoclasts and osteoblasts.The remodeling occurs through the cellular activity within the basic multicellular unit and it includes five consecutive steps; resorption, activation, formation, reversal and termination (Seeman and Martin 2019). At this time the woven bone is substituted by another type of bony tissue called lamellar bone. Additionally, with osteoclastic bone resorption and creation of bone along stress lines, the additional callus is progressively resorbed and the construction of the bone is repaired as closely as likely to its preinjury level (Shenoy and Pillai,2017).

In rigidly fixation defect, the healing process is primary occurred by two ways, gap healing and contact healing (Marsell and Einhorn,2011). In gap healing of woven bone is directly arraigned in that gap then it substituted by lamellar bone that is initially orientated transversely to that of the original lamellar bone location. Afterward and over a period of weeks, this is substituted by osteons that are organized as they were before the defect had happened.While in contact bone heling where the defective gap is less than 0.1mm and strain is less than 2%, osteons develop directly through the fracture area and parallelly to the long axis of the bone (Shapiro, 1988).The osteoclasts pile out a tunnel in the dead bone leading to creation of a cutting cone passing through the fracture

area to facilitate the blood vessel growing.This brings in osteoblasts which lay down lamellar bone (McKibbin,1978).

Several factors could significantly and systematically influence on bone healing process, including diabetes, malnutrition, osteoporosis and rheumatoid arthritis. In specific, through the first week of bone tissue repairing, cytotoxic agents, nonsteroidal anti-inflammatory medications and steroid medications can have very damaging effects (Kalfas, 2001). Numerous factors, including fibroblast growth factors (FGF),TGF- β, platelet derived growth factors (PDGF),vascular endothelial growth factors (VEGF), BMPs and insulin-like growth factors (IGF), have been demonstrated to interact during the later stages of reparative callus development and remodeling (Lieberman *et al*.,2002).

2.11. Bone defect

Bone defects are representing a current challenge in orthopedic neurosurgery, reconstructive and maxillofacial surgery, these defects can be categorized into two distinct origins, congenital and acquired.The congenital one is generally due to skeletal abnormalities during development.While, the acquired type may be resulted from various conditions including pseudarthrosis of a fracture, osteomyelitis ,sever comminuted diaphyseal fractures, malunion, nonunion, resected bone tumor, bone cysts, spondylolysis, vertebral body collapse and further acetabular of bone defects from synthetic joint restoration.The maxillofacial surgery and the alveolar bone loss are the most frequent causes of graft failure (Paderni *et al*., 2009; Bernabe´*et al*., 2012).The huge segmental or critical sized of bone defect is consider as most risky condition in which the healing occurs with some difficulties and it can be caused by many affections as developmental deformities, diseases, high energy trauma, revision surgery, osteomyelitis or tumor resection

(Reichert *et al*., 2009).There are plenty of factors that influence bone defects and their critical size is dependent on the circumferential loss of bone, absolute versus comparative size, anatomical position (articular, metaphyseal diaphyseal) and the soft tissue environment including injury to the surrounding muscles, periosteum, existence of chronic diseases, age, and other anomalies (Nauth *et al*., 2011).

2.11.1.Critical size defect

The critical size bone defect can be defined as the smallest bony defect which cannot be healed naturally during the animal lifespan (Spicer *et al*., 2012; Cheng *et al*., 2013). New studies proved that the critical size bone defect does not heal within the duration time of the study because it is really impossible to monitor the healing characteristics process of defect during lifespan (Gosain *et al*.,2000). Or demonstrations fewer than 10% bony defect healing through the lifespan of the animal (Spicer *et al*., 2012). Although, size of bone defect is not the only limitation to describe a bone tissue defect as critical (Lasanianos *et al*.,2010), it has been found that in several types of animals, a distance of critical bone size defect ranges between 2-2.5 times the width of the defected bone can be measured the minimum size of bone defect (Gugala *et al*.,2007).The nonunion fracture produced by such defects can extremely affect the value lives of animals because of the postoperative and prolonged treatment expenses besides the socio-economical, major surgical and investigate challenges (Reichert *et al*., 2009).The extensive bone loss in this defect has been shown to directly affect tissue differentiation and new vascularization and eventually leads to impulsive bone fracture, which cause non-union without surgical intervention(Claes *et al*.2003).

2.11.2. Tibial bone defects

Bone xenografts are kind of substitution of autogenous bone implant that must have properties of uses biocompatible and osteoconductive. In a comparative histological evaluation study for rabbit tibial bone compensation treated with xenografts to repair the bone defect by using different materials of bone substitutes with beneficial value and without fibroplasia. A study has demonstrated that the chemical and physical properties of commercially existing hydroxyapatites used routinely as bone substitute or as filling materials has allowed progressive bone apposition as a result of the bioactivity and absorbable character of hydroxyapatites (Calasans-Maia *et al*.,2009).

Autologous mesenchymal stem cells was used to evaluate the progress of allograft healing in adult rabbit critical sized tibial defects, the results indicated that autologous MSCs enhanced union of host implant connections as well as, enhanced the biological integration of the allograft piece in term of osteocyte index, new bone creation index and resorption Index (Nather *et al*.,2010).

Likewise, the use of marrow-copied osteoprogenitor cells in a sheep model to boost compensation of critical-size tibial openings upon autologous movement on a hydroxyapatite ceramic (HAC) mover was evaluated and the outcome proved that their use with a combination of HAC-based movers enhanced superior bone repair compared to hydroxyapitate ceramic unaccompanied.Potentially this combination could be used clinically in the repairing of extreme long bone implants(Kon *et al*.,2000).

2.11.3.Repairing of bone defects

The repairing of bone defects especially the critical-sized one which result from trauma, nonunion, revision, surgery of prosthetic grafts and tumor excision is a difficult challenging issue in veterinary medicine (Reichert *et al*., 2009; Lyu *et al*., 2013). Repairing huge bone defects still to be an obstacle task especially for orthopedists that make them prefer using autologous bone implant over xenogeneic and alloplastic implants for restoring bone morphology as a result of their osteogenic character and being easier in incorporation (Finkemeier,2002). Several bioactive materials have been successfully applied to repair bone defects including bone grafting and other bone alternatives(Salgado *et al*.,2004).

2.12.Bone grafting

Grafting of bone is an ancient surgical procedure that belongs to many decades earlier. The first documentation for using of bone implant was in 1668 by the Dutch surgeon Job Van Meek'ren, who succeeded to achieve the first bone implants using a canine xenograft for restoring a cranial defect (Walsh *et al*., 2003). The 2nd world war makes the bone grafting to became critical issue that inspire the US navy to establish bone banks as a continuous supply to restored repair fractures during fight (Blitch and Ricotta 1996). Also, church documents indicate the first transplantation of a bone tissue implant in 1682 with dog's cranial bone in a Russian soldier (Heppenstall,1980). While Schroeder (1938) was the first one to use a bone implant procedure in veterinary orthopedics.

Bone implants represent a high demanding market due to its high request in practical training for compensation of bone defects and recovery of atrophic bone area (Deev *et al*.,2015). In dogs and cats, the bone tissue implanting is one of the greatest common surgical procedures

to supplement the regeneration of bone tissue to repair missing bone in many bone fractures or fail to heal that are seriously complex and can even threat life of the patient. Bone implants can be used for repairing various orthopedic abnormalities including nonunion and delayed union of fractures, osseous defects from injury, congenital pseudoarthrosis, cancers and infection. Besides, they can also be useful for rebuilding facial and plastic surgery (Dimitriou *et al*., 2011;Hung, 2012). Approximately above two million of bone tissue grafting techniques are accomplished yearly worldwide that represent another most recurrent material movement right after transfusion of blood, Clinically, autografts represents the most spread strategy due to its biological properties in spite of their associated significant problems such as the limited harvestable quantity, the need for a second surgical procedure and the pain that could be occasionally persisted at the harvesting site over a time. The implant that collected from bone tissue banks or further animals are still to be like a source of threatens such as disease transmission or immunological response(Oryan *et al*.,2014;Campana *et al*.,2014). However,the demanding for various bone grafting segment during the last few years have increased significantly like a result of the highly versatile bone tissue reconstruction demanding techniques, like techniques of spinal fusion, revision arthroplasties and leg salvage techniques subordinate to injury, cancer or bone skeletal disorders (Dinopoulos *et al*., 2012).

The clinical outcomes of the grafting methods are highly variable according to many variables including the mass bone, type of fixation and implant, value of removed bone, the host area of transplant, immunogenetics between the host and donor, bed preparation, the protection techniques, local and systemic disease and structural

characteristics that depend upon the shape, size and type of implant (Khan *et al*.,2005).

2.12.1. Purposes of bone grafts

Hung,2012; Dinopoulos, *et al*., 2012 documented that bone implants may be used for the following purposes:

1. To plug cavities or defects resulting from many affection-like cysts, cancers or others.
2. To consider as link joins and so deliver arthrodesis.
3. To consider as a connection for main defects or found the continuity of an extended bone.
4. To deliver bone blocks to cut joint motion (arthrorisis).
5. To found mixture in a pseudarthrosis.
6. To improve combination or as a substantial material of defects in malunion, delayed union, osteotomies and new fractures.
7. To plastically arthrosis of acetabulum for congenital abnormalities of the hip and perthes illness.
8. To be considered as connection for main defects of bone of multifragmentary breaks by founding of bone sections continuity and filling of cortical bone tissue defects, so inspiring and hasten the primary creation of callus bridging.
9. To replace whole cortical segments missing from fracture disintegration or from removal caused by tumors and other within the space occupying lesions.

2.12.2. Principles of bone grafting

Various variables are essential for bone grafting including:

1) The osteogenic capability or action of the transplanted implants.
2) The capability of implants to multiply and survive.
3) The immune response of animal.
4) The experience that the new transplanted material could need for the grade of initiation.
5) The host tissue exhibition to the spaces of the grafted tissue which means affinity (Joshi *et al*.,2010).

2.12.3. Properties of a perfect bone graft substitute material

The main characteristic that bone implant substitutes must have been various and expanded including bioresorbable, biocompatible, osteoinductive, osteoconductive close to bone structure. Besides, they should be easy for usage, effective cost and resistant to mechanical services on the surgical site. As well as should not require a repeat operation, should be the existing in adequate amount and should be moldable to imitate to and fill irregular defects. Furthermore, they should be implantable through a minimal surgical contact, it should improve new bone development and combination by the host and it should be as firm and solid as intact bone for instant load–bearing ability. Additionally, they can be kept for a long period, easily formed desired into the proper shape and form, should not possess an antigenic property and finally it should be fully synthetic. However, the materials that are used today can only meet some of these properties (Joshi *et al*.,2010; Haugen *et al*.,2019).

Generally, the bone graft alternatives should have the following properties:

1. Their interconnected porosity should have suitable pore size that permit the perfusion within all size bone implant to exchange of waste products, nutrients and bone cells. The very small pore size is typically about 100 μm, while pore sizes >300 μm are suggested for permitting new bone formation and revascularization (Saito *et al*.,2012).
2. Should have a surface that allows bone cell attachment, vascular ingrowth, proliferation and migration.
3. Should have adequate elastic properties and the mechanical compressive strength power to enable the absorbance of the surrounding load for both hard and soft tissues in non-contained defects.
4. Should be biodegradable control to guarantees resorption through the tissue remodeling process whereas keeping the defect volume for bone ingrowth.
5. Should have a sufficient dimensional constancy for permitting the chairside adaptation of the bone implant to the defect (Haugen *et al*., 2019).

2.12.4. The physiology of bone grafting

Many essential variables are responsible for obtaining the ideal bone implant or replacements as Osteoconduction, Osteoinduction and Osteogenesis (Greenwald *et al*.,2001).

2.12.4.1. Osteoconduction

It can be defined as a three-dimensional process when capillaries, osteoprogenitor cells and perivascular tissue from the host bed infiltered into the sit to provide mechanical, physical support and proper direction to the repair process. During this process the new viable bone replaces old necrotic ischemic tissue that is mechanically and dynamically

important for bone graft known as creeping substitution then and there substitutes the graft with new bone formation to create a practical skeletal part. An osteoconductive surface is that one which allows bone growth on its surface or down into pipes, pores and channels (Urist, 1989; Khan *et al*., 2000; Albrektsson, and Johansson, 2001; Khan *et al*., 2005; Joshi *et al*., 2010; Dinopoulos *et al*., 2012).When the bone implant material consider as a implant the process of the osteoconduction occurs and new bone formation is perpetuated by the innate bone. This implant permits in the development of osteoblasts, vessels and MSCs so that union happens with the host skeleton. Osteoconduction depends however, on many factors such as cellularity of the recipient bed, size of defect, passive ingrowth of new bone from recipient bed, contact with donor tissue, and host controlling of remodeling and resorption (Cypher and Grossman,1996). In the boundary of the defective site, the osteoblast that are going to be in future as grafted scaffold use the bone implant material as a framework upon which to generate and spread new bone. All bone-implant substitute materials and bone implant may be pointed through these processes (Hung,2012). Likewise the bioactive materials ability to permits ingrowth of bone into its bone structure depends on porosity of implant for new bone formation (Zhang *et al*., 2018).

2.12.4.2. Osteoinduction

This element of bone graft means stimulation of primitive osteoinductive factors including pluripotent and undifferentiated cells to form osteogenic elements and induce the different phase of bone regeneration (Khan *et al*.,2000; Albrektsson and Johansson,2001; Dinopoulos, *et al*., 2012). During osteoinduction, the MSCs at and round the host area are recruited to distinguish into different cell types as osteoblasts and chondroblasts. This differentiation and recruitment are

controlled by many growth factors named graft matrix–derived growth factors whose activity is triggered when bone mineral is detached. The main controlling growth factors for bone formation are interleukins, platelet-derived growth factors, fibroblast growth factors (acidic and basic), insulin-like growth factors (I and II), TGF-B (B1 and B2) epidermal growth factor, granulocyte-macrophage colony–stimulating factors. Besides angiogenic factors such as vascular endothelial-derived growth factor, retinoic acid, granulocyte colony-stimulating factors; all these factors have a specific role in proliferation and osteoblast differentiation (Khan *et al*., 2005). As well as, bone morphogenetic proteins (BMPs) -2, -4, and -7, which are memberships of the transforming growth factor-β superfamily that are ability of inducing differentiation of stem cells into bone and cartilage creating native bone cells. A bone implant material that is osteoinductive and osteoconductive elements serve as scaffold as well as trigger the creation of osteoblasts and accelerate incorporation of implant (Reddi *et al*.,1987; Hung,2012).

2.12.4.3.Osteogenesis

Osteogenesis mean the formation of new bone tissue and during this process the vital osteoclasts and osteoblasts formed from implant bone material are playing important roles in bone formation and growth that is essentially cancellous than the cortical graft via its spongy structure of bone (Reddi *et al*., 1987; Khan *et al*.,2000 ; Joshi *et al*., 2010; Roberts and Rosenbaum, 2012; Dinopoulos *et al*., 2012; Hung, 2012). Osteoblast precursors differentiated into mature osteoblasts under suitable host status.The MSCs are responsible for an important portion of new bone creation. The osteoblast precursors are present in bone marrow, and other tissues such as periosteum (Cypher and Grossman,1996). Cells from cancellous and cortical grafts transport to the host area and create new

bone tissue that is potential in the primary stage repairing of bone.The characters of cancellous implants segment which composed of structures as an intimate trabecular and lined with osteoblasts and a major surface site make them very attractive at areas where new bone creation is favorite (Khan *et al*, 2005).

2.12.4.4. Osteopromotion

It's defined as speeding up of osteoinduction elements without including osteoinductive characteriscs. Like, enamel matrix derivative to improve the osteoinductive effect of demineralized freeze-dried bone allograft but will not stimulate alone new bone repair (Hung, 2012).

2.12.4.5. Creeping substitution

It involves the transport of the new formed bone tissue during channels which constituted from blood vessels that attacking a movement of bone. Axhausen in 1907 was the first person who used this term to explain the characteristic dynamic healing and repairing process transplantation of bone.Axhausen firstly demonstration that bone transplants undergo necrosis which replaced by new bone during this mechanism (Cypher and Grossman,1996).

2.12.4.6. Mechanical supports

They are weight bearing supports that fill in major bone tissue defects then supplying strength and structure (Millis and Martinez, 2003).

2.12.4.7. Osteointegration

It is the ability of grafted implant for firmly attaching with the creation of new bony tissue around the grafted border without the formation of fibrous tissue or a procedure whereby clinically asymptomatic firm

fixation of alloplastic materials is maintained and achieved in bone through functional loading (Albrektsson and Johansson,2001).

2.12.5. Structures of bone grafting

Both the mechanical support and osteogenesis of the used cortical and cancellous bone implanted graft are the most essential advantage of bone implant. These factors differ with the bone structure as most of the cellular elements in graft decease and are gradually substituted by creeping substitution. In compact bone this procedure exhibited for replacement is significantly slower than in cancellous bone. Firstly, the implant would fuse strongly with the native host to permit the use of the unprotected portion, and then the remodeling of the bone tissue construction occurres corresponding with functional demands. However, in dogs, if mechanical strength is needed and cortical bone implant must be used, the process of replacement products resorption begins as early as for six weeks after grafting, and it may take up to one year before the complete original structural strength happens. Drilling pits in the implant does not appear to improve the process of repair, but it may lead to the early development of biologic pegs that improve implant fusion to native host bone (Hung, 2012).

2.12.6. Sources of bone grafting

Autogenous implant when the bone segment is taken and harvested from the ilium, fibula and tibia of the same animal.These bones deliver whole cancellous bone, bone transplants and cortical implants. If external and internal fixation practices are not used, the strength is essential in the used implants for linking a defect in a long bone or even for the repairing of pseudarthrosis. The excellent source of cortical grafts is anteromedial border of tibial bone.On the other hand, there is no advantage from

leaving the periosteum attached with implant but suturing to the periosteum has beneficial value in the grafting process (Hung, 2012).

2.12.7. Types of bone grafting

2.12.7.1. Natural bone grafts

2.12.7.1.1. Autogenous bone graft

The autogenous bone grafts are defined as harvesting of bone from one anatomic area to another area in the same animal.These implants have osteogenic properties (marrow-resulting preosteoblastic precursor cells as well as osteoblastic cells), osteoconductive characters (bone mineral and collagen) and osteoinductive characters (noncollagenous bone matrix proteins with growth factors) that integrated more rapidly and totally into the host bone.Autologous bone grafts categories into many forms, including cortico-cancellous, cancellous, non-vascularized cortical, vascularized cortical. Histopathologically though, there are three main differences: 1) cancellous autografts repaired totally with time while cortical autografts remain combination of viable and necrotic bone 2) cortical grafts undergo reverse creeping substitution while cancellous autografts undergo creeping substitution. 3) cancellous autografts are more rapidly revascularized than cortical grafts.The autografts regarded as the golden standard in repairing post-traumatic conditions such as fracture, bone graft, malunion. nonunion, delayed union.There is whole histocompatibility, no chance for transmission of disease and low risk of the graft rejection. As a result, autografts are the perfect and favored implant material in restoration of musculoskeletal system. However, there are disadvantage of using of autogenous bone graft including less amounts of implant materials, especially in newborn animals and in revision reconstructive technique and the probability of high incidence of postsurgical morbidity at the donor area (iliac crest, rib, fibula) due to

pain, muscle weakness, hemorrhage, increased surgical time and nerve injury. The autograft implant sometime can be achieved without hard bony structure (Cypher and Grossman,1996; Khan *et al*.,2005;Calori, *et al*.,2011;Roberts and Rosenbaum,2012; Hung,2012; Chiarello *et al.*,2013; Egol *et al*., 2015).

2.12.7.1.1.1. Cancellous autobone grafts

As a form of autologous bone grafting, cancellous autobone grafts are the most commonly used type that can be obtained from different places such as posterior superior iliac spine, the iliac crest, proximal tibia, femur, distal olecranon and radius.This type of bone implant is usually used to fill bony defect and to adjunct some external and internal fixation because this type does not provide significant mechanical support for defective bone. Cancellous autograft comprises of large amount of mesenchymal stem cell, osteoblasts, growth factors and bone morphogenic protein while its matrix act as an excellent scaffold for transplantation of osteoblastic cells and vascular ingrowth discussing to itsosteoconductive,osteoinductive,osteogenicelements(Finkemeier.,2002).

Because autologous cancellous bone implants have spaces within their construction that allow for the distribution of nutrients and materials and the revascularization of their circulating blood vessels through the process of microanastomosis, they have been deemed to be more osteogenic components than cortical bone grafts as well as the high surface area size of a cancellous autograft that permits to the so many incorporation and revascularization of the implant locally to the native host bone. This implant possesses proteins materials that contributed to the osteoinduction of the implant, beside, it can be presented and preserved when the autografts are suitably treated (Goldberg and Akhavan,2005).

At first time following transplantation of autograft, inflammation and hematoma are created rapidly with the recruitment of mesenchymal stem cells to put down formation of fibrous granulation tissue. At the same time, the necrotic material implant and the dead tissue are gradually removed by macrophages also neovascularization happens. Next, through the autograft incorporation, with the production of new bone by accumulating hematopoietic cells through the transplanted bone, osteoblasts produce layers of osteoid around the dead tissue. After this treatment, the implant is completely replaced and resorbs within 6–12 months (Khan *et al*.,2005).

One of most advantages of such implant are low risk of disease transmission and their excellent success rate, whereas their disadvantages include the high risk of wound infection, limited quantities, prolonged anaesthetic time and increased blood loss (Bhatt and Rozental,2012).

2.12.7.1.1.2. Cortical autobone grafts

These grafts have an excellent mechanical supportive and structural integrity with less amount of osteoprogenitor cells. They are mainly osteoconductive character with very less osteoinductive properties. These implants can be collected and transferred with or without vascular nutrition and exhibit excellent mechanical support. The common areas for harvesting such grafts are the distal radius, iliac crest for non-vascularized autograft, while the iliac crest, fibula, ribs and distal radius are used for vascularized grafts (Goldberg and Akhavan,2005).

The vascularized autografts have been used to fix small fractures with compromised cortical vascular supply like acutely displaced femoral neck and carpal bone defects and in addition for radical fixation procedures such as the reformation of forearms following injury of upper-

extremity.While vascularized implants experience immediate bone repair, nonvascularized implants experience mortality and resorption at the implant host that interact with accompanying revascularization within the first six weeks. During this period, vascularized implants are stronger than non-vascularized implants due to the resorption of dead and necrotic osteons from the cortical bone before the synthesis of appositional new tissue. Revascularization and healing of the nonvascularized cortical implant depend on the reparative phase of resorption. It causes the implant's porosity to grow and its torsional strength to diminish. Cortical autografts are ideal for defects larger than 6 cm that require some structural support due to their unique features, however vascularized cortical autografts are preferred for defects larger than 12 cm due to the higher incidence of failure rate within non-vascularized grafts in defects of such sizes (Sutherland and Bostrom,2005;Roberts and Rosenbaum,2012).

The cortical autograft has the creeping substitution which mediated by the presence of osteoclasts followed by swift hematoma creation and inflammatory reaction in the first stage of bone regeneration then the remodeling and revascularization procedures are severely disadvantaged by the thick construction. Due to osteoclast resorption, the overriding method for incorporating the cortical autograft is through appositional bone development over a necrotic core and these serious processes take 12 months according to implantation area and the implant size (Wang and Yeung, 2017).

2.12.7.1.2. Allogeneic(homologous) bone grafts

It refers to the transplantation of a bone fragment from one genetically distinct member of the same species into another. This graft, which is the best alternative to autografts, has been successfully employed in clinical

practice in a variety of circumstances, especially for those who have established nonunion, poor healing potential, and extensive union following fractures. Allograft is obtained from living donors or human cadavers and represent the second most common bone-grafting process worldwide (Goldberg and Akhavan,2005;Faldini *et al*.,2009).

The advantages of allograft implant that are obtained from cadaver sources are the obtainability to be prepared in different sizes, shapes and endless amount. In allograft there is no donor-site injury or killing animals and damage host structures. The allograft may be customized and machined, therefore they can be obtainable in a variety of kinds involving morcellised, cancellous chips, cortico-cancellous or cortical grafts, highly processed bone products, osteochondral (DBM) and several bone parts depending upon the recipient area supplies. Bone allografts have mostly osteoconductive and less in osteoinductive characters but there is no osteogenicity because there are no viable cells since they are devitalized through deproteinization, decalcification, freeze drying processing or even irradiation. However, there are risks of transmission of bacterial and viral infections, toxins, malignancy, rejections and immunogenicity in addition to high cost from obtaining, conservation and processing of allogeneic implants (Roberts and Rosenbaum,2012;Dinopoulos *et al* ., 2012).

The allografts are immunogenic and have a higher incidence of failure rate, which is supposed to be produced by many factors including triggering of high histocompatibility complex antigens, the sterilization methods, storage technique, freezing and the freeze- drying process.All these factors which applied to lengthen their shelf life and decrease rates of disease spread lead to limitation the osteoconduction efficiency of allograft (Bhatt and Rozental,2012; Wang and Yeung, 2017).

2.12.7.1.2.1.Demineralized bone matrix (DBM)

It is a highly processed derivative of an allograft which contain approximately 40% mineral content of the bone matrix and it is derived from human allograft bone that are removed by mild acid.The available commercial DBM usually include 0.5-0.6 M of hydrochloric acid as a demineralizing agent while collagens, growth factors and noncollagenous proteins remain. The matrix of demineralized bone is primarily utilized as a material for filling bone deficiencies, as evidenced by its inferior structural integrity and structural characteristics(Boyce *et al*.,1999;Finkemeier,2002).

Demineralized bone grafts have osteoinductive characteristics but no osteoconductive properties and it is produced by carefully selecting mineral from the bone without destroying the growth factors and other bone morphogenetic proteins. DBM is provided in a different forms like flexible piece or putty or is joined with powder, cortical chips, segmental blocks that are widely used in human orthopedics(Cammisa *et al*., 2004;Khan *et al*.,2005).

Their benefit includes the capacity to supplement and expand autologous cancellous bone implants when the availability of autogenous bone is constrained or the size of the lesion is great. On the other hand, where stable fixation is available, composites of DMB with autologous bone marrow can also be employed with excellent outcomes. DMB has been used with good outcome to induce bone formation in different applications to repair the bone defects caused by cavities and bone cysts, filling of huge bone tissue defects, craniomaxillofacial repairing and treatment of defects and high danger fracture (Wang *et al*.,2007;Laurencin and El-Amin,2008).

The osteoconductivity of the DBM is made by providing a work context for new bone cell generating and formation after the demineralization process.The DMB has certain drawbacks, including its allergic material, radiolucency, the potential to transmit infectious disease, lack of strength and inherent rigidity, besides its need for meticulous care in its preparation and the degree of osteoinductivity of DBM implants pales in comparison to that of recombinant BMPs or purified. Another disadvantage is the high cost of repairing major bone defect.The determination of osteoinductive of DBM by preserving growth factors which are related with preparation techniques that can be affected by processing, storage, and sterilization methods and vary from donor to another animals.The formation and preparation of the DBM is auxiliary to that of the autogenous graft, with growth factors triggering an endochondral ossification event and culminating in new bone creation at the area of grafting. In evaluation study of DBM efficacy in spine union surgery, the mixture of matrix of deminerlized bone and autograft at contralateral side representing the important part of DBM like a bone implant extender (Katz *et al*.,2009;Nandi *et al*.,2010).

2.12.7.1.3.Xenobone grafts

Xenografts (heterologous) include the transplantation of bone tissue from one species to another.They are considered as one of the best bone implant substitutions to use in clinical field (Liu and Lv,2018).The animal harvested bone implants (xenograft) are only osteoconductive implant that direct the new bone tissue formation into the osseous defect(Reynolds *et al*., 2010). Bone xenograft are made from the inorganic part of animal bone following being classified as Osteoconductors (Calasans-Maia *et al*.,2009). Demineralized bone matrix xenograft is most commonly harvested from cancellous and cortical bone

of pigs, bovine origin and has less inflammatory tissue reaction and trabecular bone formation (Zhang *et al*.,2018).The advantages of xenografts are their geometric and architecture structure is similar to bone, predictable clinical outcome, well documented. As well as preserves augmented bone volume and slow bio-absorbability, however the transplantation of xenograft has a number of biological problems including the hazard of disease transmission (e.g. Retroviruses and prions), its ability to initiate an immune response after grafting, the lack of living cells and the reduced osteoinductive characteristics because of manufacturing processes. Other drawbacks also include biological components, highly variable of its resorption rate, reduced future availability due to European regulatory changes. Bovine xenografts have been established for cranio-maxillofacial applications because of the limitations use of autografts. Presently, the xenografts are used for reconstruction of segmental bone critical-sized defects to deliver mechanical strength, osteoconduction and osteoinduction (Bhardwaj and Kundu, 2010;Haugen *et al*.,2019).

Both, the bovine and porcine derived xenografts were used in a comparative study to repair rat calvarial defect.The outcomes concluded that the porcine-derived xenografts were favorable cell response and bone-regeneration capacity similar to synthetic bovine bone mineral(Bae *et al*.,2019).

2.12.7.1.4.Bone marrow grafts

They are an osteoinductive and osteogenic cellular implants that comprising of growth factors, cytokines, pluripotent stem cells which all stimulate bone tissue formation by transferring mesenchymal stem and osteoprogenitor cell.These MSCs have the capability to distinguish into different cells involving osteoblasts ,chondrocytes, tenocytes, adipocytes

and bone marrow stromal cells.The main benefits of bone marrow aspiration is being safe, simple, low-cost, clean and it permits transfer of cells into the defect area and could be used in mixture with bone substitutes to accelerate and treat bone defects. As well as, the bone marrow graft (injection, aspiration, application and harvesting) can be achieved percutaneously with minimal patient's morbidity (Dallari *et al*.,2007).

2.12.7.1.5. Osteochondral grafts

Osteochondral implants are mechanical allograft consist of a articular cartilage and bone in animals and have been used experimentally and clinically to treat intra-articular fractures, highly comminuted, osteochondrosis, severely osteoarthritic joints and malignant bone cancer (Hung,2012).

2.12.7.1.6. Composite grafts

They have been used as graft substitutes in a traumatic osseous defects and craniofacial reconstruction surgery that reached complete combination at 6 weeks in all animals (Khan *et al*.,2000).

2.12.7.1.7. Collagraft

It is a synthetic implants consist of mixture of (35% tricalcium phosphate and 65% hydroxyapatite) with suspended, deantigenated bovine fibrillar collagen.It is available in a soft strips or as paste form and can transport antineoplastic agents and antibiotic locally to treat bone abnormalities however, it lacks structural integrity therefore it is contraindicated in the repairing of intra-articular fractures (Khan *et al*.,2000).

2.12.7.1.8. Biomaterials agents

These substances are made to interact with biological systems and can be employed for either therapeutic or diagnostic medical objectives. As alternatives to synthetic bone grafts, a variety of biomaterials have been proposed, ranging from metals like tantalum or titanium to ceramics made of calcium phosphate (CaP), calcium sulfate, and cements, bioactive glass, and polymers like polylactides or hydrogel-based materials. However, because they resemble the mineral component of bone, bioceramics, particularly calcium orthophosphates, are the most often employed. Bioceramics synthetic materials are superior for bone repairs due to their improved strength bioactivity and biocompatibility (Wu *et al*.,2014; Habraken *et al* .,2016). Numerous calcium orthophosphate elements are available in solid state or aqueous solution compounds at high temperatures, and they can be obtained either by precipitation at body or room temperature.The only CaP that can be obtained by solid state reaction (sintered HA) and precipitation in aqueous environments at low temperatures is stimulatingly hydroxyapitate(Ginebra *et al*.,2018).The synthetic substance must permit the proliferation and integration of host MSCs and must reproduce the cancellous bone structure of the host (Salgado *et al*.,2004;Baldwin *et al*.,2019).

2.12.7.2.Synthetic bone graft substitutes

This type of graft substitute used for tissue engineering.The perfect implant scaffold must be have structural integrity and procompetitive property and must maintain a proper balance between degradability and mechanical properties porous architecture and act as transit framework for cells until generation of new bone formation (Bhardwaj and Kundu, 2010).

2.12.7.2.1.Calcium phosphate (CaP)

They are founded by hydroxyapatites,in a fixed ratio between tricalcium phosphate (TCP) and (HAp). Chemically, they are associated to the mineral stage of calcified tissues. They are synthetic mineral salts and usually formed by sintering at high temperatures with the elimination of water vapor and prepared by high pressure compaction. They are available in various forms e.g., porous, non-porous implants and granular particles.The CaP prepare certain mechanical provision mostly in the method of compressive power, though their main privilege in their osteoconductive characteristics. This implant accelerate bone creation by helping as an osteoconductive matrix for host osteogenic cells to form a new bone formation under local osteoinductive growth factors.The most CaP compound used is TCP (Scheer and Adolfsson,2009; Zwingenberger *et al*.,2012).

2.12.7.2.2.Tricalcium phosphate (TCP)

It has biocompatible and bioabsorbable properties. Its chemical and crystal features are like to those during the mineral stage of bone.The structural composition of the implant is Ca3PO4. It could be found in either α or β-crystalline forms. It is highly biodegradable compared with HAp. It formed by combining osteoclastic resorption and dissolution. Tricalcium phosphate implants have been used for two decades as dental applications and synthetic bone void fillers.The small particle size and the interconnected sponge like microporosity are believed to enhancement osteoconductive characteristics and promote time resorption besides the used procedure of remodeling(Ghosh *et al*.,2008; Nandi *et al*.,2010).

Although it has more porous structures that are related to one another and can help to facilitate fibrovascular invasion and bone replacement, it has very poor mechanical properties.After grafting, some TCP would

undoubtedly transform into hydroxyapatite due to the physiological pH's thermodynamic instability,which would only partially impede the breakdown of TCP.The typical occurrence is being reabsorbed by phagocytosis after 6–24 months, while some events might linger for years. For that reason, these types of TCP are effectively used as filling materials in case of bone defects caused by benign tumors and trauma but are not favored as a bone-implant substitutes owning to their random biodegradation shape(Ogose *et al*.,2006). In case of segmental tibial defect, the biocoral is superior TCP for repairing this defect (Gao *et al.*,1997).

2.12.7.2.3.Calcium phosphate cements (CPC)

There are two combinations in it, one of which is an aqueous cure. To fill defects of several shapes that later solidified by mixing with an aqueous stage through isothermic reaction, CPC can be injected directly. CPC is mechanically supportive, highly microporous, self-hardened with excellent biocompatibility and low bending strength that have a crystalline structure-dependent rate of resorption.Apatitic CPCs and brushite CPC can be distinguished based on the composition. Apatitic CPCs are viscous, indicating a relatively poor ability to inject them, but a setting reaction can take place at physiological pH levels, and they have slightly more robust mechanical qualities than brushite CPC. The implants show more degradability, however unpredictable degradation has been noted, because of the hydroxyapatite kinetically advantageous transition. CPC is clinically preferred for bone replacement, notably in percutaneous vertebroplasty and kyphoplasty, based on its flow behavior before setting, but not as bone substitutes (Alkhraisat *et al*.,2010;Verron *et al*.,2014).Their main drawbacks are the poor degradation in physiological environment, the lack of structural strength due to their

granular structure and the absence of osteoinductive characteristics that in turn make these implant unable to induce osteogenic differentiation of osteoblasts and stem cells to stimulate new bone creation, which is important for big bone defects regeneration, confused tissue engineering and bone regeneration (Wang and Yeung,2017).

2.12.7.2.4.Biphasic calcium phosphate (BCP)

This compound consists from mixing tricalcium phosphate with hydroxyapatite in a changed concentrations to get the beneficial of both calcium salts. By using the perfect formulation, structural characteristics and the dissolution rate can be measured within ranges and then applied in the majority or as implant coverings (Williams,2014).

2.12.7.2.5.Calcium sulfate compounds (CaS)

It is also known as plaster of Paris that has biodegradable and osteoconductive properties. Gypsum with a proprietary alphahemihydrate crystal structure could be heated to create hard pellets, injectable viscous fluids that harden in vivo, cement, or another form. After vivo, the material disintegrates in 30-60 days when osteoblasts integrate the implant. After metaphyseal bony defects, CaS combinations have been demonstrated to be effective as filling materials for bones., traumatic bone defects following fracture and during reductions, filling of benign bone lesions, bone cysts and cavitary and expansion of implants used for spinal fusion. However, they are not suggested in situations requiring mechanical support. It is totally resorbed and substituted gradually as new bone changes and restores the structural characteristics.It is bioactive, biocompatible and resorbable after 3 months. Possible indications of calcium sulphate implant material include the significant injury of its structural characteristics that occurs upon its degradation therefore, it is a uncertain choice for load-bearing applications.Injectable CaS has been

established to be safe and effective in enhancing the repairing of calcaneus fractures and tibial plateau fractures during internal fixation and open reduction. However, this implant is associated with inflammatory reaction response due to resorption of such compound so persistent wound drainage complications may occur after implantation. Reports documented that in 4-51% of animals, a drainage occurs more commonly in high volumes of CaS or when used in subcutaneously of ulnar and tibial bone tissue (Peters *et al*.,2006; Bibbo and patel,2006;Yu *et al*.,2009;Roberts and Rosenbaum,2012).

2.12.7.2.6.Hydroxyapatites (HAp)

Hydroxyapatite is a naturally occurring component that constitute approximately 70% of bone mineral therefore considered as a safe material when inserted into the internal body structures without any risk (Sheikh,2010).

Hydroxyapatite is a naturally occurring mineral form found as formula of Ca5(PO4)3(OH) but is generally written as Ca10(PO4)6(OH)2 to denote that the crystal unit cell includes two entities. Hydroxyapatite is the hydroxyl end participant of the composite apatite group. The OH -ion can be changed by chloride, fluoride and carbonate creating chloroapatite or flouroapatite. It crystallizes in the hexagonal crystal system.It is chemically found as brown, white, green and yellow powder colorations similar to the discolorations of dental fluorosis (Sheikh,2010)

2.12.7.2.6.1.Characteristics of HAp

1 .It is bioactive materials that have the ability to integrate in structures of bone and support process of bone growth without dissolving or breaking down.

2 .Because of its stoichiometry, it is thermally unstable that could be decomposed at temperature from 800-1200C.

3. HAp does not have the structural strength that allows to succeed in long period load bearing application (Sheikh,2010).

2.12.7.2.6.2.Purposes of HAp

1 .It is a constituent of bone that is also present in sea coral and rocks.

2 .Used as implants for plastic surgery e.g., facial reconstruction because of the construction and chemical similarity between them.

3 .The implant is accepted by the body and permits normal tissue incorporation to happen.

4 .The implants are immunologically inert and do not provoke sensitive reactions because of the high heat method that used to produce the constructions.

5 .The hydroxyapatite can used as an injectable paste that does not have porous therefore the soft tissue and bone ingrowth cannot happen (Sheikh,2010).

2.12.7.2.6.3.Safety and side effect events of HAp

HAp was widely used in orthopedics as bone implant for more than four decades due to their similarity of bone structural matrix including mineral composition, biocompatibility, bioactive material , osteoconduction, slow-degradation, osteoinduction and osteointegration. HAp is commercially available either from a natural or a synthetic sources. Several methods have been reported to prepare synthetic HAp powders which involve wet and solid state chemical methods. There are so many natural sources for HAp like bovine bone, human bone, coral,

fish bone, chitosan and egg shell. However, the natural preparation of HAp without deproteinization is accompanied with transmission of infection and diseases.While synthetic HAp is available and free of disease transmission and more commonly used in orthopedics surgery(Chetty *et al*.,2012).

Estimation of HAp in vitro exhibited the increase in osteoblastic and the reduction of osteoclast activities and characterized by the doubling of the synthesis of collagen (Col) type I, osteocalcin and alkaline phosphatase. Both Col and HAp had received value for bone regeneration as they activate the bioactivity reaction of the scaffold by providing a source of phosphate and calcium ions that could be used by osteogenic cells to form new bone tissue (Wang *et al*.,2016).

Hydroxyapatite (HAp) is good has cytocompatibility properties and used as covering for orthopedic and dental scaffold in spite of its limited use because of its low solubility through the body and structural characteristics and vary from one and neighboring tissue (Santos *et al*.,2007) .

To avoid problems correlated to the using of PMMA114,the plasma spray HAp coating is used for fixation of metallic femoral stem as well as hydroxyapatite-coated pins improve fixation of pin nevertheless of the loading conditions and bone kind and decrease loosening and the rate of infection through internal fixation (Moroni *et al*.,2005).

Hydroxyapatite is considering as a nonbiodegradable ceramic usually used for bone tissue replacement that is fine tolerated and enhances healing process by osteoconduction.These implants have a network of interconnecting channels and pores like the mineralized inorganic construction of bone and available in granules, blocks and cylinders

therefore the formation of bone is headed by vascular ingrowth into the pores and gaps of the hydroxyapatite.The osteoconductive characteristics of HAC-based materials can be enhanced by manipulating structural properties of the complete implant device to the porosity and desired anatomical shaping.We recently have shown that specific mechanical features that carry the capability of the HAC material to undergo portion into progressively less massive elements and finally improve their osteoconductive presentation (Boyde *et al*.,1999).

HAp is an excellent osteointegrative and osteoconductive characteristics and have calcium-to-phosphate structure ratio of 1.67 and calcium hydroxyapatite/tricalcium phosphate (60/40) and this ratio deliver a scaffold or structure which can possess a close border with neighboring bone and have a incomplete clinical uses in the restoration of load-bearing bone defects during the early stages of grafting. Hydroxyapatite has excellent carrier of osteogenic cell populations and osteoinductive growth factors which greatly enhance to their effectiveness as bioactive material distribution vehicles in the future(Noshi *et al*.,2000).

The HAp forms involve HAp cement, porous HAp, nonporous HAp, bio-ceramics and Nano-sized HAp. Bioceramics have more dense construction compared to other stated forms.A bioceramic's ideal pore size should resemble spongy bone as closely as possible. It has been shown that microporosity (pore size <10 μm) allows bodily fluid to circulate, macroporosity (pore size 50μm) provides support, and bone-cell formation uses pore sizes of 100-200 μm and porosities of 60–65%. The ideal macropore size for bone tissue ingrowth is stated to be 565 μm in diameter, as opposed to a lesser size (300 μm). In this regard, HAp is similar to cancellous bone in terms of its fundamental structural features; it is brittle and extremely weak under strain and shear but resilient to

compressive stresses, and it may drop by 30–40% in situ after being grafted for a few months (Gauthier *et al* .,1999).

The pore interconnectivity and macroporosity of synthetic HAp permit the proliferation, adhesion and the revascularization as well as differentiation of osteoprogenitor cells and then ingrowth of new bone tissue in grafting surgical technique moreover the relationship between Ca/P especially in high level and crystallinity could slow the resorption degree of HAp–a process determined by macrophages and giant cells in this way , HAp alone is more often useful as a covering on external fixator pins implants and in places with low structural strain (Bhatt and Rozental,2012;Saputri,2018).

Recently the disadvantage of HAp could overcome by using of Nanocrystalline HAp.This great surface increased resorption degree and significantly decreased the sintering temperature of HAp ceramic while this increasing is not visible in clinical signs. On the other side, labors were also being made to accelerate the structural presentation of Nano-HAp by integration of carbon Nanotubes(CNTs)(Kattimani *et al*.,2016;Mukherjee *et al* .,2016).

The beneficial of Nanocrystalline hydroxyapatite was introduced for augmentation trials in intrabony defects procedures As well as, bioresorbable materials, osteoconductive. Fast healing of critical size defects was detected in animal experimentations and in human applications when it is used as a bone implant substitute and it stimulates bone healing and bone binding by stimulation of osteoblastic action(Silber *et al*.,2003). Nanocrystalline hydroxyapatite is perfect product in healing of osseous defects and new bone formation specially

in mandibular defect in dog as compare to another porous titanium granules(Ahmed *et al*.,2015).

More recently the attention has been turned on the development of an absorbable and nonabsorbable biocompatible of bone substitute.The series of bone creation by osteoblasts and resorption by osteoclasts continues at the bone tissue surface when the site of a bone defect is filled with HAp and leading to the creation of new autologous bone tissue .The data suggested that small size of pore materials and high population of porosity of HAp granules play an essential role in increasement the action of osteoblasts, resulting in more fast adhesion, proliferation, and differentiation of bone tissue cells and therefore it interferes with ingrowth of bone and resorption of the implant and this difficult can be overwhelmed by use of a collagen/ porous HAp compound containing calcined HAp Nanoparticles that are totally replaced and then absorbed by bone tissue (Yamasaki *et al*.,2009;Sotome *et al*.,2016).

The resorption degree of this Nano size material was not fully examined and the enhanced structural characteristics is inadequate to widen the application of HAp in veterinary clinic and therefore recently there have been labors in emerging fixed biomaterial ceramics to accelerate their structural and biological characteristics in addition to cytocompatibility for use in clinical application of tissue engineering and this led to improved bone tissue formation such as acceleration of distal femur bone defect of rabbit, while toxicity of HAp was not showed in the liver and kidney (Wang and Yeung,2017).

Nano-size HAp filler have been used for regeneration and vascularization of the soft tissue in cosmetic procedures and these

biomaterials gave good results when used to support bone tissue regeneration, however reported consideration is now becoming increasingly absorbed of HAp Nanoparticles,which are smaller than HAp microparticles hydroxyapatite and its solubility in ethanol and water increases when the size of a particle is altered from microscale to Nanoscale hydroxyapatite (Iwamoto *et al.*,2012; Hatakeyama *et al.*, 2013).

Hydroxyapatite Nano particles are economical and acceptable available synthetic substance that is widely used in immediate implants for the bone tissue deficiency and as a biocompatible bone implant for contact with bone tissue as it has similar to mineral bone and presents a carbonated and partially substituted apatite. The advantages of HAp are being non-toxic and not be targeted by the immune system as foreign body as well as the eventual scarring and local tissue inflammation caused by an immunological response would not be a problem if hydroxyapatite Nano particle were used and these particles are stronger three times more than cortical bone. Based on Nanocrystal masses and connected with collagen, building up 3-D structures present in several bone tissue forms like cancellous and trabecullar bone (Ebenezer *et al.*,2015).

The using of HAp alone provided poor results while using of the consistent high porous structure of HAp led to fusion of posterolateral lumbar intertransverse process spine surgery (PLF) with producing fusion mass with higher cellular viability of bioceramics (Motomiya *et al.*,2007).

Nanocrystalline hydroxyapatite is perfect product in healing of osseous defect especially mandibular defect in dogs as compare with another titanium granules (Ahmed *et al.*,2015).

Sharifi *et al*.,2012 studied the effect of the structural characteristics of radial bone defects that treated with autogenous implant filled by hydroxyapatite Nano particle in rabbit the worker consider that the cancellous rib bone filled with Nano hydroxyapatite was excellent for thehealing of segmented bone defects and delivers a more fast regeneration with almost normal structural characteristics of bone tissue defects.

2.12.7.2.7.Hyaluronic acid

HA known as hyaluronan or hyaluronate with chemical formula (C14H21NO11). It is an endogenous high molecular weight (≥105 Da) polymer comprising of repeats disaccharide repeats glucuronic acid and N-acetylglucosamine,with several thousand sugar molecules in the backbone. HA is a member of the glycosaminoglyscan family and is a critical constituent of the extracellular matrix (ECM) of all tissues specially in bone marrow and connective tissue and it is manufactured in the cellular plasma membrane of every cell in the body (Aslan *et al*.,2006;Necas *et al*.,2008).

HA is existing in body tissues, such as cartilage and skin and under physiologic conditions it fixes to water and swells when in gel form resulting in fuller/ smoother tissue contours.The viscosity of hyaluronic acid solutions increases with increasing its concentrations, and its single rheological characteristics make it a perfect lubricant in the biomedical kingdom (Fakhari and Berkland,2013).

The benefits has been routinely used in dentistry medicine for the treatment of periodontitis and gingivitis. It is found commercially as (Hyadent®) in a gel or liquid form at several concentrations.Therefore, and as a result of its role in supporting and preserving the structural

integrity of extracellular matrix, HA with its high water binding volume also performs tasks such as complementary the osmotic pressure and deliver cell-matrix interactions , cell-cell, and cell motility. In addition, it also has bacteriostatic characteristics. It can also assume different tasks depending on the type of cell, nature of the tissue. It interacts with cells involved in bone metabolism (fibroblast, monocyte, osteoclast, osteoblast and osteocyte) in addition to proteins (growth factors, collagen type V, collagen type I, calcitonin, fibronectin). It also helps osteoclast, osteoblast and osteocyte cells each of which plays an important role in bone formation, fulfilling functions such as differentiation, migration, and proliferation through increased mesenchymal cell migration and differentiation (Tolg *et al*.,2014;Attenello and Maas,2015; Göçmen *et al*., 2016).

Locally applied high molecular HA has also been shown to stimulate migration and differentiation of mesenchymal and muscular cells in vivo. In general, a high concentration of HA has been established in tissue repair, and largest amounts of HA were found in the extracellular matrix of dermal and musculoskeletal tissues. Free HA concentration is relatively low. However the levels of HA are dramatically elevated directly after tissue damage . Chemical modification (cross-linking) of hyaluronan arrangements results in a material that damages more slowly (because of decreased water solubility). Hyaluronan preparations used as fillers materials have been manufactured from animal or bacterial sources and their clinical effects characteristically last along 6–12 months (Dogan *et al*.,2017; Ahmed *et al*.,2020).

Hyaluronan shares bone induction properties with osteogenic substrata such as bone morphogenic protein and calcitonin. Recent researches have confirmed that HA play an essential role in the repairing process of both

bones and soft tissues (Pirnazar *et al*.,1999). Sonoda *et al*.,2000 explored the healing response of the menisci after damage in rabbit model. They found that the meniscus overhaul 69% at the hyaluronan-treated group and 62% at the control group. This constructive effect of hyaluronan on meniscus repair may demonstrate its ability to accelerate the practice results outcome after meniscus repair and injury (Sonoda *et al* .,2000).Several studies have been showed on the effect of HA on the healing of bone tissue defects formed in different areas of the body.(Aslan *et al*.,2006) used HA in a combination with bone implant in an induced rabbit tibial defect and found bone formation and fibrocartilage tissue in the defect site after 40 days. In another the study performed by (Suzuki *et al*., 2014) for using graft material alongside with HA specifically through the initial stage of ossification, they noticed enhancement of the osteoconductive properties and concluded that HA played a dynamic important role in bone tissue formation .

Many studies have shown that application of 1% hyaluronic acid (HA) could accelerate wound healing and bone tissue formation in infected extraction sockets due to its osteoinductive, bacteriostatic, anti-inflammatory properties. Bone regeneration was encouraged in cases using beta-tricalcium phosphate/ hydroxyapatite in combination with HA during change of chemical and physical characteristics of the implant material in an experimental study. Besides, other new animal studies have reported that connection of HA and other implant materials could stimulate osteoinduction elements (Kim *et al*.,2016).

2.12.7.3. Natural bioactive materials

Due to the resemblance to the native extracellular matrix many workers used natural polymers according to their chemical composition for bone transplantation and replacement.These polymers can be divided into three

categoriesas as follows: polynucleotides (RNA and DNA) ,polysaccharides (amylose, cellulose and glycosaminoglycans) and proteins (gelatine ,collagen, elastin, fibrinogen) (Ghassemi *et al*., 2018) their similarity to native ECMs outcome in high osteoinductive characteristics.

2.12.7.4.Growth factor-based graft substitutes

Growth factors are polypeptides that fused to specific cell membrane receptors and inhibit or stimulate others cell functions such as osteogenesis properties and have been projected for use in engineering of bone tissue. Numerous bone implant alternatives lack any osteoinductive characteristics, particularly synthetic cements and ceramics.These bone substitutes' capacity to hasten bone repair is primarily dependent on their osteoconductive qualities.The osteoconduction of bone replacement would typically make it easier for progenitor cells to migrate and connect to supports, which would then cause them to generate growth factors to encourage the creation of bone tissue (Bhatt and Rozental,2012; Roberts and Rosenbaum,2012).

2.12.7.4.1.Bone morphogenic protein

BMPs are low-molecular-weight proteins polypeptides that have been derived from the bones of a variety of mammalian types involving rat, mouse, monkey, bovine and man. They are also formed by clonal osteogenic sarcoma cell shapes (Wolfe *et al*.,1999).

BMPs recruit endochondral bone formation, presumably by accelerate bone collagen formation and stimulating osteoblastic differentiation of mesenchymal cells and include a collection of 20 distinct cytokines that are all members of the transforming growth factor beta (TGF-) superfamily and all contain various levels of osteoinductive components.

For the regeneration of bone tissue, they have received substantial research.These chemicals stimulate the osteoprogenitors' and other MSCs' mitogenesis as well as their differentiation into osteoblasts.While BMP-7 can directly enhance angiogenesis, BMP-2 can induce osteoblastic development from mesenchymal stem cells. Open fractures, non-unions, joint fusions, avascular bone necrosis, and serious bone abnormalities are only a few of the clinical situations in which they are used.Though, as they are soluble, they can easily diffuse away from the non-union area when administered alone and inactivate in vivo.Then, recombinant human (rh)-BMPs are joint with a carrier matrix (collagen) that serves to retain and releases constant concentration of them over time. Presently, the only drug that accepted BMP for use in an injury location In order to treat open tibial shaft defects stabilized with internal fixation as a nail and treated within 14 days of the initial injury, recombinant human bone morphogenic protein-2 (rhBMP-2) is used. The use of rhBMP-2 in the treatment of post-traumatic arthritis of the lower leg has been discovered to considerably accelerate union rates, and detectives have been looking into other potential applications for its usage in trauma (Giannoudis and Einhorn,2009 ; Chan *et al*.,2014;Einhorn and Gerstenfeld, 2015). Recombinant bone morphogenetic protein-2 (rhBMP-2) is delivered by a composite of synthetic biodegradable polymer and interconnected-porous calcium hydroxyapatite ceramics (IP-CHA), which is an excellent combination (carrier and scaffold). IP-CHA strongly activates rhBMP-2's clinical uses in bone tissue regeneration (Kaito *et al*., 2005).

2.12.7.4.2. Insulin like growth factor (IGF-I, IGF-II)

It is a single chain polypeptide involves three ligands insulin, IGF-I and IGF-II and three insulin receptor [IR] the IGF-I receptor [IGF-IR] and the mannose 6-phosphate IGF-II receptor [M6P/IGF-IIR]), as well as six

IGF-binding proteins (IGFBPs). IGF-I is a 70 amino acid, IGF-II has a like structure and comprises of 67 amino acids. IGFs have about 40–50% homology among themselves and with insulin. IGF-I is expressed by almost tissues of the body and have four types designated as C, B, D, and A.In evaluation, proinsulin involves the C, B and A types, while mature insulin secreted and produced by the pancreas involves the A and B types. IGFI synthesis is secreted by the ovaries, kidneys, placenta, testes, skin, pancreas and the lungs and the circulating IGFI is mainly resulting from the fat and liver. It is derived from chondrocytes, osteoblasts and mostly modulate the synthesis of the cartilage matrix and stimulates chondrocyte formation. IGFs I and II are existing in fracture healing area and both decrease collagenase synthesis and stimulate type-1 collagen synthesis (Salgado *et al*.,2004;Bonefeld and Møller,2011).

During fifty years the IGF-I has been known to be the primary mediator of the effects of growth hormone (GH).The clinical practice of IGF in combination with recombinant human PDGF (rhPDGF) and β-tricalcium phosphate has been used as a gel delivery system for the repair of skeletal reconstructive surgery, periodontal defects and in critical size calvarial defects in irradiated rats, however the effect of local practice of IGFs alone in craniofacial bone defects has not yet exposed a clear potential for acceleration of bone formation in the informed dosages. In implantology most of the studies had estimated the combined use of platelet derived growth factor(PDGF) with IGF-I to encourage bone ingrowth into titanium grafts and to increase the bone contact rate of grafts located into extraction sockets as well as encouraging bone regeneration in dentoalveolar defects around implants or after periodontal bone loss. In another side IGFs may be useful as adjunct factor in an attempt to improve bone regeneration by the application of a combination

of cytokines that resembles as carefully as possible the natural shape of growth factors that released through bone tissue healing (Hollinger *et al.*,2008).

2.12.7.4.3. Basic fibroblast growth factor (b-FGF)

It is formed locally in bone through the primary stage of fracture healing and was found to stimulate bone and cartilage forming (Tang *et al.*,2004).

2 .12.7.4.4. platelet-derived growth factor (PDGF)

It is harvested in blood platelets and acts as a local tissue growth regulator on bone healing of unilateral tibial as reported in an animal model and revealed that this growth factor had a stimulatory effect on bone defect healing (Dallari *et al.*,2007).

2.12.7.4.5. Vascular endothelial growth factor

It is one of the most parameters affect bone tissue formation and regeneration at the fracture site and It is a prominent pathway in the VEGF pathway and the angiopoietin pathway, the two main hormonal pathways governing angiogenesis. It has also been shown that VEGF promotes bone growth. The main source of VEGF release is a hematoma, which also helped endothelial cell growth to promote vascular penetration in a hypoxic environment. In the process of mending a bone fracture, hypertrophic chondrocytes in the epiphyseal growth plate generate VEGF to encourage blood vessel invasion of cartilage and blood flow that encourages the production of new bone through the endochondral ossification process (Gerstenfeld *et al.*,2003 ;Wan *et al.*,2008) .

2.12.7.4.6.Transforming growth factor-beta (TGF-) It is responsible for stimulated matrix formation and cell proliferation.

It-is release by platelets and is further synthesized by mesenchymal cells and existing in the graft hematoma (Sutherland and Bostrom,2005).

2.12.7.4.7.Fibroblast growth factors (FGFs)

There are 22 members of fibroblast growth factors family and 4 fibroblast growth factor receptors (FGFRs) being recognized and are produced by monocytes, macrophages, mesenchymal stem cells, osteoblasts, and chondrocytes, and are reported to be secreted beginning in the early stages of bone repair and continuing throughout the entire healing process. The properties of FGFs in fracture healing is not well understood, but it has been demonstrated that FGFs play an important role in wound healing, neovascularization and angiogenesis but also have strong mitogenic effects on mesenchymal progenitor cells and all of which are mediated by the FGFRs /FGFs signaling. Among all those FGFRs and FGFs, FGF2, FGF1and FGFR1-3 were present closely related to bone regeneration by different studies, in which FGFR2 and FGFR1 and have stronger expressions in osteoblasts and osteoprogenitors while FGFR3 is mostly associated to chondrogenesis (Collin-Osdoby *et al*.,2002;Sutherland and Bostrom,2005;Su *et al*., 2010 ;Coutu *et al*.,2011).

2.12.7.4.8.Parathyroid hormone (PTH)

Is a naturally occurring endocrine that regulates the equilibrium of calcium and phosphate in humans. It has been shown to promote bone mass, strength, and prevent bone loss (Babu *et al*.,2015).

2.12.7.4.9. Platelet-rich plasma (PRP)

PRP is primarily created using commercially available technologies to concentrate and isolate platelets from peripheral blood vessels. The portion of autologous blood with an above-baseline platelet concentration

is the plasma fraction. It contains a number of important mitogenic and chemotactic growth factors, such as VEGF, platelet-derived growth factor, transforming growth factor-beta, insulin-like growth factor, and platelet-derived growth factor. Utilizing the cascade of growth factors generated by the degranulation and aggregation of platelets in the native haematoma of fracture to regenerate bone through platelet-rich plasma (PRP) poses problems (Sampson *et al*.,2008;Nauth *et al.*,2011).

While, autologous platelet concentrate is a portion of blood plasma that is high in the growth factor associated with platelets. Platelet-rich plasma is an appealing therapeutic alternative for several musculoskeletal diseases because platelets carry a rich reserve of cytokines and factors within their dense granules and alpha granules. The degranulation of platelets causes the release of many growth factors, including PDGF and TGF-b, which are well known as the first steps in the cascade of bone regeneration. These growth factors help to promote the chemotaxis, proliferation, and speed up the development of bone tissue by stimulating osteoblasts and mesenchymal stem cells(Alsousou *et al*.,2009).

2.12.7.4.10. An autologous growth factor concentrate (AGF)

It enhances formation of new bone tissue in lumbar spinal fusion after used in mixed with autografts and it is prepared by the ultra-concentration of platelets contains such growth factors. Also when mixed with an allograft carrier, AGF was present to be equivalent in clinical and radiographic results to autograft in 1 or 2 level lumbar interbody union with additional posterior fixation (Jenis *et al*.,2006).

2.12.7.5.Cell-based bone graft substitutes

2.12.7.5.1.Mesenchymal stem cells(MSCS):

They are undifferentiated or immature cells which are accomplished of creating any undistinguishable identical cells.Embryonic and somatic (adult) stem cells are the primary sources of stem cells. Bone marrow, stromal mesenchymal stem cells (MSC), haematopoietic stem cells, dermal stem cells, neural stem cells, fetal cord blood stem cells, and many other types of stem cells are all examples of somatic stem cells. An array of various phenotypes, including adipocytes, haematopoietic precursor neuronal cells, myocytes, and muscle cells, have been produced in mice using embryonic stem cells, pancreatic islets, chondrocytes and osteoblast(Nandi *et al*.,2010).Many workers used autogenic bone marrow or MSCS with porous ceramic for rat or canine segmental bone tissue defects, their outcomes exposed an osteogenic possible.(McGonagle *et al*.,2007;Papathanasopoulos and Giannoudis,2008).

2.12.7.5 .2.Collagen

It is an osteoconductive of bone substitutes with minimal mechanical provision and its use as a transporter for different growth factors such as BMPs. It can be used in binding with other bone substitutes like tricalcium phosphate or hydroxyapatite. Collagen donates to vascular ingrowth, mineral deposition, growth factor binding and supplying a favorable environment to bone tissue regeneration (Nandi *et al*.,2010).

2.12.7.5.3.Gene therapy

It involves the transfer of genetic information to the cells then the cell creates the protein fixed by the targeted gene, cell's genome permits the appearance of exact proteins from the cells themselves for a prolonged period .The genetic material can be presented directly to exact anatomic area in vivo technique or specific cells can be obtained from the patient, expanded, genetically operated in tissue culture and then reimplanted (Chen,2001).

2.12.8.Impairment of bone graft repair

There are many aspects which have been connected with impairment of bone implant incorporation involving firstly using of systemic steroid which inhibit of the progenitor cells differentiation and down the osteoblastic trail. The nonsteroidal anti-inflammatory drugs (NSAID) are well recognized to prevent prostaglandin formation leading to reduced local blood flow thereby delaying graft resorption. Secondly malnutrition especially phosphorus and calcium deficiencies have been connected with late mineralization of new bone tissue (Khan *et al*.,2005).

2.12.9.Problems of bone grafting

The main complications are rejection, non-union, premature fracture, fracture (as fixation loosens), infection, infected sequestrum, exostosis, osteomyelitis, broken plates, rotational malalignment, loose of screws and degenerative changes in the associated joints.The rejections of immunological implant can be diminished by good preparation and treating technique.The mechanism of autobonegraft refusal appears to be associated powerfully to cellular before humoral immunity and the infection may be an appropriate signs of allograft rejection.The non-union may be occurred because of improper immobilization, suboptimal bony tissue contact, and form of bone refusal (Millis and Martinez,2003;EL-Keiey,2009;Joshi *et al*.,2010).

2.12.10.Drawbacks of bone grafting

The main drawbacks are immune mediated rejection, limited resources, increase anaesthetic period, technical difficulties specially in vascularized grafts, surgical invasion, post-operative hemorrhage, iatrogenicity and morbidity. Dehydration reduces the healing process' consistency. Preserved grafts take time to grow bone, and there is a risk of infection,

structural weakness, discomfort, bleeding, permanent deformity, pain and nerve damage in the donor location (Martinez and Walker,1999).

2.13.Immune rejection

Rejection is still the major obstacle to successful transplantation. The immunoreaction against the various grafts is an ongoing dialogue between the innate and adaptive immune system that if left unchecked will lead to the rejection of transplanted cells, tissues, or organs. Activation of elements of the innate immune system, triggered as a consequence of tissue injury sustained during cell isolation or organ retrieval and ischemia reperfusion, will initiate and amplify the adaptive response. T cells require a minimum of two signals for activation, antigen recognition, and costimulation. The activation requirements of naive T cells are more stringent than those of memory T cells. Memory T cells are present in the majority of transplant recipients as a result of heterologous immunity.The majority of B cells require help from T cells to initiate antibody production. Antibodies reactive to donor human leukocyte antigen molecules, minor histocompatibility antigens, endothelial cells, RBCs, or autoantigens can trigger or contribute to rejection early and late after transplantation (Wood and Goto,2012)

Rejection of graft is not common in autografts, nevertheless the risk of rejection in xenografts is real (Hutchinson, 2001). Prevention of graft rejection is achieved through the use of drugs for example the use of immunosuppressant drugs which will suppress the immune system of the recipient. Allograft rejection is more as compare to auto graft rejection. This could be that nature of allograft tissue coming from either a living donor or cadaver necessitates the rejection. Allograft tissue is regularly processed in ways to assist in decreasing the possibility of immune response that may lead to graft rejection. Sensitivity of the synthetic

material used in the implant or device can result in graft rejection (Rabin, 2005).

2.14.Deproteinization

It is crucial for the process of removing antigenicity from xenograft bones. For xenografts, hydrogen peroxide (H2O2) is used as a deproteinizing agent, repairing bone defect and shows incomplete osteoinduction action (Lei *et al*.,2015).

For xenogenic bone tissue implantation, removing the antigens that could possibly elicit an immune response is crucial.The main sources of immune response antigens for bone implanting are MHC-II and MHC-I, which are produced in osteoblasts, osteocytes, bone marrow cells, and osteoclasts. Contrary to the matrix and trabecular bone, which lack antigenicity, the bone gap has multiple expression stages of antigenicity in osteoblasts, osteoocytes, chondroblasts, chondrocytes, blood cells, bone marrow cells, and adipose tissues. Deproteinized bones have been found to retain their osteoinduction and osteoconduction functions while losing their immunological reactivity. Deproteinized bones' trabecular porosity architecture serves as a mechanical framework for bone cell growth and blood arteries, both of which are crucial for bone ossification. (Castro- Ceseña *et al*.,2013).

2.15.Porosity

A 3D porous morphology is one of the greatest vital factors affecting bone organic activity because the pores permits proliferation and migration of the mesenchymal stem cells, osteoblasts as well as vascularization that are required for tissue engineering. It has been exposed that both macro and micropores are important for engineering of bone tissue.The larger macropores are essential for proliferation, cell

attachment, ingrowth and the tissue creation whereas microporosity is important for the transport of nutrients, oxygen, metabolic waste and ions to and from the implant. In overall pore sizes smaller than1 μm activates the bioactivity that confirms communication with proteins, whereas pores between 1 – 20 μm controls the cell type that is concerned to the scaffold and assist with cellular development, vascularization, orientates and direct the cellular in-growth.Cellular-growth and bone in-growth happens in the pores between 100 – 1000 μm. Pores larger than 1000 μm confirm the aesthetics, shape and functionality of the graft (Dorozhkin,2007).

2.16.Nanotechnology

It is an autonomic scale and the study of the controlling of material during molecular matter.Nano word means very small matters that derived from the Greek civilization which means dwarf and usually related to Nanobot, Nanometer and Nanotechnology. A noun and describes something about 109 and refers to the capability to manipulate ,measure and organize matter at the Nanoscale level.Generally Nanotechnology deals with structures that having size 100 Nanometers or smaller in at least one dimension, besides devices or developing materials within that size (Chakravarthi and Balaji,2010; Boulaiz *et al*.,2011).

Nanotechnology is a modern development field of science and tissue engineering that has led to original and advanced approaches in many aspects of physics, medicine, engineering, chemistry and biology. The scale classically refers to matter in the size range of 1–100 nm but it is often extended to involve materials below 1 μm in size and manipulation of modern phenomena and characteristics (biological, chemical and physical) at that length scale . Nanotechnology is using the techniques and philosophy of the Nanoscale to transform and understand

biosystems, which use biological ideas and constituents to form new Nanoscale protocol and devices. These substances adhere to quantum physics' principles rather than those of classical mechanics.They behave significantly differently as a result from their more traditional counterparts who have comparable anatomical structures but grain sizes higher than 100 nm. Successful manipulation of Nanotechnology in bone regeneration is challenging given that the hierarchy of bone is generated from Nanoscopic collagen fibers between 100nm -2,000nm in length which deliver the architecture for Nanoscopic (20-40nm in length) and hydroxyapatite Ca2(PO4)3OH to build upon. In response to the lack of an ideal management option for bone healing of severe or non-union fractures, Nanotechnology is a new subject in orthopedic surgery (Mendonça *et al*.,2008;Brannigan and Griffin,2016).

Nanotechnology materials can be categories into three main groups: raw materials, Nanostructured materials and the group collected by fullerenes and Nanotubes.The raw material involves Nanocrystalline and Nanoparticles materials that are readily substituted and industrial for less performing bulk materials.Nanostructured materials are classically treated methods of raw material that deliver special forms and functionality. Examples of Nanostructured materials involve the dendrimers and the quantum dots. Fullerenes and Nanotubes can produce constituents that are 100 times more conductive than copper and stronger than steel and can be carefully used in some medical applications(Chakravarthi and Balaji,2010).

2.16.1.Biomaterials and nanotechnology for bone healing

Bone is complex in its anatomical makeup and bioactivity, which limits the efficiency of bone grafts and implant fields.Therefore, it is difficult to replicate synthetic implants.While implants can somewhat replicate the

structural support that bone offers, they are unable to replicate bone's bioactivity. It is necessary to have a material that, in addition to being sceptic, can supply the biological, mechanical, and structural complexity of bone. Nanophase materials' surfaces more closely resemble the surface of trabecular bone than their conventional counterparts. In addition to the smaller material grain size seen in Nanophase materials importantly increases their surface area to volume ratio. Both of these properties results in enhancement osseointegration properties of the graft which decreases the fibrous encapsulation and the danger of joint replacement loosening compared to conservative implants (Webster and Ahn,2006).

2.16.2. Nanotechnology applications in the veterinary area

The use of Nanotechnology in veterinary medicine has exposed a notable development in recent years. For veterinary care, animal health, and other sites of animal production, Nanotechnology holds great potential. Nanoparticles are used in the area of veterinary medicine to distribute heat, medicines, other substances, and light to particular types of cells in animals.Various Nanomaterials are being researched today for their potential use in a wide range of applications, including Nanoshells (for the use of IR radiations to destroy cancer cells), aluminosilicate (to stop bleeding), carbon Nanotubes (sensors and drug delivery), gold Nanoparticles (for diagnosis and labeling agents), Nanocrystalline silver (disinfectant agent), Nanorobots (for the treat of individual cells), and iron oxide Nanoparticles (improved MRI imaging) (Meena *et al*.,2018).

Nanotechnology is utilized to create Nanoscale medications, detect contaminants, create controlled delivery systems, and build Nanodevices for molecular and cellular biology.It will play a significant role in the domains of veterinary science, animal welfare, and other animal production and will also act role in controlling of infectious diseases

through enhancing immunogenicity and protect our patients from viral or bacterial infections and the implementation of a smart drug delivery system, but also improve wound healing process and used for can alleviation the pain. Also these new compounds could carry genes and drugs in a further targeted method then these systems will have an effect on the rate of distribution, absorption, excretion and metabolism of drugs or other substances in the body thus permitting us to control the organize pharmacodynamic of drugs (Underwood and Van, 2012) in addition to Nanoparticles used for treatment disease, diagnosis, improve animal breeding ,delivery of drugs and reproduction which involve magnetic Nanoparticles ,quantum dots, polymeric Nanoparticles ,Nanopores, fullerenes, Nanoshells, dendrimers and liposomes. However, veterinary medical science is still in the early stages of applying Nanotechnology, which is one of the major advances currently used in many fields compared to other sister disciplines (Woldeamanuel *et al*.,2021).

With highly adjustable surface chemistry and huge active surfaces that allow for binding to imaging labels, tiny molecule medicines, and ligands including nucleic acids, peptides, and antibodies, Nanoparticles stand out from bulk materials. Additionally, due to their tiny size, tumor tissues have enhanced retention and permeability, extravasation via endothelial cells, and other intracellular interactions (Goel *et al*.,2017). According to predictions, Nanotechnology will lead to a significant number of inventions in the twenty-first century that will develop veterinary medicine and other animal breeding facilities as well as the practice of clinical veterinary medicine. Animal history from birth to consumer table, cellular and molecular breeding, animal nutrition scenarios stretching from nutrient uptake and use animal waste change as ejected from

pathogen detection, livestock, and much more will all be accelerated by veterinary Nanotechnology (Wilczewska *et al*.,2012).

2.17.Nanotopography

It exhibited to influence proliferation, cell adhesion, cell specific adhesion and differentiation. Related variations in Nanostructure and chemistry convey important chemical variations and permit biomimetic dealings between tissues alloplastic and surfaces. Nanoscale modification of an implant surface could contribute to the mimicry of cellular environments to service the process of rapid bone tissue regeneration (Mendonça *et al*.,2008).

The result may be changed in physical characteristics involving improved catalytic ,magnetic, electrical, optical, biological and mechanical characteristics when compared to conservative formulations of the same material. Nanostructured surfaces have unique characteristics that change cell adhesion by indirect (affecting protein–surface interactions) and direct (cell–surface interactions) mechanisms. The changes in initial protein–surface interaction are supposed to control osteoblast adhesion (Balasundaram *et al*.,2006).

2.18.Alkaline phosphatase

Alkaline phosphatase (ALP) can be defined as a glycoprotein that, at basic pH levels, catalyzes the hydrolysis of phosphate monoesters and functions as an ectoenzyme attached to the matrix vesicles and the outer surface of cells. In humans, four genes produce ALP isozymes that are expressed in the bone, kidney, and liver as well as the intestinal, placental, and germ cell tissues.Numerous studies demonstrate that ALP stimulates extracellular mineralization by causing the mineralization inhibitor inorganic pyrophosphate to release inorganic phosphate. Crystal

development, primarily in the form of crystalline hydroxyapatite, follows the accumulation of calcium and inorganic phosphate, which initiates mineralization.ALP incubation in solution can degrade calcium phosphate as a biomaterial component (Zhou *et al*.,2019).

Despite the fact that ALPs have been studied for a while and are found in numerous mammalian tissues, little is still understood about them.The intestinal isoenzyme is believed to be involved in the transport of phosphate into gut epithelial cells, and the bone isoenzyme may be involved in the calcification of mammalian bones. Continuous and substantial research has been conducted on alkaline phosphatase (ALP), especially in bone which is essential in the normal skeletal mineralization.The alkaline phosphatases levels used extensively in routine diagnosis of bone and liver affection and diseases in healthy individuals of different ages. Since ALP is a consequence of osteoblast activity, as in the case of bone Paget's disease or a condition that affects blood calcium level (hyperparathyroidism), vitamin D insufficiency, or damaged liver cells, an increase in ALP suggests an active bone building process (Sharma *et al*.,2014) .

ALPs have been linked to a number of biological processes, including the control of protein phosphorylation, cell proliferation, apoptosis, and cellular migration throughout embryonic development.The different signals that control ALP genes are demonstrated by the crystal-clear distinctions in their expression profiles (Tsai *et al*.,2000).

One of the earliest significant participants in the process of osteogenesis to be recognized is ALP.When evaluating the phenotypic or developmental maturity of mineralized tissue cells, tissue engineering and investigations of osteogenic stem cells, ALP has emerged as the preferred

marker. It contributes in promoting of mineralization process. Additionally, research into the crucial interactions between ALP and matrix vesicles, cell membranes, the intricate interactions of lipids, proteins and ions that ultimately lead to the nucleation and growth of mineral crystals promises to shed new light on how cells make use of ALP's special properties to form minerals. The intense activity in this field will undoubtedly lead to the discovery of novel and important knowledge about the fundamental mechanisms underlying the development of hard tissues, as well as therapeutic opportunities for the treatment of bone diseases and an improvement in the production of useful bone biomaterials (Golub and Boesze-Battaglia,2007).

The detection of specific biochemical markers of bone formation in serum, like ALP activity, can be clinically useful in evaluating the progress of the healing process because there was limitation of using advance diagnosis technique in veterinary medicine (Komnenou *et al*., 2005; Sousa *et al*.,2015).

The biochemical indicators give a dynamic picture of the underlying process of bone resorption, invoving its turnover, etiology, and the ability to distinguish between normal and delayed healing as well as any healing process impairment. Bone resorption and bone production markers are the two main subclasses of biochemical markers of bone turnover.The tartrate resistant acid phosphatase and breakdown products of type I collagen in protein matrix, including hydroxyproline, telopeptides, etc., are associated to osteoclasts' resorption of matrix and are examples of bone resorption markers. Osteocalcin and bone-specific alkaline phosphatase, which are produced by osteoblasts, are indicators of bone development and are examples of bone production markers (Kumar *et al.*,2018).

Chapter Three

Materials and Methods

3.1.Experimental animals

In the present experimental study, thirty six adult local breed of both sexes adult dogs were used. To be sure of being healthy, all animals were inspected to be free from any infectious diseases as well as vaccinated with rabies vaccine (Biocan DHPPI+LR, biovita, Czech Republic) then injected with Ivermectin 1% (promectin, spanish) at 0.3mg /kg S/C to control the external and internal parasite besides acclimatization for 14 days (Paterson *et al*.,2014).The average weight was (21 ± 0.3)kg and the age was (2.1±0.9) years. All animals were housed at the animal's house of Veterinary Medicine College \University of Mosul during the whole period of experiment.They kept and numbered in an individual cage under the same manegmental protocol of housing, feeding, and health care.The experimental design was approved by Ethics Committee of the Faculty of the College of Veterinary Medicine, Mosul University No UM.VET.2021.060.

3.1.1. Experimental design of animals

In all animals,a rectangular bone defect of (2.5 × 0.7cm) at the proxomedial portion of tibial bone was experimentally induced. Then these defects were closed with the deproteinized ribs xenograft derived from 2 months new born lamb ages for all groups except the control group.Then the experiment animals were allocated randomly into 4 groups of 9 animals for each as followings :

Group 1: The rectangular bone defects of the tibial bone were lifted without any treatment and the same excised autograft was repositioned

and fixed with 2-0mm sterile cerclage stainless wire suture material as a control group .

Group 2:The rectangular bone defects of the tibial bone were reconstructed with xenograft rib from new born lamb.

Group 3:The rectangular bone defects of the tibial bone were reconstructed with xenograft rib from lamb and furtherly supported with 1ml of 1% hyaluronic acid gel.

Group 4:The rectangular bone defects of the tibial bone were reconstructed with xenograft rib from lamb and furtherly supported with 1ml of 33% hydroxyapatite Nano gel.

All animals undergo the similar conditions and surgery along the whole period of operation. Clinical, radiological, serological, biochemical, macroscopical, histopathological and histobiochemical, examination were demonstrated when necessary during the period of study.The gross examination focus on the presence of swelling, seromas, firm application, fixation of implant, the progress of healing process, inflammatory reaction, infections and wound dehiscence.While the clinical observation studied the pain, swelling, infection and lameness degree of the operated leg at 0 time presurgery and 7, 14, 30 and 60 days post-surgery. On the other hand, the radiological examination observed the presence or absence of lucent line, periosteal reaction and bone formation around the bone implant at 14,30,60 days post surgery ,whereas the biochemical examination measured the levels of alkaline phosphates enzyme and insulin like growth factor in serum post-surgery.The histopathological and histobiochemical investigations done after collection samples in different periods of healing. The experimental design is illustrated in the following diagram (Fig.4).

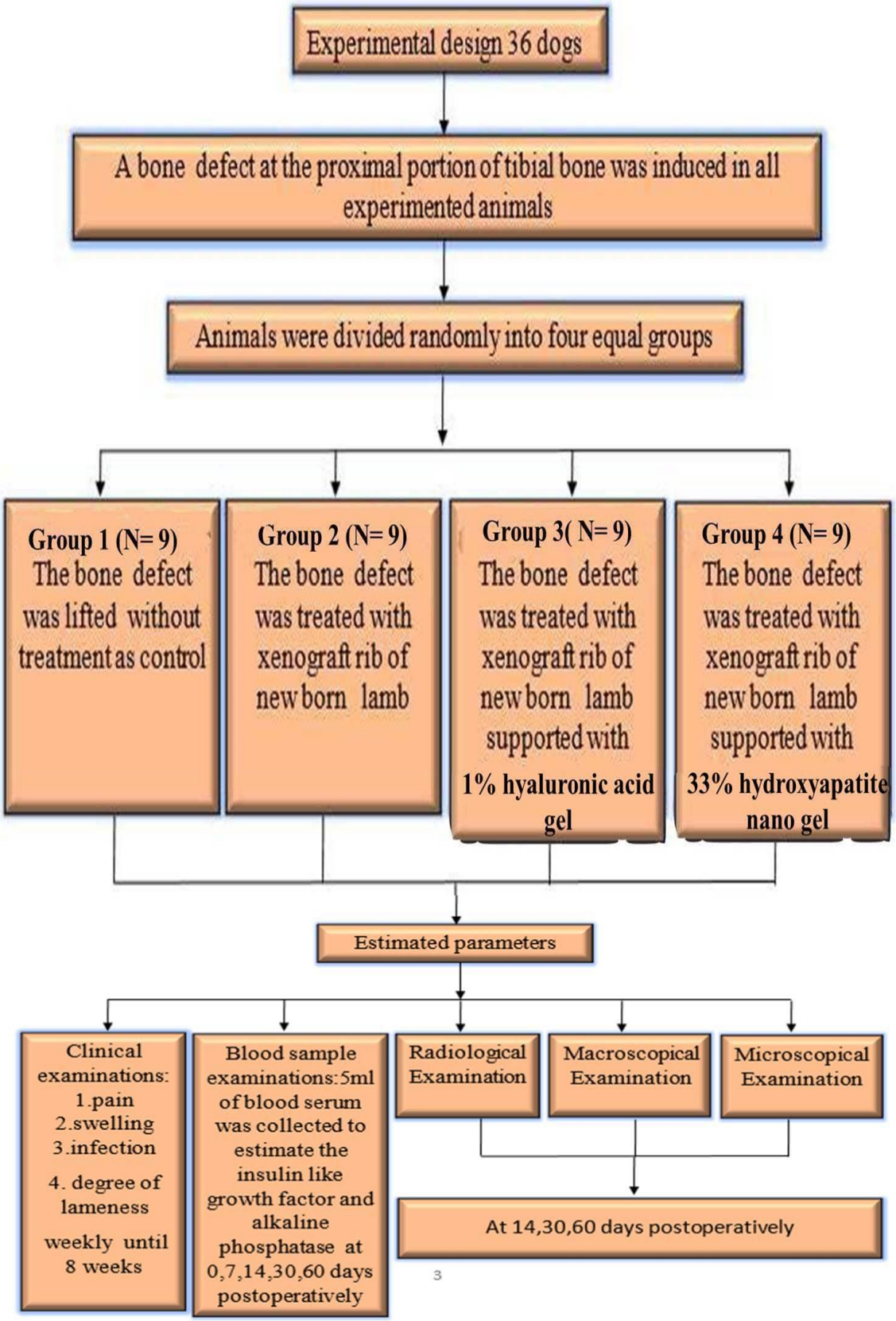


Figure 4:Diagram of experimental design.

3.2. Preparation of the bone xenograft

The ribs of two months small lambs were aseptically harvested from the abattoirs and put in 0.9% sterile solution of normal saline then the fascia, soft tissue including cartilage, periosteum and the surrounding muscles were removed by a blade and clean cotton. Then rinsed with distaled water and cut into a suitable size of (2.4 × 0.6 cm) using a bone cutter (Fig. 5). To remove the fat, the obtained segments were treated with chloroform and 96% ethanol at (1:1)ratio at 25 °C for 12 hours (Hu *et al*., 1991),then deproteinization was performed by adding 1% pepsin (UK) (Lei *et al*.,2015). Later, the grafts were passed through a water bath at temperature 37°C for 48 hours and washed with distal water. Finally, the prepared segments were allowed to dry at room temperature for 24 hours (Fig.6).

Following 24 hours of dryness of these bone segments,the bone then sterilized by autoclave at 121°C for 30 minutes (Draenert and Delius, 2007) and dried by oven at 60°C then kept in the disposable sacs and freezed on -80°C.At the time of using these pieces were thawed and the segment was ready to use for bone repairing (Fig. 7) (Heo *et al*.,2011).

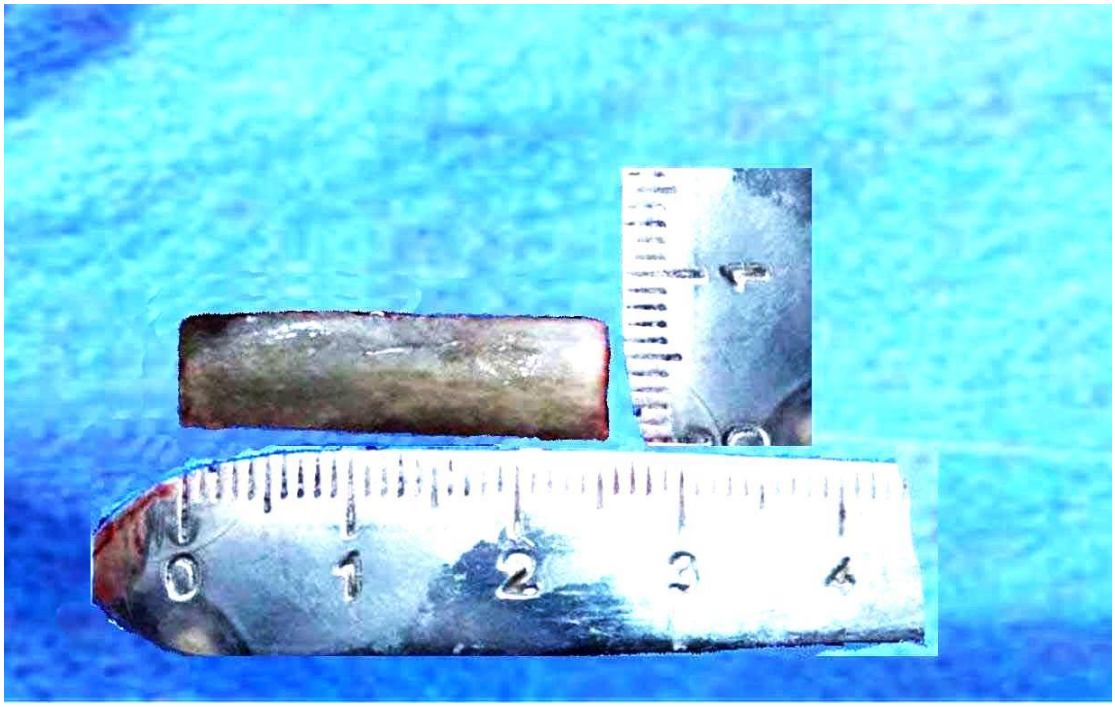


Figure 5:Photographic image shows segment of lamb rib bone xenograft of (2.4 × 0.6 cm).

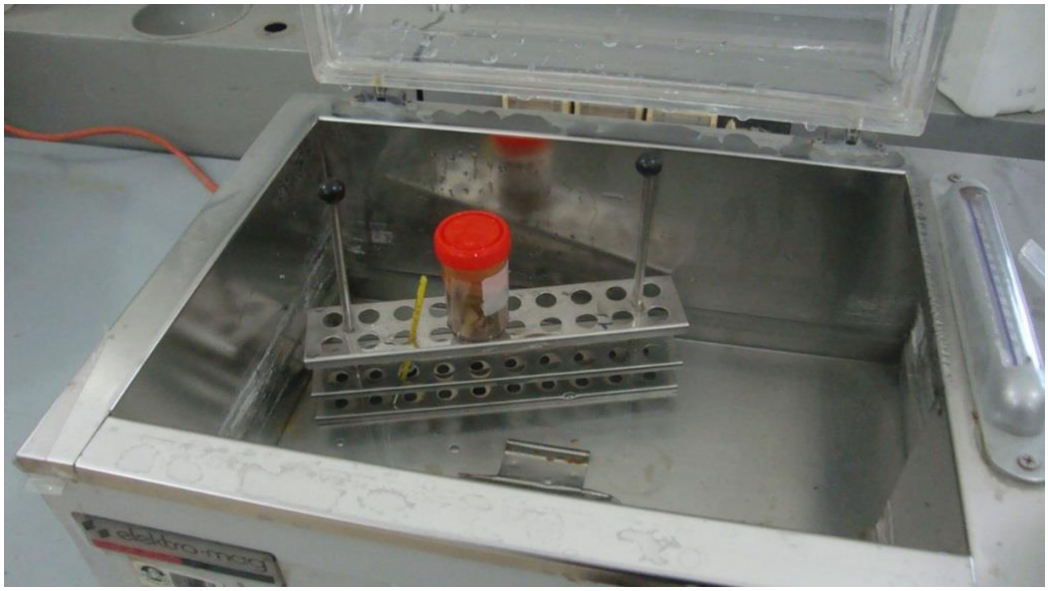


Figure 6:Photographic image shows the keeping of the segments in bath room at 37C°.

3.3.Preparation of pepsin

The used Pepsin solution was prepared as follows:

A 10 mg of 1% of Pepsin powder (Sigma-Aldrich, USA) was dissolved in 1ml distilled water and added to a 10mg cold hydrochloride acid (HCL) (Sigma-Aldrich, USA) to reach the result up to PH (4.4) which remain active (about 90%) and stable for 2-3 months when preserved at -20°C (Budavari *et al*.,2001).

Figure 7:Photographic image shows keeping the sterilized lamb rib bone xenograft in a plastic sac.

3.4.Preparation of hydroxyapatite Nano gel

3.4.1.Muco-adhesive gel Preparation

Preparation of hydroxyapatite Nano gel was performed at Pharmacology laboratory,College of Veterinary Medicine,Baghdad University according to (Şenyiğit *et al*., 2014)and (Tuğcu-Demiröz *et al*., 2015) setting. A 0.125 g of Carbopol was dispersed in distilled water 6.25 (w/w) 2% by stirring at 800 rpm for 60 mins and adjusted by adding dropwise of 10% sodium hydroxide (NaOH).The substance(hydroxyapatite Nano powder Ca5(PO4)3OH)) of (Hualanchem. Co.China) mixed until a transparent gel formed and the

gel PH was adjusted to 4.5. Finally, the substance was sterilized using ethanol.

3.5.Experimental animals

3.5.1.Anesthetic protocol and preparation of animals

All animals were fasted 6-8 hours of food and 6 hours of water before the operation. Preoperatively all dogs were premedicated with atropine sulphate(Vabco-Jordon) intramuscularly at a dose of (0.04 mg/ kg B.W.), A 10 minutes later, a mixture of Xylazine hydrochloride 2%(Interchemi-Holland) and Ketamine 10%(Dutchfarm - Holland) intramuscularly injection at a dose of (5 mg/ kg B.W.,10 mg/ kg B.W.) respectively was used to induce general anesthesia at the surgical level (Green and Thurmon,1988).

The animals were restrained on the operating bench on right lateral recumbency and their legs were restrained to the operating bench to allow easy exposure of the medial aspect.The medial right hind limb from the upper part of the stifle joint to below the tarsal joint was prepared aseptically by clipping, shaving and antiseptic using 70% ethyl alcohol and povidone iodine. The operative area was allowed to dry for 3 minutes then covered with drapes and towels to be ready for surgical interventions .

3.5.2.The surgical procedure

A 3-5 cm longitudinal skin incision was done at the proxomedial aspect of the right tibia of each animal then another incision in the deep crural fascia over the medial shaft of tibia was made to separate the fascia from the muscle and to expose the bone. The tibialis cranialis and the medial digital flexor muscles were retracted by bluntly dissection, whereas, the fascia along their borders was separated from tibia (Fig.

8). A rectangle of (2.5 × 0.7 cm) bone defect was experimentally induced using electrical saw (Total company -china) with continuous irrigation with 0.9% sterile normal saline solution (Fig.9).

In the control group, the same harvested bone segment is re-implanted at the experiment defect in the same animal then, a two bony holes were mady by electric drill, the implanted piece was firmly fixed with 2-0 mm sterile cerclage stainless steel wire suture material (Fig.10,11). In the first treatment group, the surgery was done as same as the first group except using the rib lamb xenograft for repairing that experimentally induced bone defect (Fig.12). For the second treatment group the same as in the first treatment group was performed except the implanted segment was additionally reinforced with 1ml of 1% hyaluronic acid gel (Mc Cosmetic-Spanish) as a filling materials along the edge of the repaired defect (Fig.13). Besides, in the third treatment group also the same as in first treatment group was carried out but the implanted segment was reinforced with 1ml of 33% high viscosity hydroxyapatite Nano gel as a filling materials along the border of the repaired defect (Fig.14). Following filling of bony defects in all four groups, the fascia and muscles were closed by lockstitch simple continuous suture pattern using No.1 polyglactin 910 suture (Yangzhou Super Union- China) then the skin was closed by suture of simple interrupted using surgical No.1 silk (Yangzhou Super Union- China). Finally spraying of operation site with wound spray (Gordon) and proper immoblizaton of the limb by application of gypsona (Plaster of paris) to restrict the movement of limb. All animals were monitored daily until week 8 to evaluate the clinical, serological and radiographical observations. Later, the animals were anesthetized without euthanasia and the biopsies were collected to evaluate macroscopical and histopathologicaland histochemical

investigation from 3 animals for each group at (14, 30 and 60 days) post-surgery to determine the healing process and the bone formation in the site of operation.

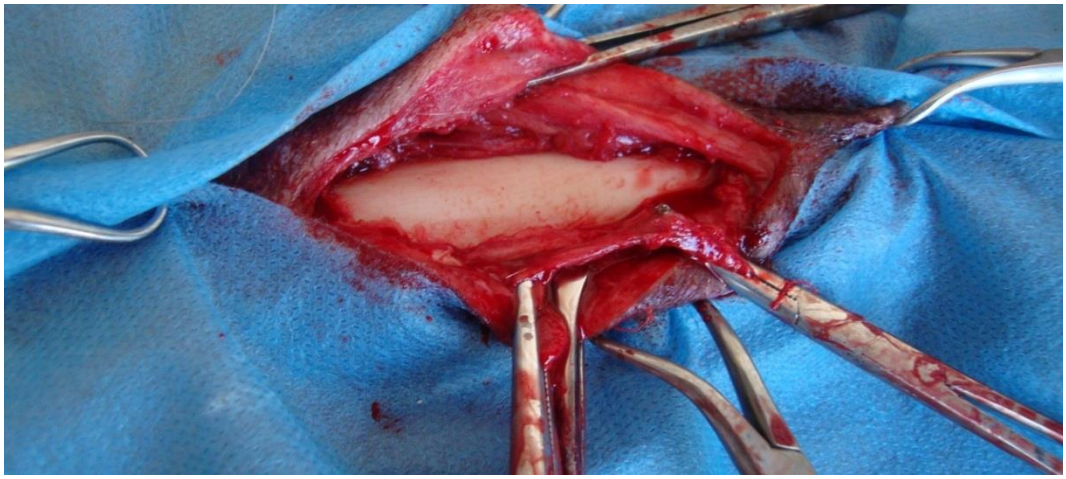


Figure 8 : Photographic image shows the operative site.

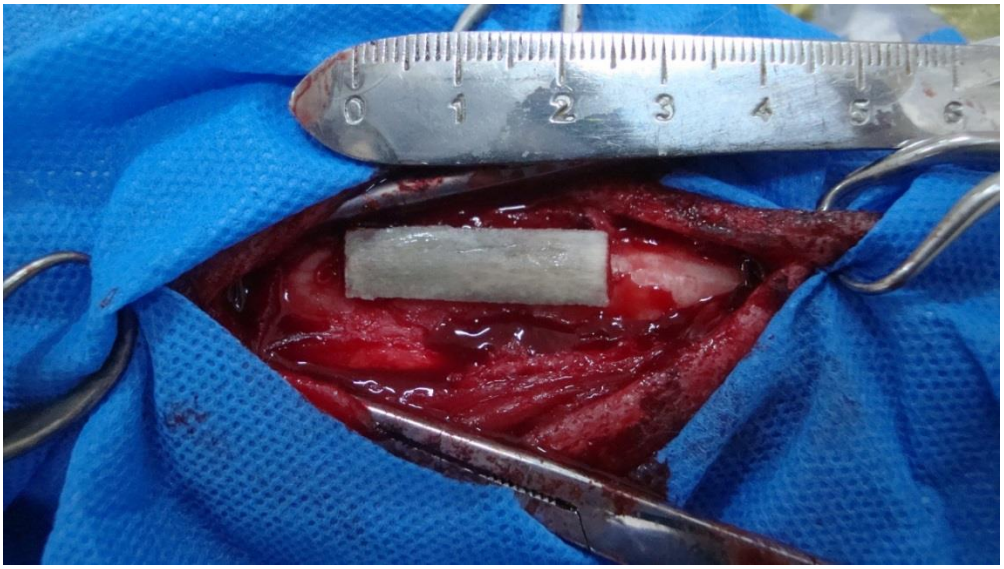


Figure 9: Photographic image shows the determination of experimental bone defect (2.5cm × 0.7cm).

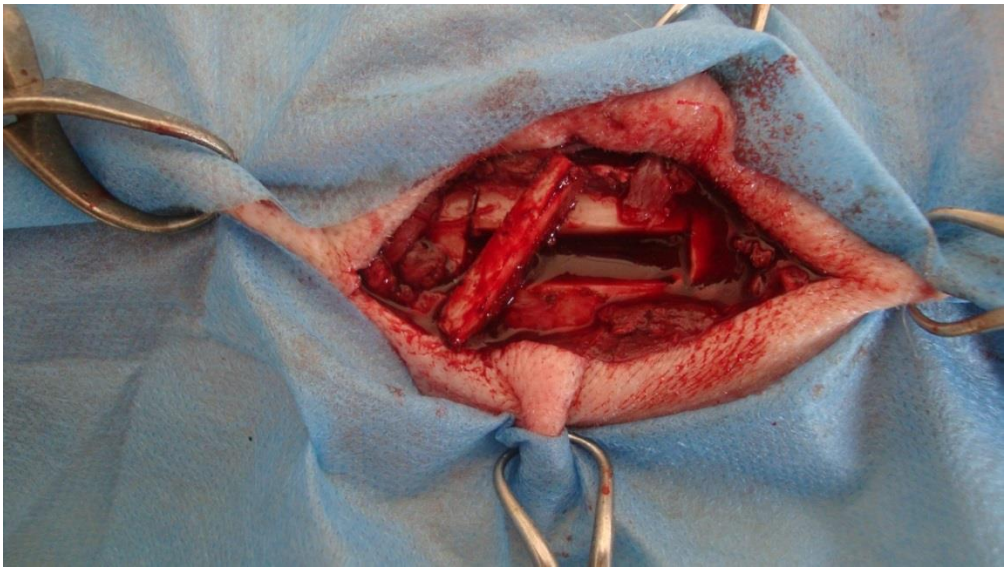


Figure 10: Photographic image shows the rectangle bone defect in the control group after incision.

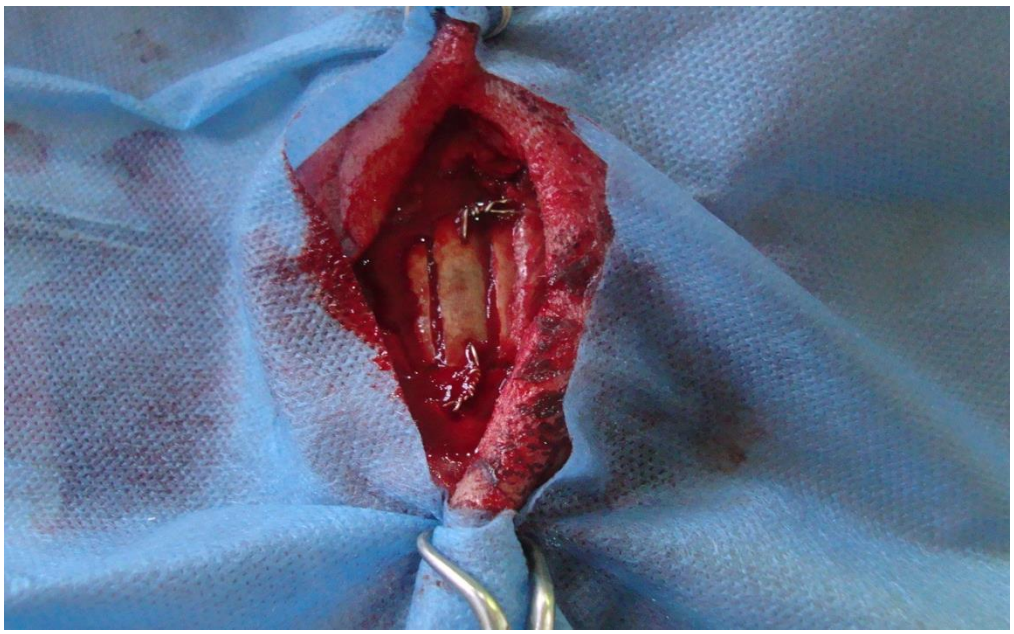


Figure 11: Photographic image shows the fixation the proximal and distal bone graft with 2-0 mm sterile cerclage stainless steel wire suture material in control group.

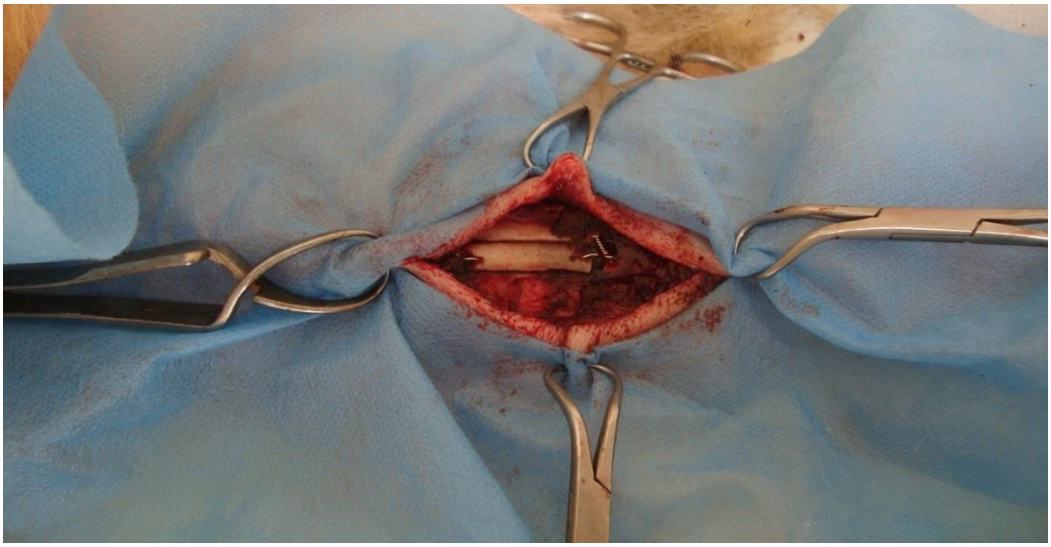


Figure 12: Photographic image shows the fixation the proximal and distal bone graft with 2-0 mm sterile cerclage stainless steel wire suture material in xenograft group.

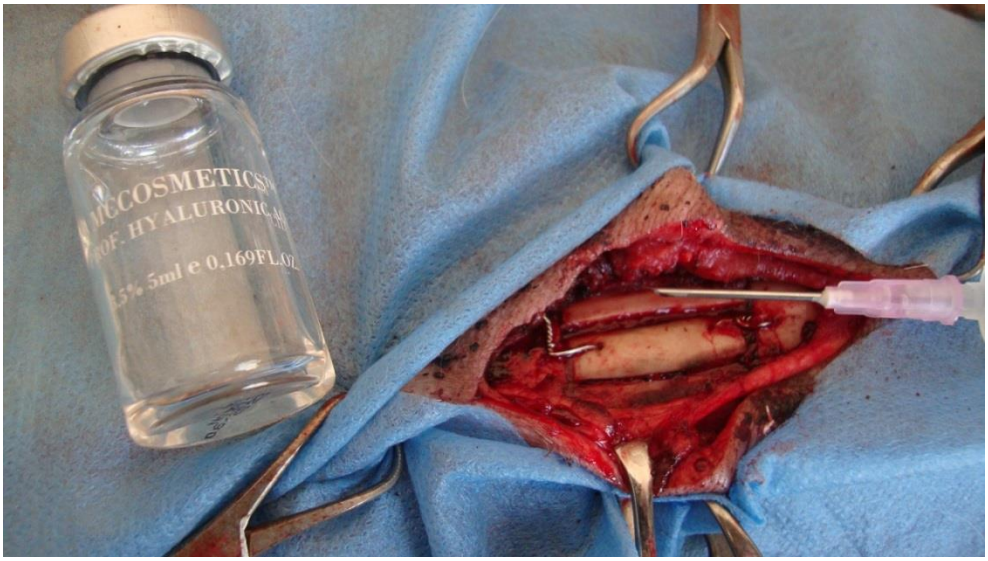


Figure 13: Photographic image shows repairing the defect using lamb rib bone xenograft and fixed with 2-0 sterile cerclage stainless steel wire suture material reinforced with 1ml hyaluronic acid 1% as a filling material.

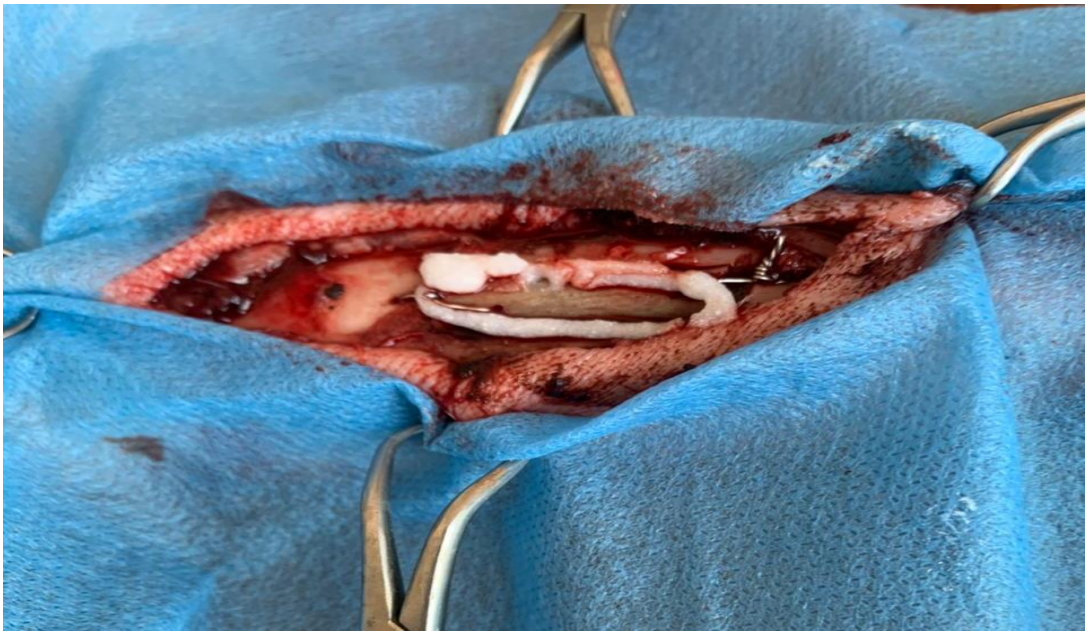


Figure 14: Photographic image shows repairing the defect using lamb rib bone xenograft and fixation with 2-0 sterile cerclage stainless steel wire suture material reinforced with 1ml hydroxyapitate Nano gel as a filling material.

3.5.3.Postoperative care

A course of of pencillin-streptomycin antibiotic (Interchemi-Holand) at dose of 3-5 mg/Kg BW was injected intramasscurally for three successive days and the suture stitches were removed on the 10th day post-surgery.

3.6.Assessments

The clinical, radiological, serological, macroscopical, histopathological and histochemical examinations were investigated during the whole period of experiment in all experimental animals.

3.6.1.Clinical observation

The external fixation with gypsona was removed in about 7days post-surgery.The operative animals examined carefully and observed for any abnormal signs as seroma, odema, hematoma, wound dehiscence, loss of function, infection and weight bearing capacity.

3.6.2.The degree of lameness

The degree of lameness was estimated weekly till the end of the experiment on day 60. Lameness scores (0 to 5) were allocated based on the following scale as mentioned with modification by (Arias *et al*., 2013) (Table 1).

Degree	Description
5	No extremity usage.
4	intermittent and Infrequent support of the extremity. No weight bearing.
3	Walk show lameness in the functioned extremity at trot and walking. Incomplete weight bearing with promotion of the extremity at gallop.
2	Walk show lameness in the functioned extremity at gallop and trot . Whole weight extremity bearing.
1	Normal walking, intermittent lameness at gallop and trot without extremity promotion.
0	Normal.

Table 1:Shows the degree of lameness according to(Arias *et al*., 2013).

3.6.3.Radiographic examination

For all experimental animals, the radiographs (Shimadzu corporation-Japan) and digital radiography (DR-nexus -varex imagining corporation) of the tibial bone defect were performed at 14,30,60 days post-surgery using exposure factors of 60 kVp, 0.04 mA at 0.25 seconds and 90cm F.F.D in three views mediolateral, anterioposteriorly and oblique one of the operated limb.The radiographic examination included the assessment of periosteal reaction with the presence or absence of the bridging of the segmental bone defect and the callus formation of the operated limb.

3.6.4.Biochemical analysis: Alkaline phosphatase (ALP)

The concentration of the alkaline phosphatase was determined through colorimetric calculation using spectrophotometer device manufactured by (Jiangsu-China). A kit(DEA) manufactured by France company (Biolabo

reagents) was used to measure the concentration of the ALP in serum at day 0 presurgery and 7, 14, 30, 60 days post -surgery. Blood samples (5 ml) were collected from the canine Jugular vein. Then at every selected interval time, the test tubes containing blood samples left for 30 minutes to clot.Then these tubes were centrifuged (Gemmy industrial corp-Taiwan) at a speed of 3000 rpm for 10 minutes in order to obtain the serum (Appendix 1)(Tietz,2006).

3.6.5.Insulin like growth factor(IGF-I)

3.6.5.1.Evaluation of growth factor

To detect the level of insulin like growth factor in serum, a 5 ml of blood samples were collected aseptically at day 0 presurgery and 7.14.30,60 days post-surgery from jugular vein and prepared in an anticoagulant test tube by using 5ml sterile disposable plastic syringe. The blood left in room temperature for 30 minute to permits formation of clot then centrifuged at 3000 rpm for 10 minute to remove the clot.Then the serum transported into 0.5 Eppendorf tube and stored at -20 °C.The serum sample were analyzed by enzyme -linked immunosorbent assay technique (ELISA) with using of specific dog insulin like growth factor 1, (IGF1) ELISA Kit(Appendix 2) (mybiosource-USA) (Fig.15).

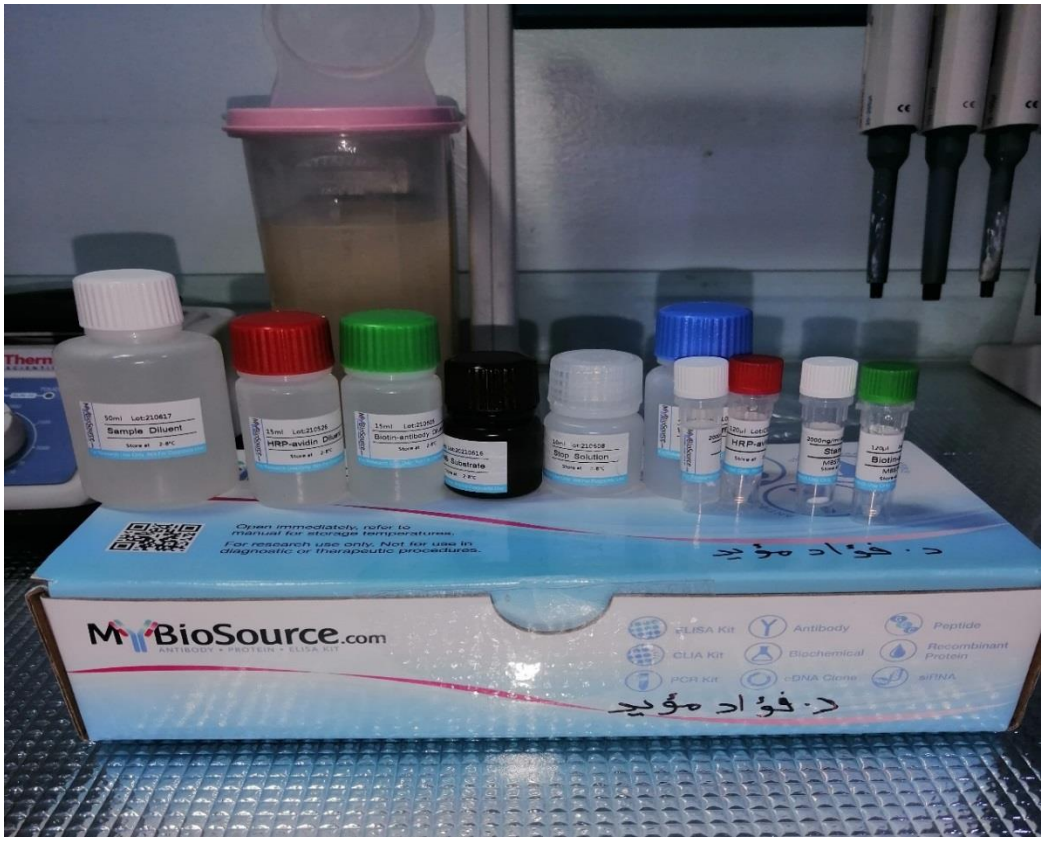


Figure 15: Photographic image shows insulin like growth factor kit(IGF-I).

3.6.6.Pathological examination

3.6.6.1.Macroscopical examination

The expermental animals were premedicated and anesthetised as same as the surgical technique for tibial bone grafting to observe the macroscopical findings in the site of bone grafting and to dissect the grafts from the surroundings tissue by electrical saw for collecting the histophalogical sample in which the site were prepared aseptically then a longitudinal skin incision was made over the operated bone defect at the proxomedial aspect of the right tibia to reach to deep crural fascia and muscles to expose the region of bone defect and inspection of abnormality changes and different tissue associated with healing process over bone defect at the end of 14, 30 and 60 days posoperatively. After

observation of the changes in field of operation specimens for histopathological examination were collected from the graft and edges of original bone. To get full data for the healthy and operated edges of tibia for both the bone graft and the abnormalities in the site of operation, another incision was made between the healthy and operated edges of bone defect then, the gap was filling with a suitable amount of bone cement and finally the skin and muscle were sutured with routine methods and give pencillin -streptomycin intramusculary injection for 5 days and follow up until return of the animal to normal healthy case (Fig.16).

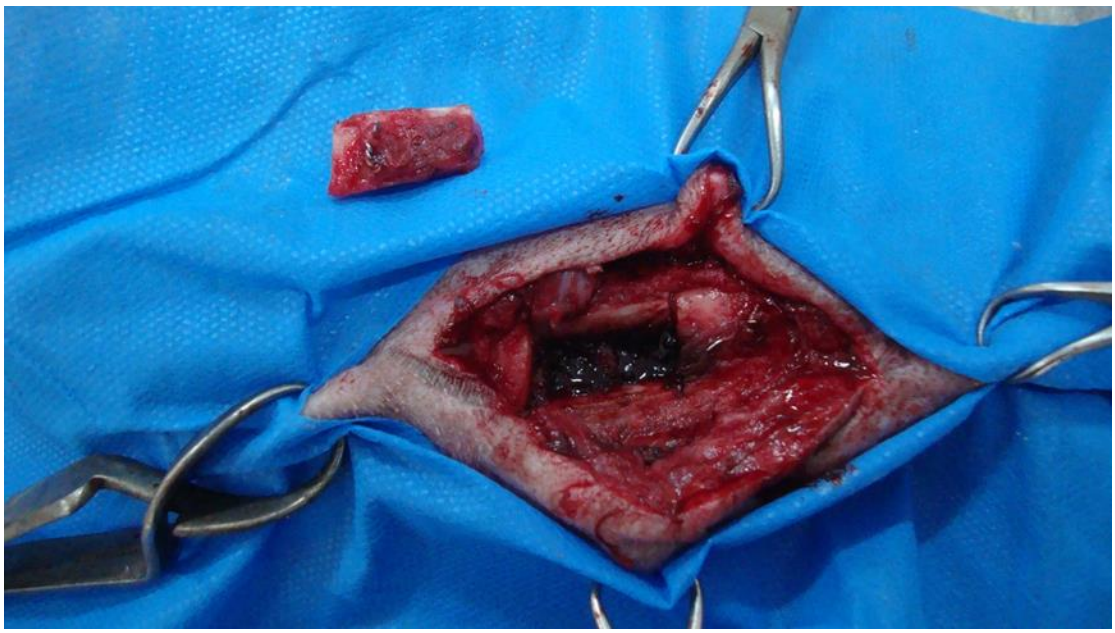


Figure 16:Macroscopical image showes excision of bone grafting biopsy for histophalogical examination.

3.6.6.2.Histopathological examination

To identify the most important histopathological changes including union or nonunion, rejection of xenograft with original bone and stages of graft healing in the operated bone defect, biopsies from 3 animals for each group were obtained at 14, 30 and 60 days post-surgery.These

samples then fixed in 10% neutral buffer formalin (NBF) for 3 days to complete the fixation process. Later decalcification was done using 10% formic acid solution for two weeks. After routine histological processing method , the samples sectioned into 5µm with a microtome (Leica SP 1600; Leica Microsystems, Germany), and finally stained with Hematoxylin and Eosin , Masson's trichrome and Alcian blue 2.5PH PAS stains(Suvarna *et al.*,2013) (Appendix 3,4,5) and view under light microscope (AX80T; Olympus, Tokyo, Japan) to evaluate the healing and new bone formation at the site of defect.The hisoplathological observations was analyzed using semi qualitative analysis according to (Lucacio *et al*.,2015) using the scores shown in (Table 2).All histological sections were photographed using microscope camera(Omax USB3,18MP,China).

3.7.Statistical analysis:

Two-way ANOVA (Analysis of Variation-ANOVA two way) for means and standard errors of the obtained data was used to detect the effect of different factors in study parameters.The analysis was done using IBM SPSS V25 program UK at $P \leq 0.05$(Handel,2013).

Table 2:Showes the histological scoring system (Lucaciu *et al*.,2015).

Histological scores	
1. Bone formation 0 – absent 1 – present at the periphery 2 – present centrally 3 – present centrally and at the periphery	**7. Mature bone** 0 – absent 1 – present at the periphery 2 – present centrally 3 – present at the periphery and centrally
2. Vascularization 0 – absent 1 – present at the surface of the graft 2 – present in the depth of the graft	**8. Bone trabeculae** 0 – absent 1 – present at the periphery 2 – present centrally 3 – present at the periphery and centrally
3. Osteoblasts 0 – absent 1 – present at the periphery 2 – present centrally 3 – present centrally and at the periphery	**9. Haversian canals** 0 – absent 1 – present at the periphery 2 – present centrally 3 – present at the periphery and centrally
4. Osteocytes 0 – absent 1 – present at the periphery 2 – present centrally 3 – present centrally and at the periphery	**10. Inflammation** 0 – absent 1 – mild 2 – moderate 3 - severe
5. Osteoclasts 0 – absent 1 – present at the periphery 2 – present centrally 3 – present centrally and at the periphery	**11. Granulation tissue** 0 – absent 1 – mild 2 – moderate 3 – intense
6. Immature bone 0 – present centrally 1 – present at the periphery 2 – absent	**12. Neo-formation of blood vessels** 0 – absent 1 – present at the periphery 2 – present centrally 3 – present at the periphery and centrally

Chapter Four

Results

4.1.Clinical evaluation

The outcome data indicated that the experimental animals were not exhibited any visible signs of complications like loss of appetite, seromas, severe hematoma. Besides no immune rejection were developed along the whole period of the study for all groups, as well as no wound dehiscence nor anesthetic death or even death or development of undesired habits were observed during the experimental though, there were signs of local temperature, swelling and pain that sustained for little days then diminished slowly.Whereas the skin wounds healed normally by the first intention within 10-15 days post-surgery in all animals.

4.1.1. Lameness

Lameness (inability to bear weight on the affected limb compared with that of the unaffected limb) was noticed in operative limbs within three hours following surgery for experimental animals and become more obvious in the next following 24 hours.The degree of lameness in all experimental animals at zero time revealed no significant differences among control and others groups at ($P \leq 0.05$) (Table 3). For all groups, the highest rate of lameness was noticed at day 7 post-surgery and the score was same for both control and xenograft groups (4.4 ± 0.24), (4.4 ± 0.24) respectively. Dogs could not bear their weight using their operative limb during that period; however that rate gradually started to decrease at day14 following operation. Besides the score was same for both control and xenograft (3.4 ± 0.24),(3.4 ± 0.24) respectively. Lateron, the rate of degree of lameness started to return to its normal value at 60 days post-surgery indicating normal weight bearing without lameness, as

well as, limb function and score were (0.0±0.0) respectively in control, xenograft and other two treatment groups.The average time for recovery of the normal ambulation of the treated groups was significantly (P≤ 0.05) shorter than that of the control group(Fig.17).

Table 3:Shows the mean values of lameness during the period of the study in all groups.

Groups / Day	Degree of lameness (Mean ±SE)			
	Control	Xenograft	HA	HAp
0	0.0±0.0	00.0±0.0	0.0±0.0	0.0±0.0
7	4.4^{Cb} ± 0.24	4.4^{Cb} ± 0.24	3.1^{Bb}± 0.26	2.3^{Ab} ± 0.2
14	3.4^{Ca} ± 0.24	3.4^{Ca} ± 0.24	2.1^{Ba} ± 0.26	1.3^{Aa} ± 0.2
30	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
60	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Within the same row of time (day), means with different small letters means significantly different.Within the same column of groups, means with different capital letters are significantly different at (P≤0.05).

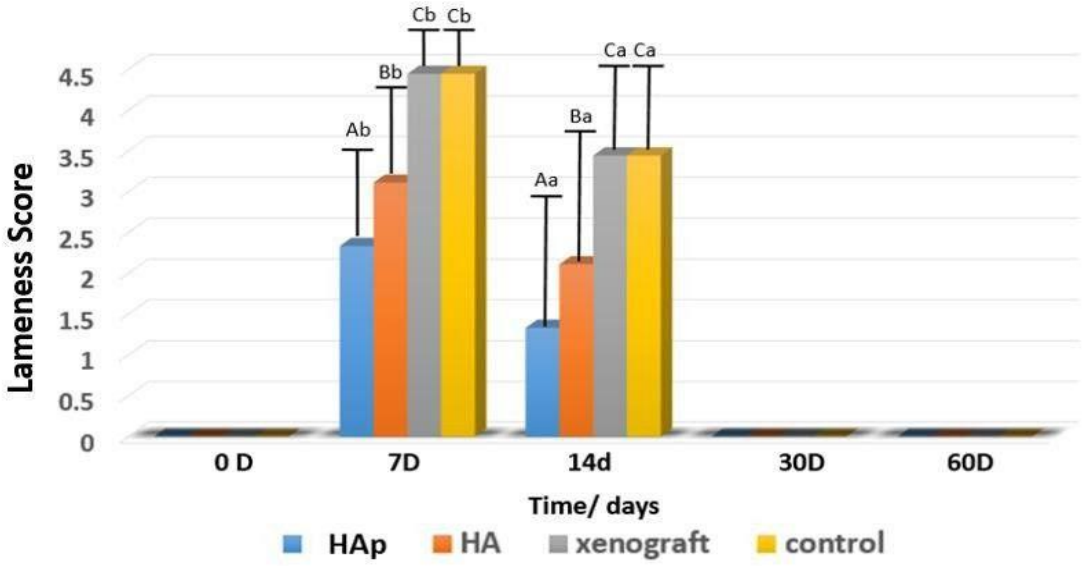


Figure 17: Graphic presention shows mean values of lameness during the period of the study in all groups.

4.2.Serum alkaline phosphatase

The results for the levels of serum alkaline phosphatase for all operative animals at zero time revealed significant differences (P≤0.05) among control and other groups.The level of serum alkaline phosphatase increased at 7 and 14 days post-surgery. In all groups the highest rates were at day 7 as (54.7 ± 3.8),(54.2± 1.86) IU/L, while those rates for day 14 were (43.8±2.5),(54.1±5.24) IU/L respectively for HA and HAp groups then these rates gradually decreased and returned near the normal values (36.3±3.9) ,(37.1±2.4) IU/L respectively at 60 days post-surgery (Table 4) and (Fig.18).

Table 4:Shows the mean values of serum alkaline phosphatase (IU/L) during the period of the study in all groups.

Groups / Days	Alkaline phosphates (Mean ±SE)			
	Control	Xenograft	HA	HAp
0	$34.5^{Ca} \pm 2.64$	$25^{Ab} \pm 2$	$29.8^{Ba} \pm 0.21$	$26.8^{Aa} \pm 1.43$
7	$37.2^{Bb} \pm 2.7$	$31.6^{Ac} \pm 3$	$54.7^{Cd} \pm 3.8$	$54.2^{Cc} \pm 1.86$
14	$42.3^{Bc} \pm 2.3$	$35.7^{Ad} \pm 2.1$	$43.8^{Bc} \pm 2.5$	$54.1^{Cc} \pm 5.24$
30	$34.1^{Ba} \pm 1.6$	$31.6^{Ac} \pm 2.25$	$31.6^{Aa} \pm 2.6$	$46.6^{Cb} \pm 2.2$
60	$34^{Ba} \pm 1.7$	$22^{Aa} \pm 5.7$	$36.3^{Cb} \pm 3.9$	$37.1^{Ca} \pm 2.4$

Within the same row of time (day), means with different small letters means significantly different. Within the same column of groups, means with different capital letters are significantly different at (P≤0.05) .

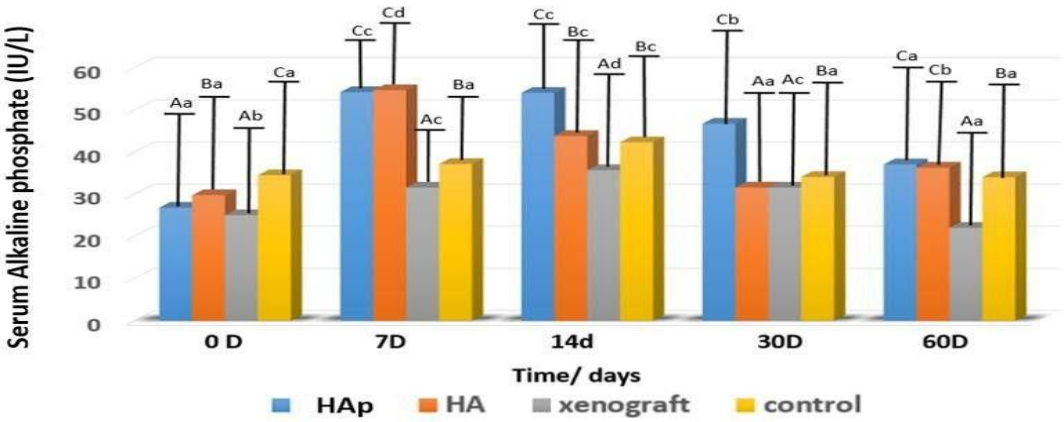


Figure 18:Graphic presention shows the mean values of serum alkaline phosphatase (IU/L) during the period of the study in all groups.

4.3.Insulin like growth factor

The data for serum insulin like growth factor levels within the control and treated groups at zero time exhibited significant differences among them (P≤0.05).On day 7 following surgery,the levels of (ILGF) increased and their highest rates in all groups were(0.29 ±0.01), (0.2±0.03),(0.25±0.01), (0.3±0.02) ng/ml ,respectively.Whereas at day 14these levels gradually started to decrease for all groups and become (0.12±0.03),(0.08±0.03), (0.17±0.02), (0.2±0.02) ng/ml, respectively. Later, these rates continued in down especially in HA after 60 days post-surgery and were (0.06±0.06), (0.03±0.03), (0.0±0.0), (05±0.03) ng/ml, respectively in all groups(Table 5) and (Fig.19).

Table 5 :Shows the mean values of insulin like growth factor(ng/ml) during the period of the study in all groups.

Groups / Days	Insulin like growth factor (Mean ±SE)			
	Control	Xenograft	HA	HAp
0	0.23^{Bc} ± 0.03	0.12^{Ac} ± 0.05	0.23^{Cc} ±0.03	0.3^{Dd} ± 0.1
7	0.29^{Bd} ± 0.01	0.2^{Ad} ± 0.03	0.25^{Cc} ± 0.01	0.3^{Dc} ± 0.02
14	0.12^{Bb} ± 0.03	0.08^{Ab} ± 0.03	0.17^{Db} ± 0.02	0.2^{Cb} ± 0.02
30	0.12^{Db} ± 0.03	0.06^{Cb} ± 0.04	0.06^{Aa} ± 0.03	0.04^{Ba} ± 0.03
60	0.06^{Aa} ± 0.06	0.03^{Aa} ± 0.03	0.0^{Cc} ±0.0	0.05^{Ba} ± 0.03

Within the same row of time (day), means with different small letters means significantly different. Within the same column of groups , means with different capital letters are significantly different at (P≤0.05) .

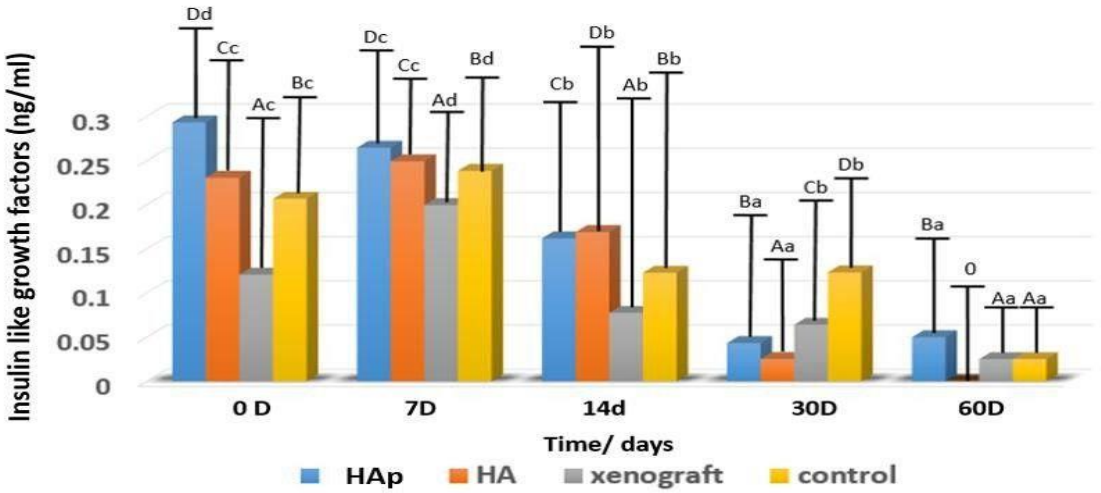


Figure 19: Graphic presention shows the mean values of insulin like growth factor (ng/ml) during the period of the study in all groups.

4.4.Radiological examination

4.4.1.At day fourteen post surgery

The radiographic mediolateral and anterioposterial views on day 14 following repairing the tibial bone defect (with harvested segment and bone xeno implant along with using cerclage wire for both control and xenograft groups) showed the presence of lucent line around the fixated bone implants with swelling of soft tissue surrounding the site,Though no positive periosteal response nor new bone formation were noticed there (Fig. 20, A, B, C, D).

In both 1st (HA) and 2nd (hydroxyapatite Nano gel) groups post repairing an experimental tibial bone defect with bone xeno implants revealed the existence of a lucent line surrounding the fixated bone implant accompanying with swelling of mild soft tissue surrounding the site, besides a relative cortical thickening denoting initial periosteal response but no new bone formation (Fig. 21, A, B, C, D).

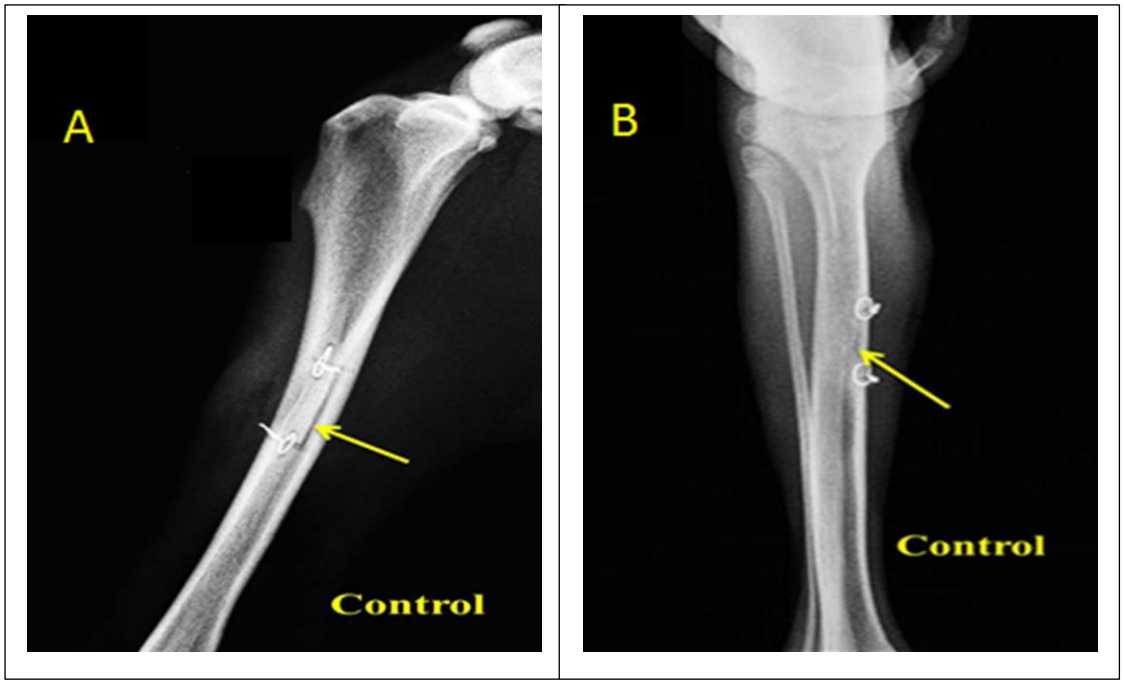


Figure 20:Radiological image in mediolateral A and anterioposterial B view at 14 days post-surgery in the control group shows lucent line and no definite periosteal reaction nor new bone formation (yellow arrows).

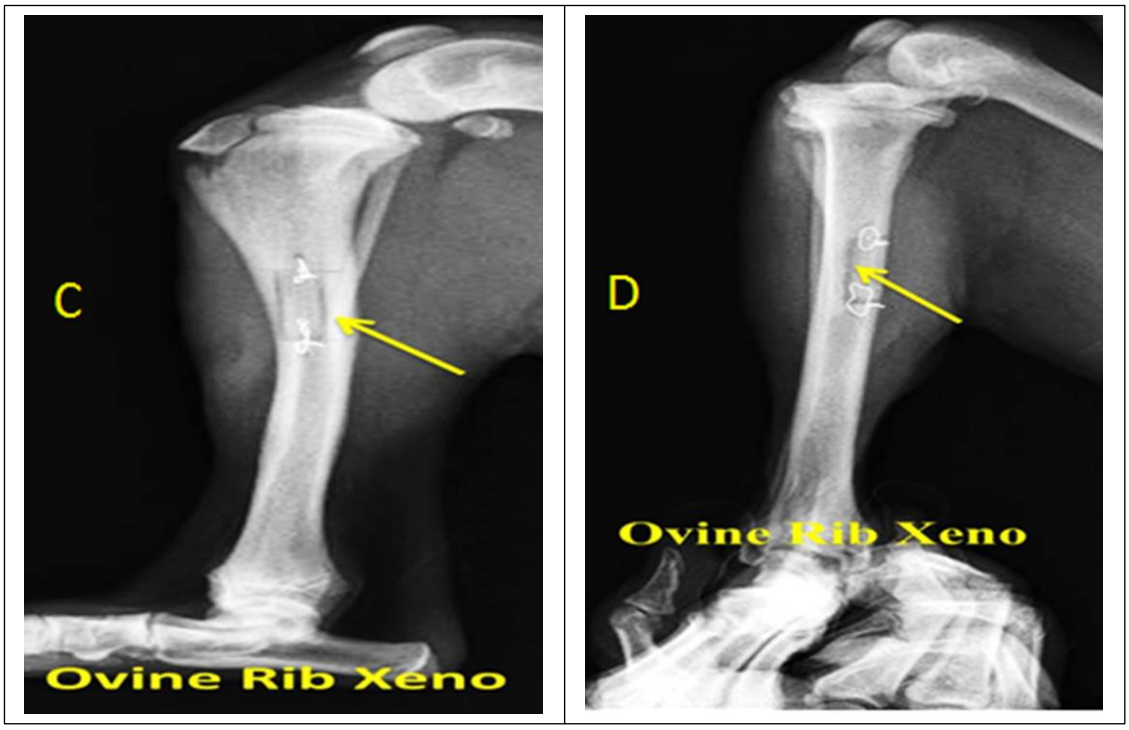


Figure 20: Radiological image in mediolateral C and anterioposterial D view at 14 days post-surgery in the xenograft group shows lucent line and no definite periosteal reaction nor new bone formation (yellow arrows).

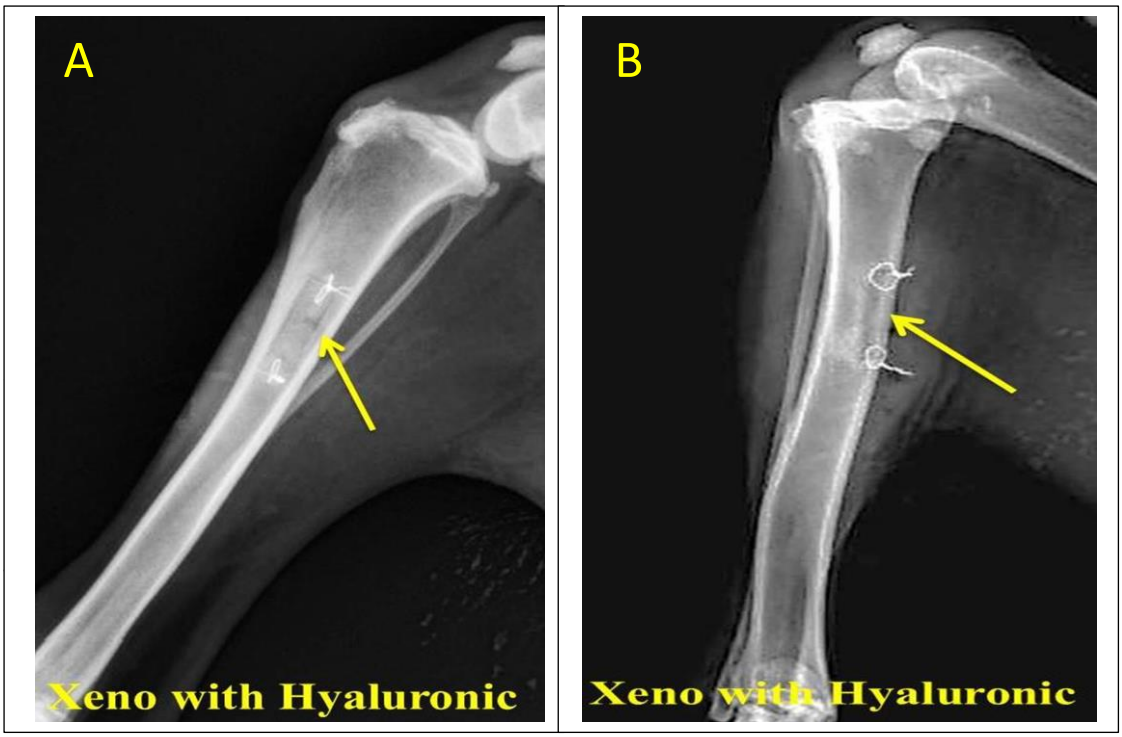


Figure21: Radiological image in mediolateral A and anterioposterial B view at 14 days post operation in hyaluronic acid group shows lucent line with relative cortical thickening denoting early periosteal reaction but no new bone formation (yellow arrows) .

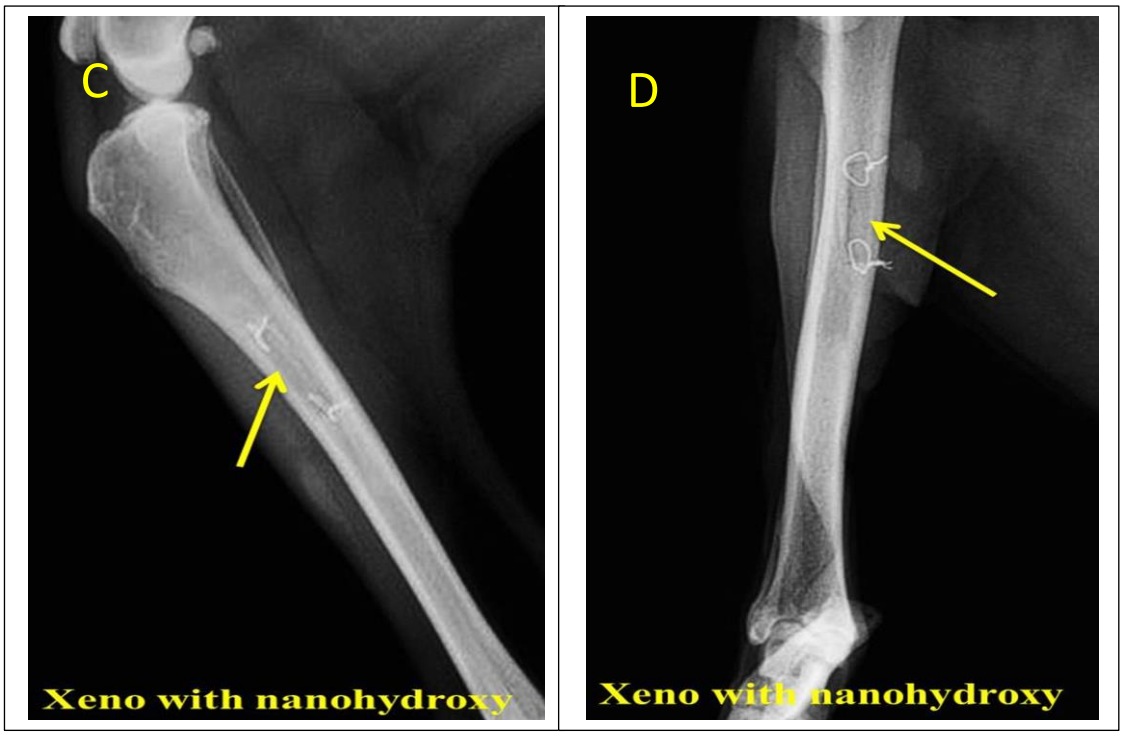


Figure21: Radiological image in mediolateral C and anterioposterial D view at 14 days post surgery in hydroxyapatite Nano gel group shows lucent line with relative cortical thickening denoting initial periosteal reaction but no new bone formation (yellow arrows).

4.4.2.At day thirty post surgery

The radiographic mediolateral and anterioposterial views on day 30 following repairing the tibial bone defect (with harvested segment and bone xeno implant along with using cerclage wire for both control and xenograft groups) showed the presence of minimal cortical irregularity and thickening with partial incomplete loss of the lucent line defect that surrounds the bone graft implants that become interrupted and hazy denoting periosteal response with initial bone formation and ongoing process of bone healing (Fig. 22, A, B, C, D).

For the 1st treatment group (HA), there was cortical irregularity and thickening with nearly complete loss of the lucent line defect that surrounds the bone graft that becomes hazy meaning near periosteal response with early incomplete new bone formation and ongoing healing process (Fig. 23, A, B).

In the 2nd treatment group (hydroxyapatite Nano gel) there was minimal cortical thickening with nearly complete loss of the lucent line defect that surrounds the bone graft that become very thin and hazy denoting near complete new bone formation and very good ongoing healing process (Fig. 23, C, D).

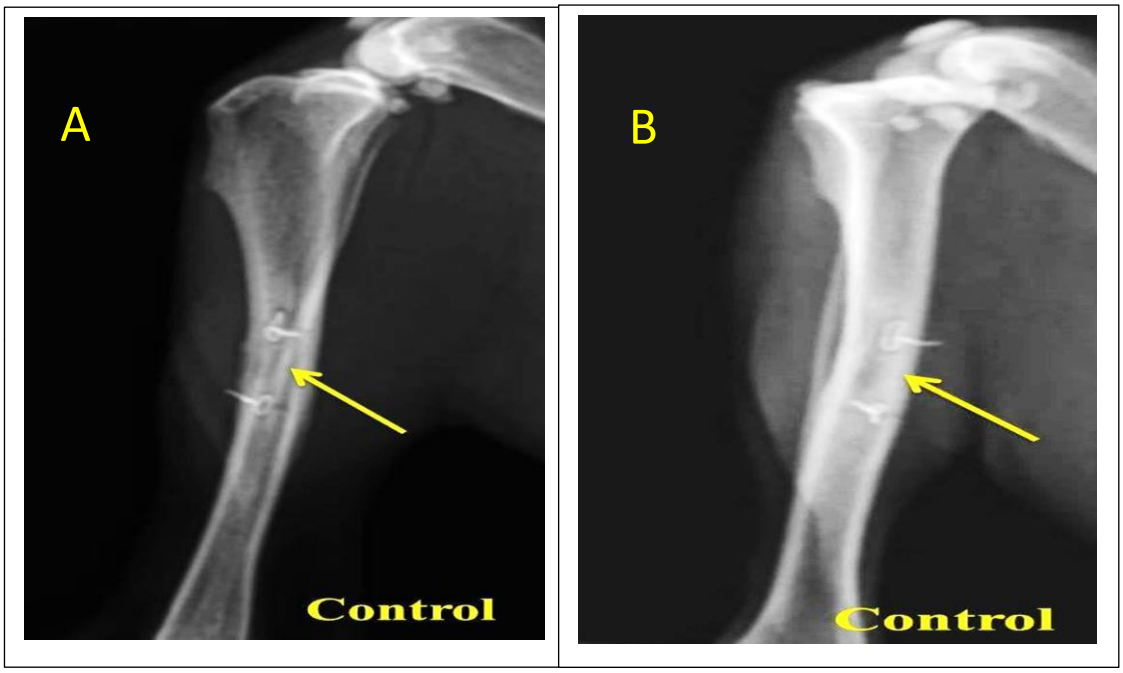


Figure:22 Radiological image in mediolateral A and anterioposterial B view at 30 days post-surgery in control group shows incomplete loss of the lucent line which become interrupted and hazy denoting periosteal reaction with early new bone formation (yellow arrow).

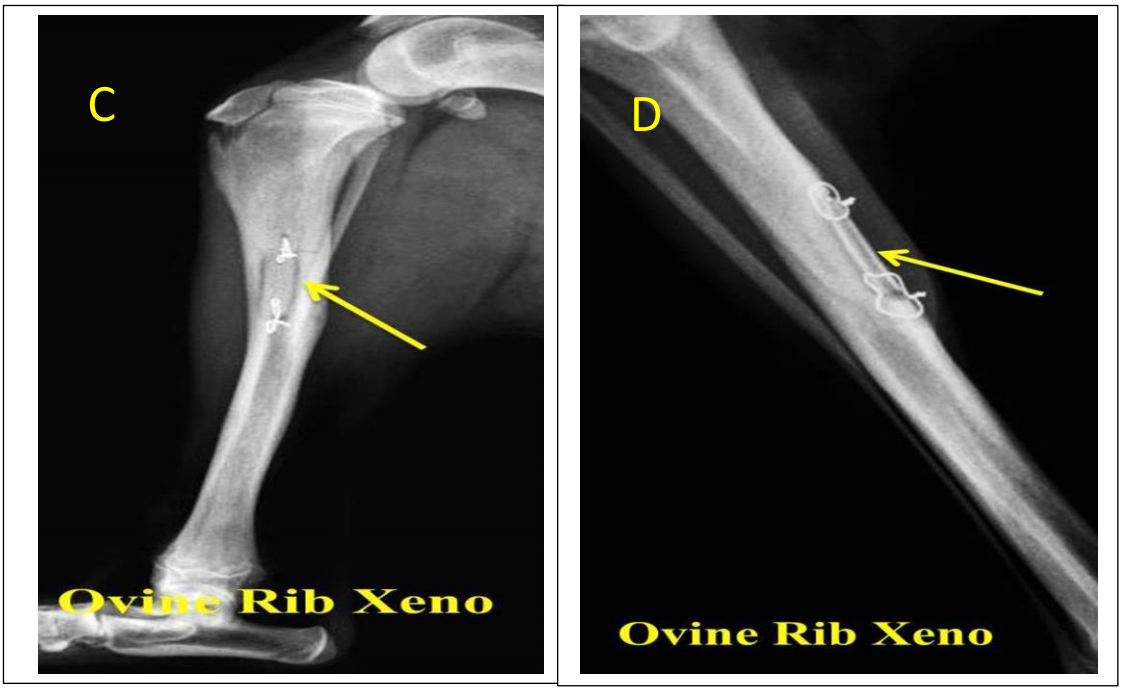


Figure22:Radiological image in mediolateral C and anterioposterial D at day 30 post-surgery in the xenograft group shows incomplete loss of the lucent line which becomes interrupted and hazy denoting periosteal reaction with early new bone formation (yellow arrows).

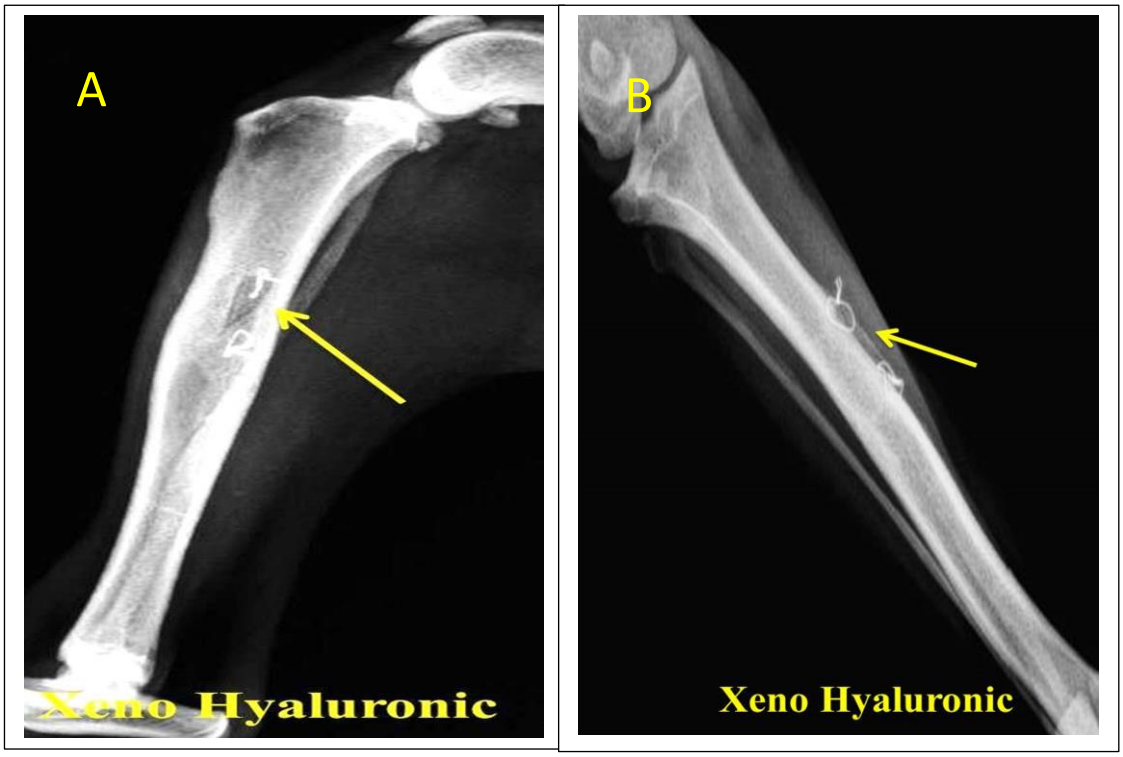


Figure: 23 Radiological image in mediolateral A and anterioposterial B view at 30 days post-surgery in hyaluronic acid group group shows complete loss of the lucent line defect that surrounds the bone graft that become hazy meaning near periosteal response with early incomplete new bone formation (yellow arrow).

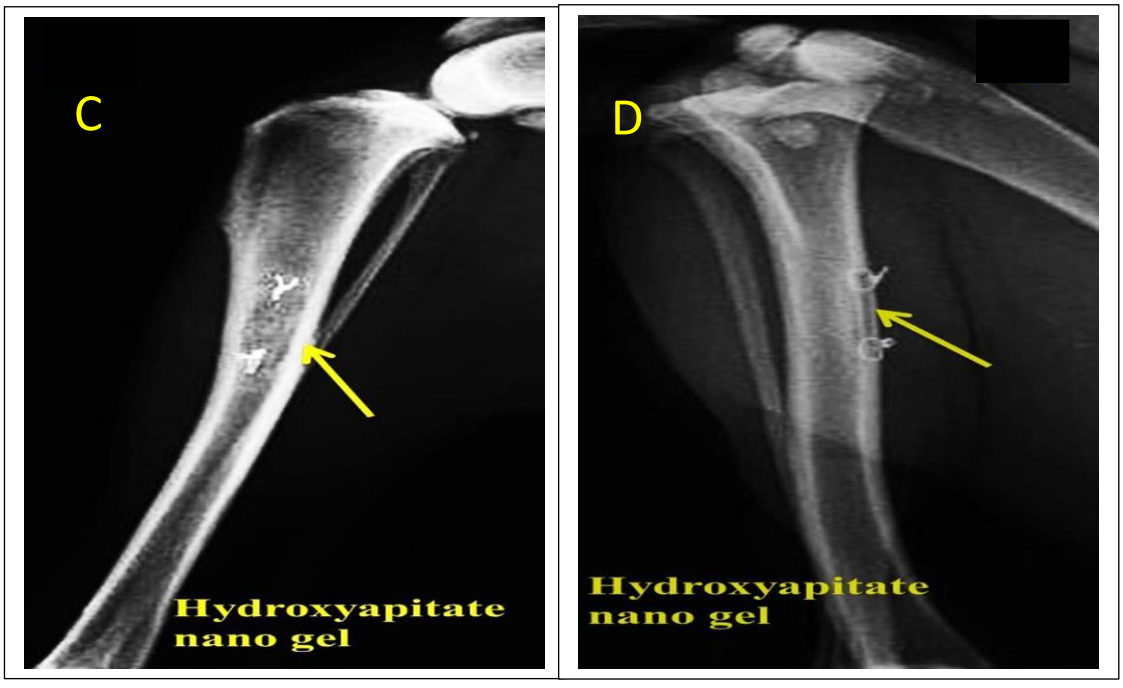


Figure23: Radiological image in mediolateral C and anterioposterial D at day 30 post surgery in hydroxyapatite Nano gel group shows complete loss of the lucent line defect which becomes very thin and hazy denoting near complete new bone formation (yellow arrows).

4.4.3.At day sixty post surgery

The radiographic mediolateral and anterioposterial views on day 60 following repairing the tibial bone defect in the control group revealed the existence of faint lucent line around the fixated bone implants with minimal cortical thickening and irregularities meaning late chronic periosteal response and new bone formation and nearly complete healing with the surrounding the area (Fig. 24, A, B).

While in (xenograft only) group, that X-Ray indicated the presence of complete healing of the fixated bone implants with the surrounding site though, certain cortical irregularities that representing chronic periosteal response and new bone formation were observed (Fig. 24, C, D).

Within the 1st treatment group (HA), there was nearly a complete healing of the fixated bone implants with the surrounding area, in spite of the presence of a hazy lucent line defect and certain cortical irregularities that meaning chronic periosteal response and nearly new bone formation (Fig. 25, A, B).

Even through, In the 2nd treatment group (hydroxyapatite Nano gel) there was progress of the healing for the fixated bone segment with the surrounding area as crossing healing, nevertheless, a hazy lucent line defect with minimal cortical irregularities that meaning chronic periosteal response and maturating new bone formation were noticed (Fig.25, C, D).

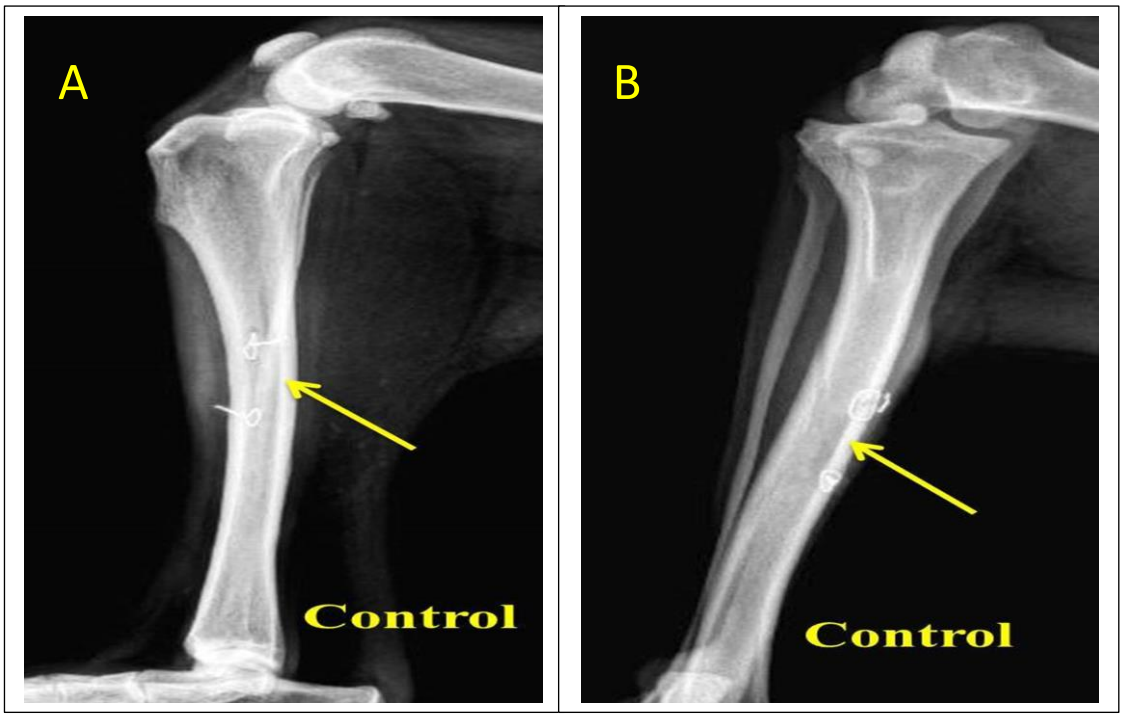


Figure24: Radiological image in mediolateral A and anterioposterial B at 60 days post-surgery in the control group shows faint lucent line with minimal cortical thickening and irregularities meaning late chronic periosteal response and new bone formation (yellow arrows).

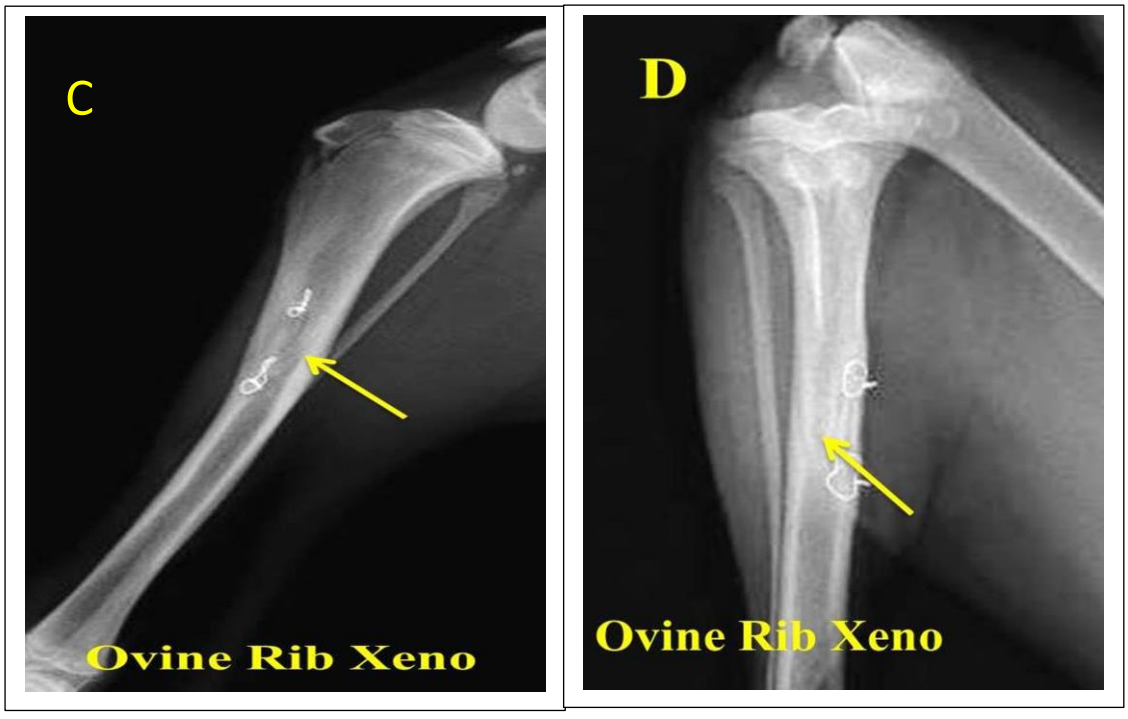


Figure 24:Radiological image in mediolateral C and anterioposterial D at 60 days post-surgery in the xenograft group shows the presence of complete healing of the fixated bone implants with the surrounding site, nevertheless, some cortical irregularities that meaning chronic periosteal response and new bone formation were observed.

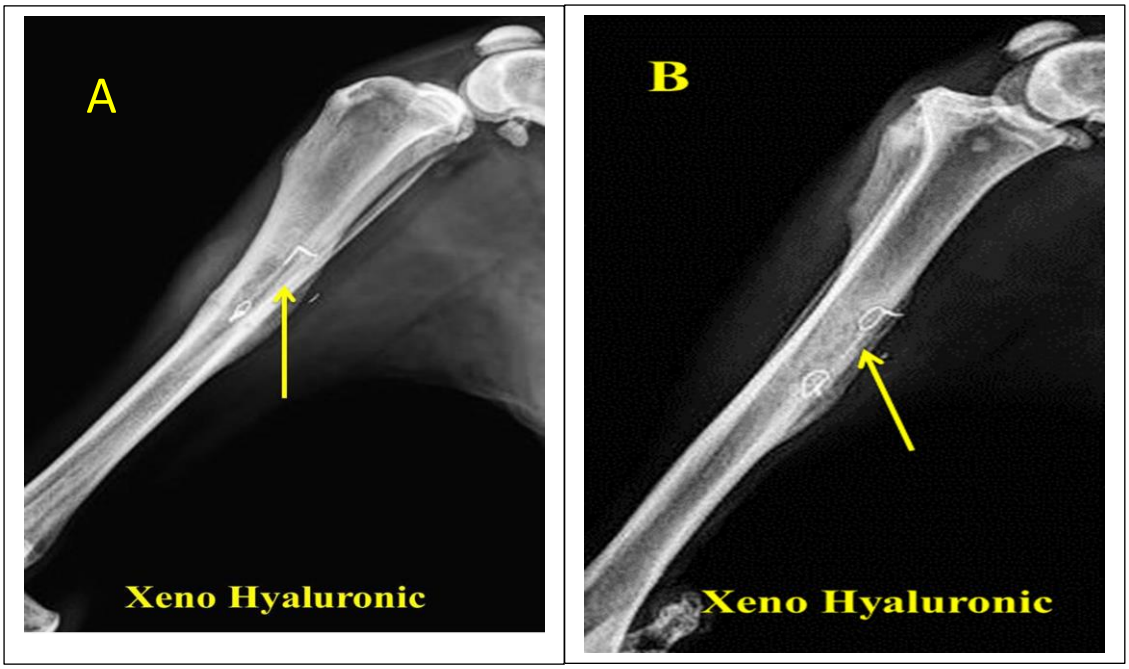


Figure25: Radiological image in mediolateral A and anterioposterial B at 60 days post surgery in hyaluronic acid group shows hazy lucent line defect and certain cortical irregularities meaning chronic periosteal reaction and near complete new bone formation (yellow arrows).

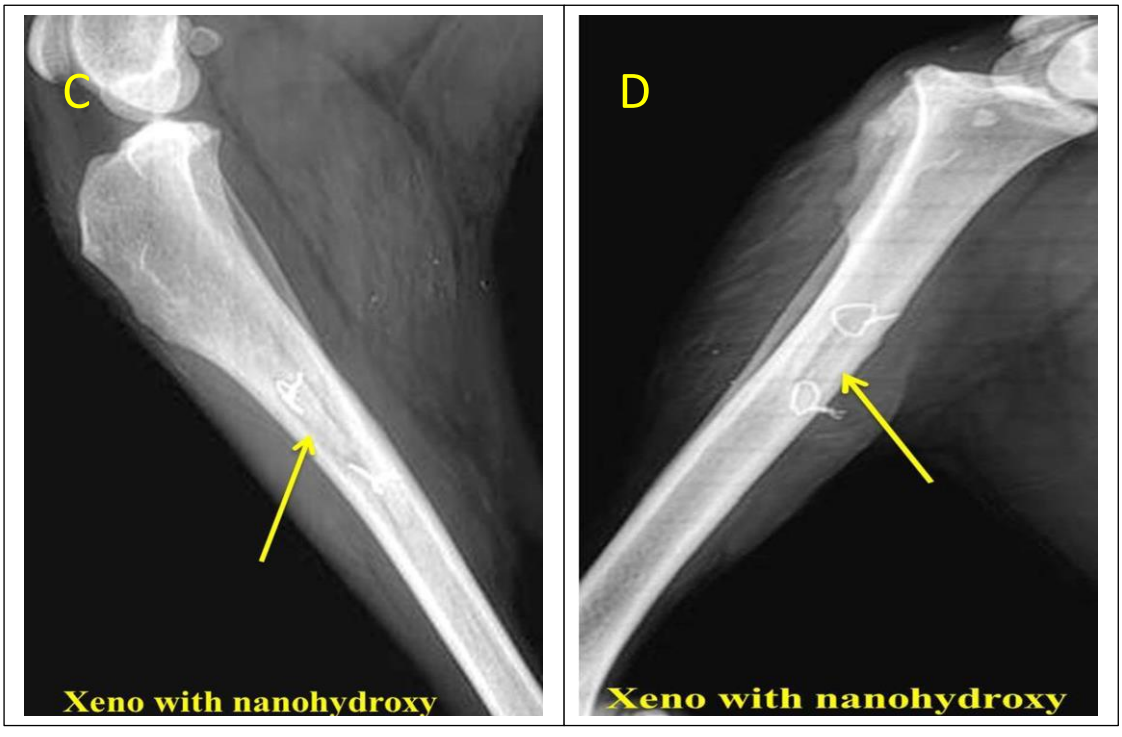


Figure25:Radiological image in mediolateral C and anterioposterial D at 60 days post surgery in hydroxyapatite Nano gel group shows with hazy lucent line defect with minimal cortical irregularities meaning chronic periosteal reaction and maturating new bone formation (yellow arrows) .

4.5.Macroscopic evaluation

The general situation of all animals was good and showed no infection post-surgery and there was a visible bony connection with host in both hyaluronic acid and hydroxyapatite Nano gel group.

Generally, the macroscopical examination for the site of bone grafting revealed no wound dehiscence, nor adverse tissue reaction or signs of inflammation, in addition to the presence of a stable fixation of the xenobone graft in all animals without signs of bone graft rejection around the implants. Results showed that the tibial bone defects in all groups healed well as follows:

4.5.1.At day fourteen post- surgery

The gross examination for the site of the operation within the control group exhibited the partial healing with the signs soft tissue inflammatory reaction around the site of operation (edema and congestion) (Fig. 26).

While in the xenograft group, the gross examination exposed that the borders of all tibial bone defects were enclosed with a dense layer of fibrous connective tissue (Fig.27).

In hyaluronic acid treated group, there was an enhancement in the healing process of the bone tissue defect at day 14 post-surgery through the presence of a mature fibrous connective tissue that filling on the borders and middle part of the bone tissue defects(Fig.28).

The hydroxyapatite Nano gel treated group showed partial healing of the defective bone (Fig.29).

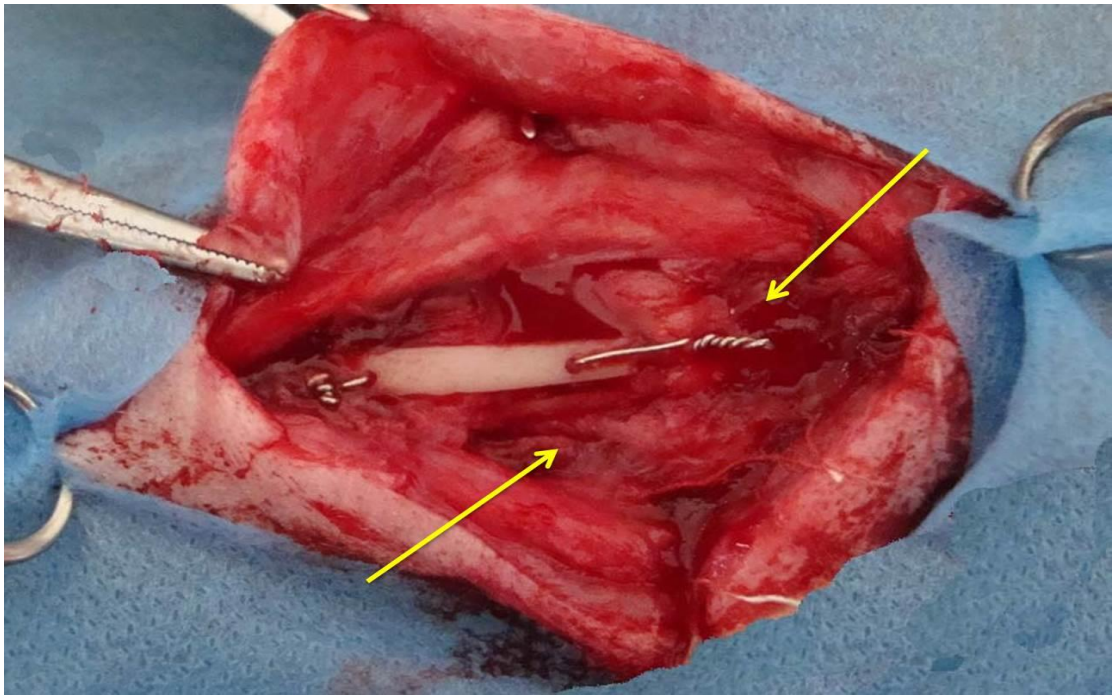


Figure26:Macroscopical image of the control group at 14 days post-surgery shows partial healing with signs of the inflammatory reaction of the soft tissue around the site of operation(yellow arrows).

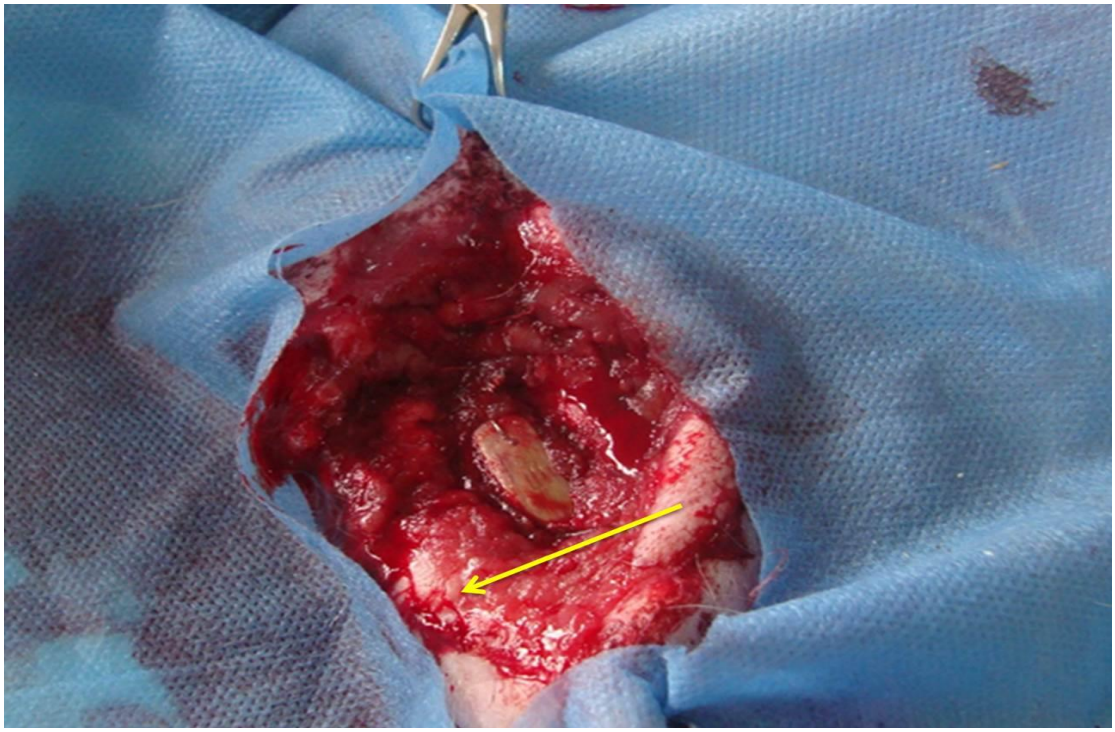


Figure 27:Macroscopical image of the xenograft group at 14 days post-surgery shows that the edges of all tibial defect bone were enclosed with a dense layer of fibrous connective tissue(yellow arrow).

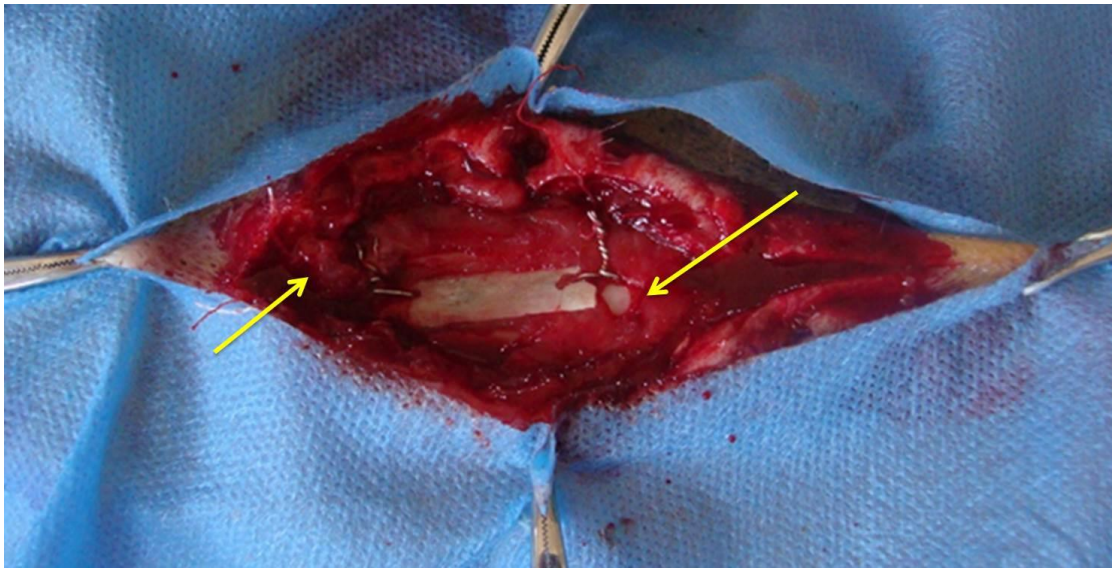


Figure 28:Macroscopical image of the treated group with hyaluronic acid at 14 days post-surgery shows mature fibrous connective tissue filling on the bordes and middle part of the bone tissue defects(yellow arrows).

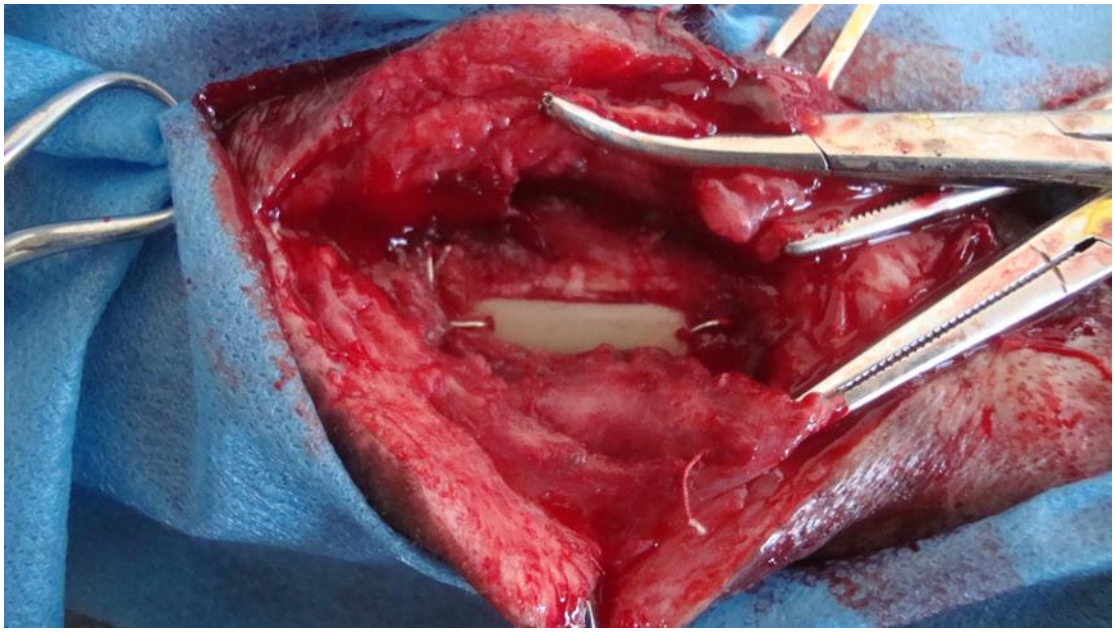


Figure29:Macroscopical image of the treated group with hydroxyapatite Nano gel at 14 days post-surgery shows partial healing of the defective bone.

4.5.2.At day thirty post-surgery

In the control group the visual investigation exhibited complete healing without inflammatory reaction (Fig.30).

Whereas, the xenograft group revealed that the borders of all tibial bone defects were enclosed with a dense layer of fibrous connective tissue (Fig.31).

On the other hand, the bone defects of the hyaluronic acid group were filled with new bone formation (Fig. 32).

However, within the hydroxyapatite Nano gel treated group, there was complete healing of the tibial defect edges (Fig.33).

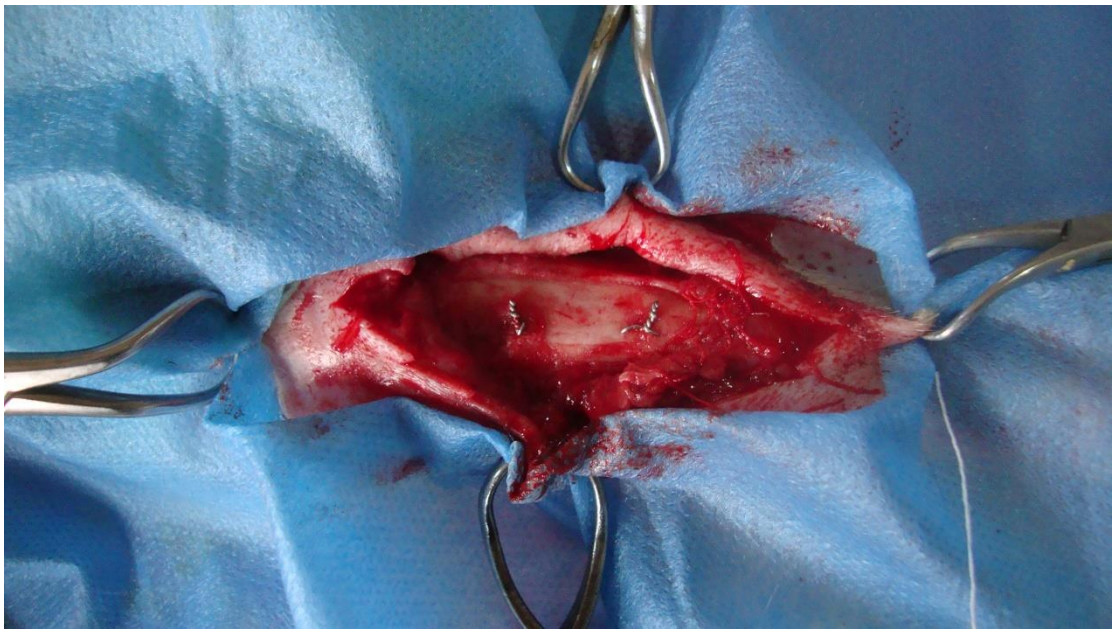


Figure30:Macroscopical image of the control group at 30 days post-surgery shows complete healing without any inflammatory reaction at the site of grafting.

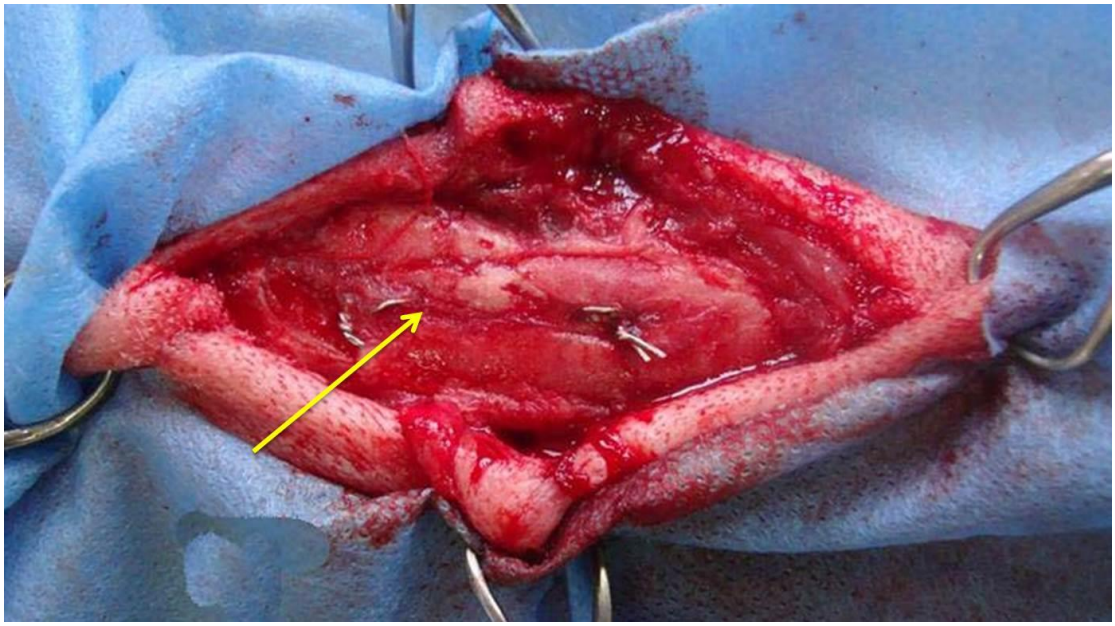


Figure 31:Macroscopical image of the xenograft group at 30 days post-surgery shows that the borders of all tibial defect bone were enclosed with a dense layer of fibrous connective tissue (yellow arrow).

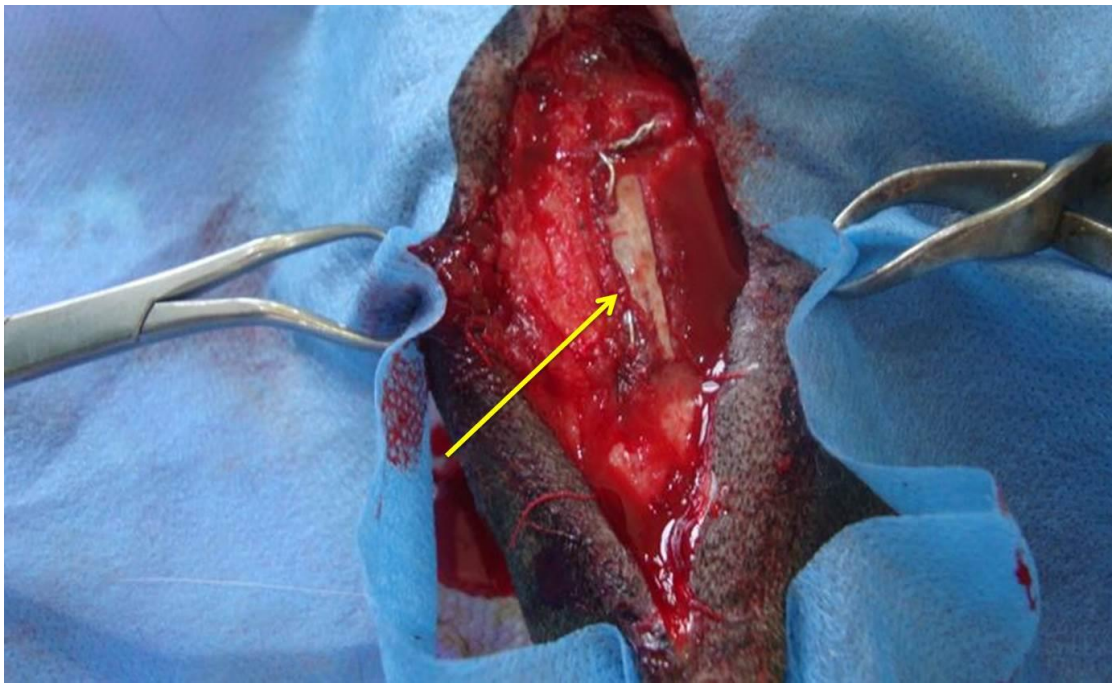


Figure 32:Macroscopical image of the treated group with hyaluronic acid at 30 days post-surgery shows the bone defect was filled with formation of new bone (yellow arrow).

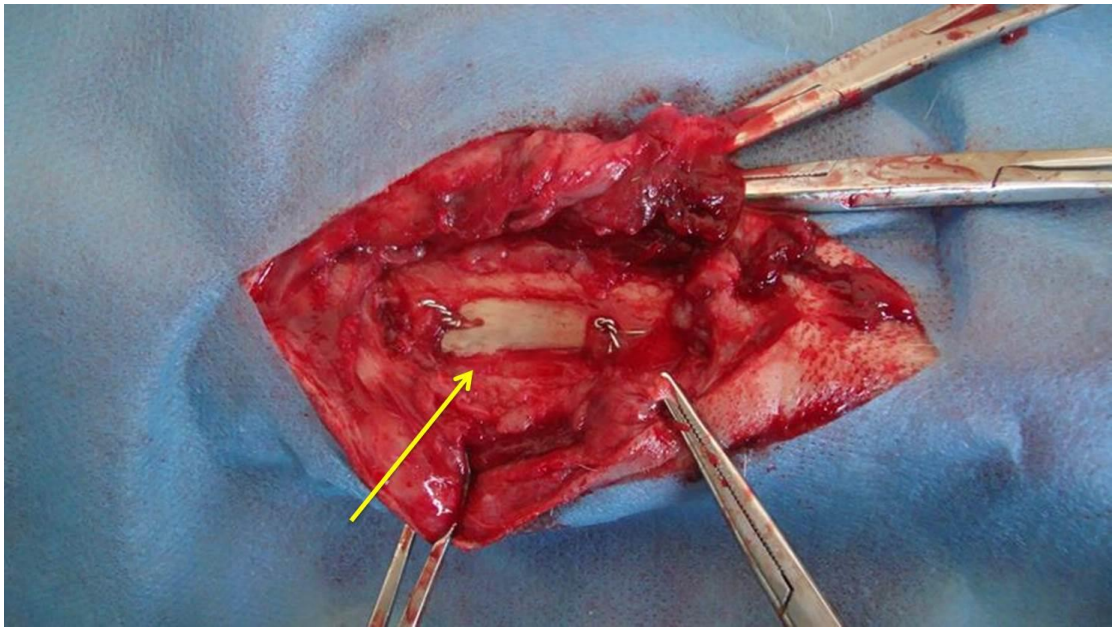


Figure 33:Macroscopical image of the treated group with hydroxyapatite Nano gel at 30 days post-surgery shows there was complete healing of the defect edges(yellow arrow).

4.5.3.At day sixty post-surgery

In the control group, the visual investigation revealed that the bone was having a healthy appearance without any inflammatory signs (Fig.34).

While for xenograft group, the macroscopical finding for the borders of the tibial defects exposed the presence of bone creation within the middle part of the defect (Fig.35).

In a hyaluronic acid group, there was an increase in the bone filling at both the border and middle part of the defect and formation of bone bridges was also found (Fig.36).

On the other hand, within the hydroxyapatite Nano gel there was complete bone healing that filling the bone defect and healthy bone appearance with no inflammatory reaction (Fig.37).

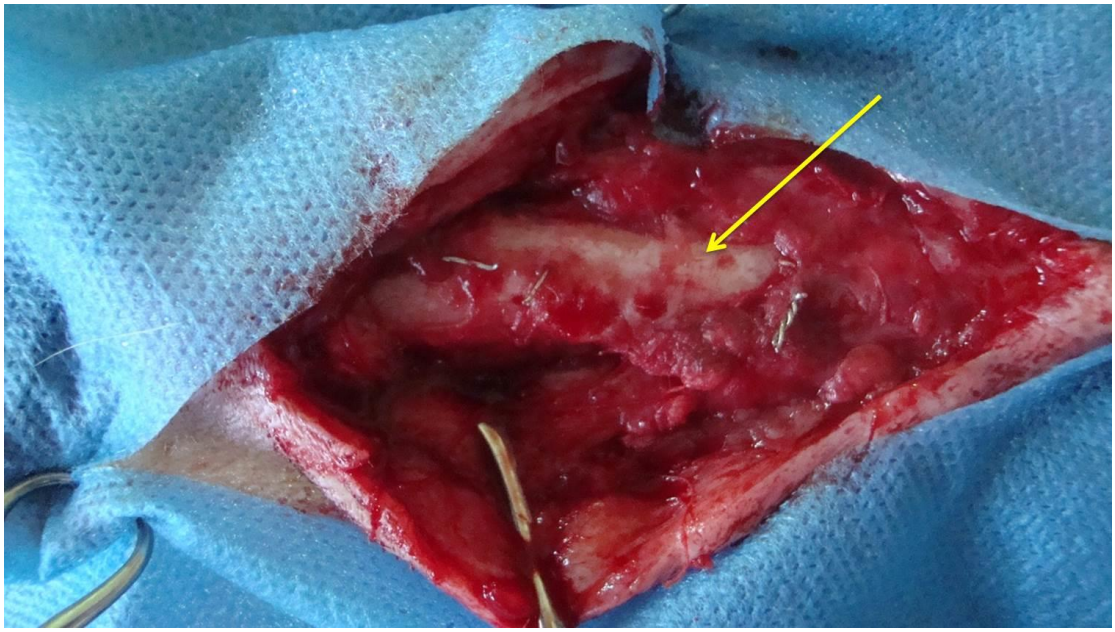


Figure 34:Macroscopical image of the control group at 60 days post-surgery shows the bone with healthy appearance without any inflammatory signs (yellow arrow).

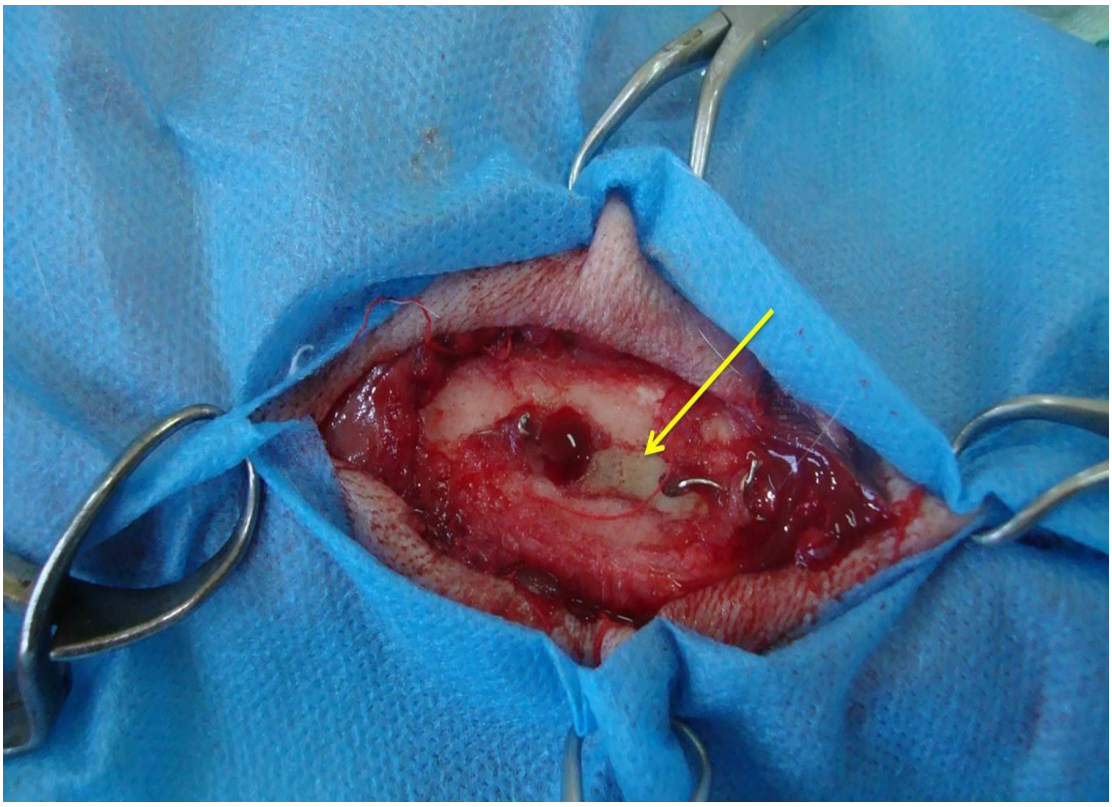


Figure 35:Macroscopical image of the xenograft group at 60 days post-surgery shows bone creation on the borders and the middle part of the defect(yellow arrow).

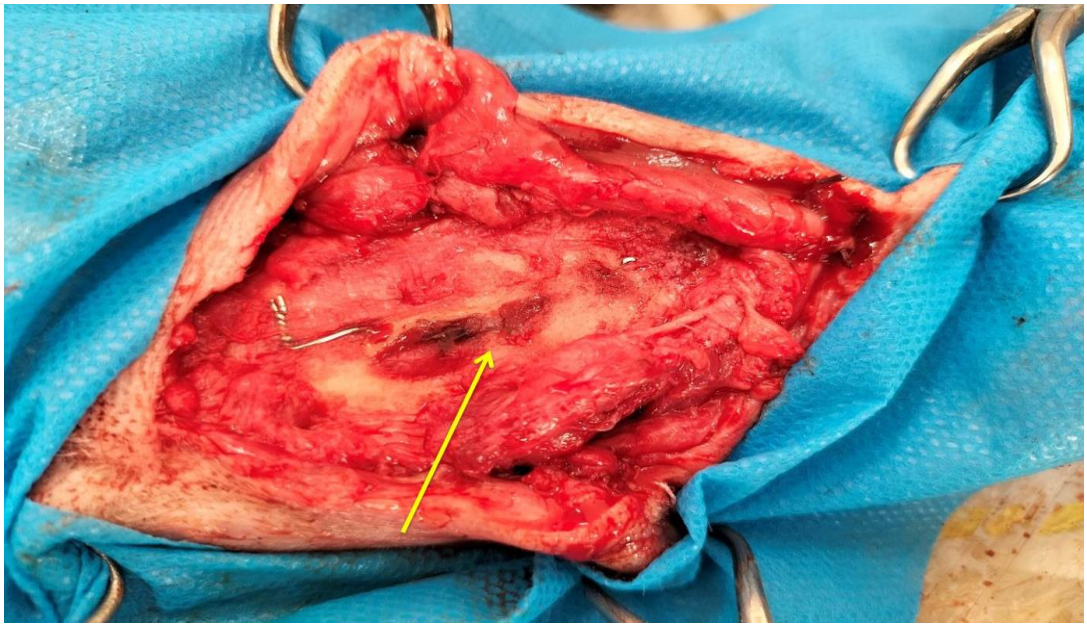


Figure 36: Macroscopical image of the treated group with hyaluronic acid shows increase in the bone filler at the border and central of the bone with formation of bone bridges (yellow arrow defect).

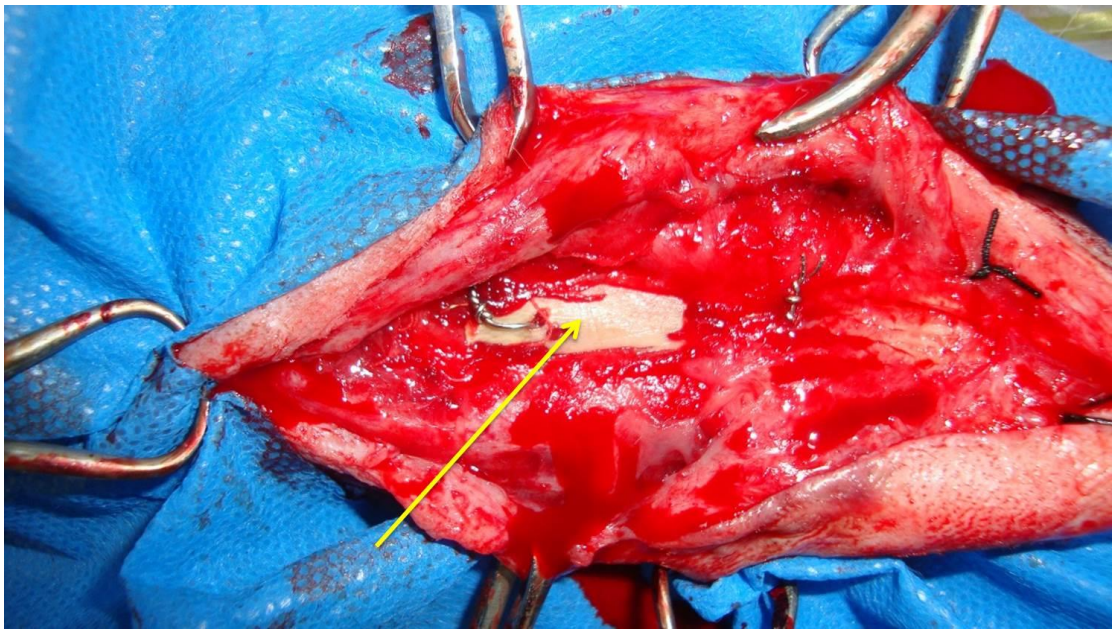


Figure 37:Macroscopical image of the treated group with hydroxyapitate Nano gel group shows complete bone healing and the bone appeared healthy with no inflammatory reaction(yellow arrow).

4.6.Histopathological findings

4.6.1.H&E stain

4.6.1.1.At day fourteen post -surgery

The histological examination for the site of bone defects within the control group indicted that these defects were filled with fibrous connective tissue with new bone lamellae formation in some places i.e. minimal bone growth(Fig.38).

Similarly, within the xenograft there was granulation tissue (fibroblast, newly blood vessels and collagen fibers) with inflammatory response but without bone formation (Fig.39)

Within the hyaluronic acid group, the histological examination of H&E stained sections at the site of defect showed inflammatory cells infiltration (mononuclear cells and giant cells) and congestion of blood vessels with extra vascular RBCs with numerous woven bone trabeculae formation lined by numerous numbers of organized osteoblasts (Fig.40).

While the bone healing was much better and more enhanced with hydroxyapatite Nano gel group compared to other groups.This was obvious clearly via the histopathological examination which showed that the bone defects were filled with newly formed bone lamellae of variable thickness, density and quantity, besides the presence of trabecular bone lamellae, bone marrow and osteoblasts without ant inflammatory reaction (Fig. 41).

The results of histopathological scoring at fourteen days post-surgery in all groups according to the (Table 6).

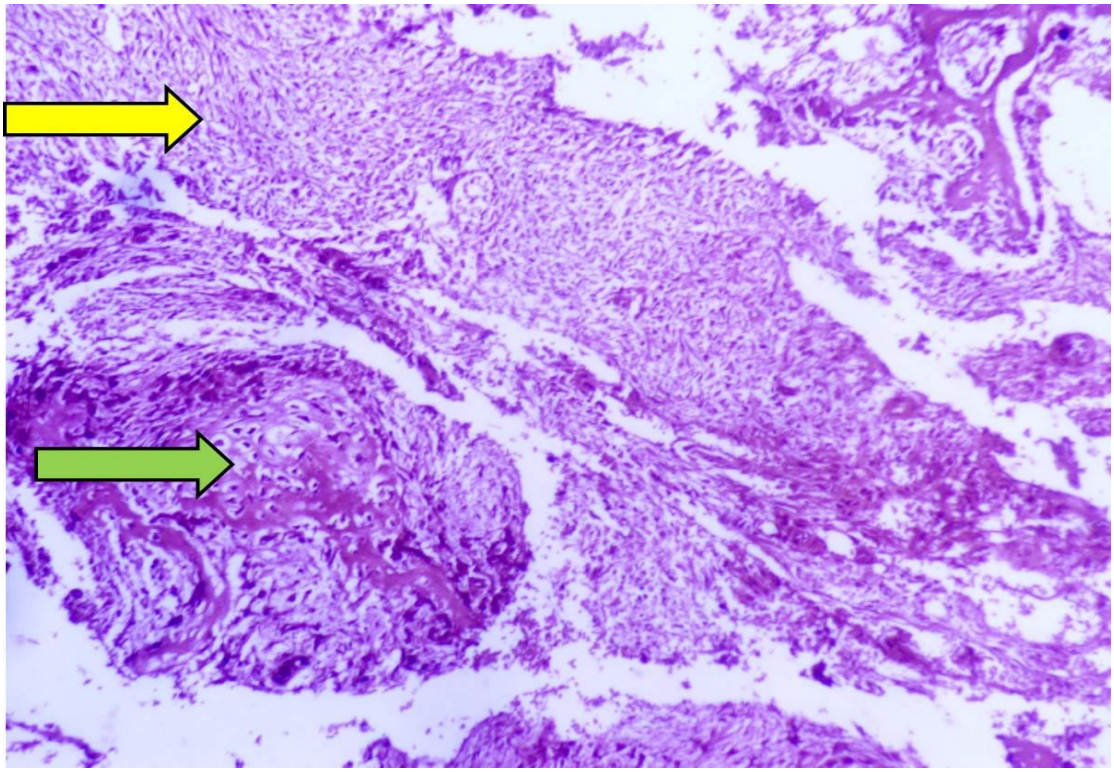


Figure 38:Micrograph of bone defect at 14th post-surgery days in control group shows the new bone formed lamellae(green arrow) with in the fibrous connective tissue(yellow arrow) H&E100X.

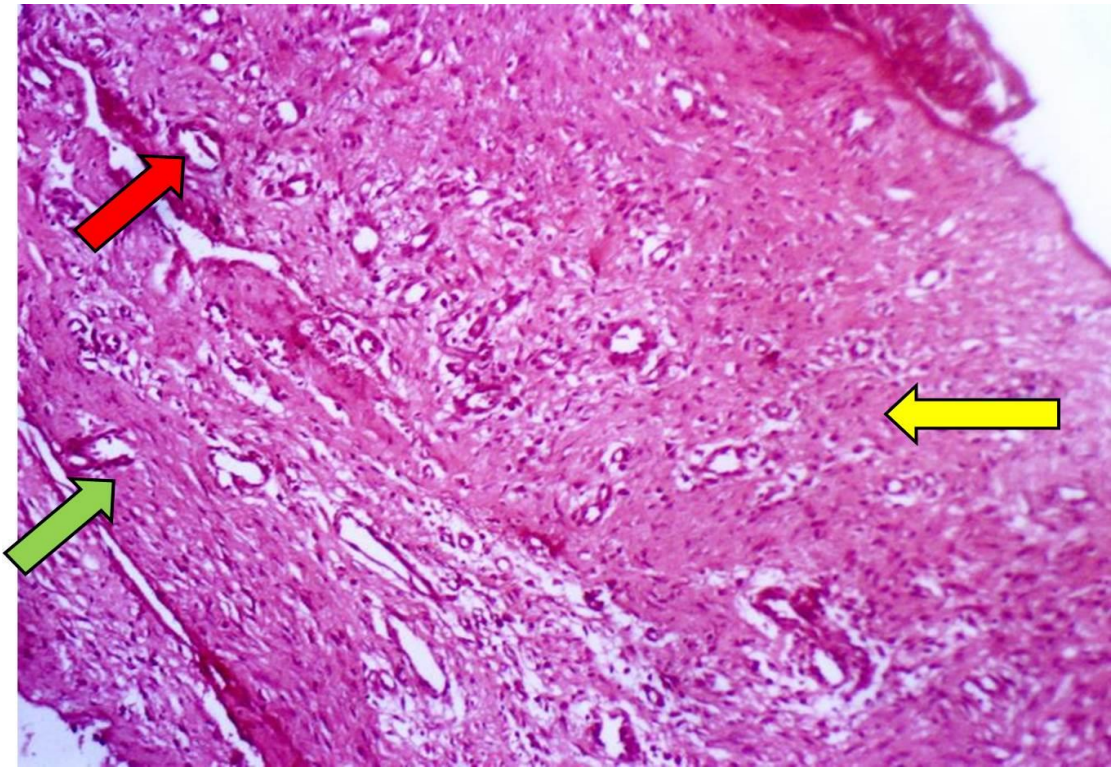


Figure 39:Micrograph of bone defect at 14th postoperative days in the xenograft group shows the presence of granulation tissue(green arrow) with new blood vessels(red arrow), tissue fibroblast and collagen fiber(yellow arrow) H&E40X.

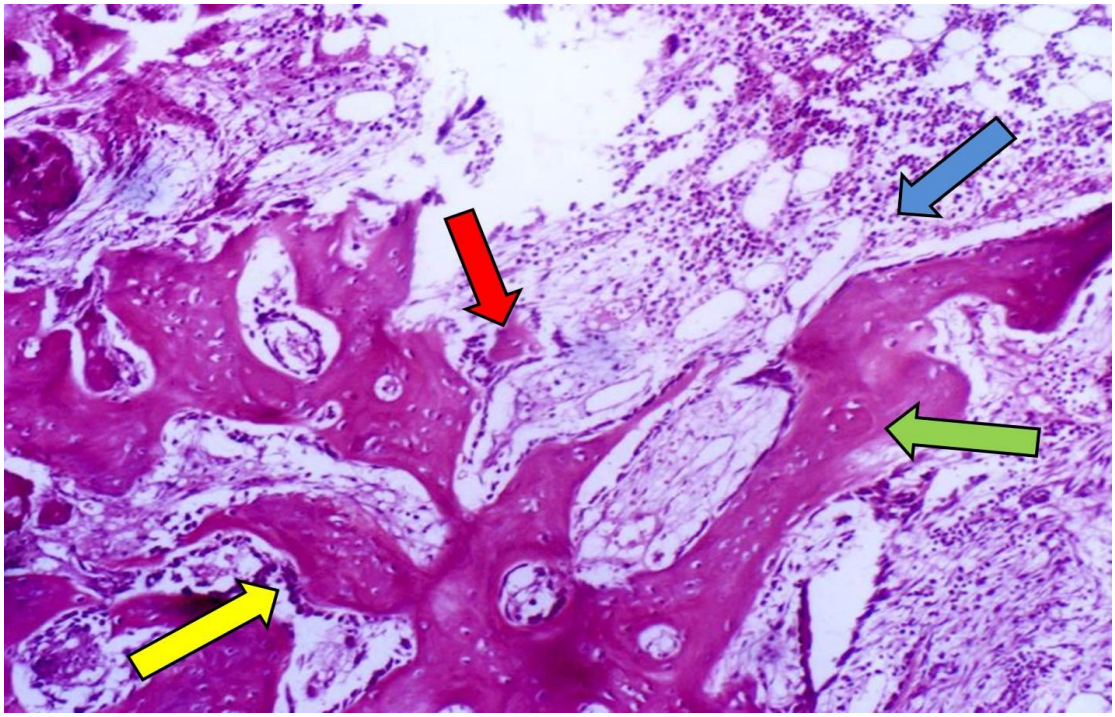


Figure 40:Micrograph of bone defect at 14th post-surgery days in hyaluronic acid group shows inflammatory cells infiltration (blue arrow) and there is congestion of blood vessels with extra vRBCs(red arrow) with numerous woven bone trabeculae(green arrow)lined with osteoblast(yellow arrow) H&E40X.

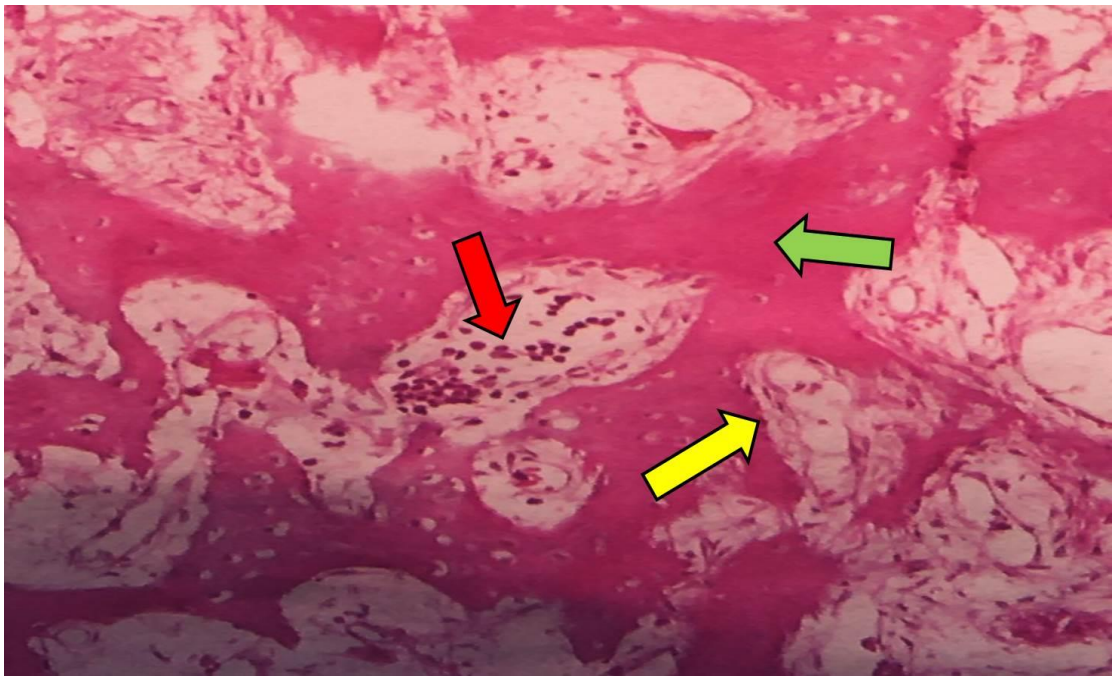


Figure 41:Micrograph of bone defect at 14th postosurgery days in hydroxy apatite Nano gel group shows the presence of trabecular bone lamellae (green arrow) and bone marrow(red arrow) and the presence of osteoblast(yellow arrow) H&E100X.

Table 6:Shows characterization of the groups using the healing score system (histological parameters) at 14th post-surgery days.

Histological bone healing parameters	The control group	Xenograft group	Hyaluronic acid group	Hydroxyapatie Nano gel group
Surface bone formation	1	0	1	2
Vascularization	1	1	1	2
Osteoblasts	0	0	2	2
Osteocytes	0	0	1	2
Osteoclast	0	0	0	0
Immature bone	1	0	1	1
Mature bone	0	0	1	2
Bone trabeculae	0	0	1	0
Haversian canals	0	0	0	0
Inflammation	3	2	1	0
Granulation tissue	2	3	1	0
Neo-formation of blood vessels	2	3	2	1

4.6.1.2.At day thirty post operation

The histological examination of the control group showed that there was woven bone formation at the site of bone defects that transformed into lamellar bone.This woven bone was lined by osteoblast cells that was encapsulated by connective tissues. Besides, inflammatory cells, red blood cells and congested blood vessels were also observed (Fig 42, 43).

Whereas,the xenograft group showed the presence of a mature connective tissues at the defect sites surrounded with the woven bone formation with the inflammatory response (Fig. 44).

At the hyaluronic acid group,the histological examination displayed mature connective tissues at the site of defect with mature bone lamellae formation lined with organized osteoblasts (Fig. 45,46).

While within the Nano hydroxyl apatite gel group,the site of defect after implantation showed formation of mature compact bone with high numbers of active marrow space with osteoblast lining and development of haversian canal (Fig. 47).

The results of histopathological scoring at thirty days post-surgery in all groups according to the (Table 7).

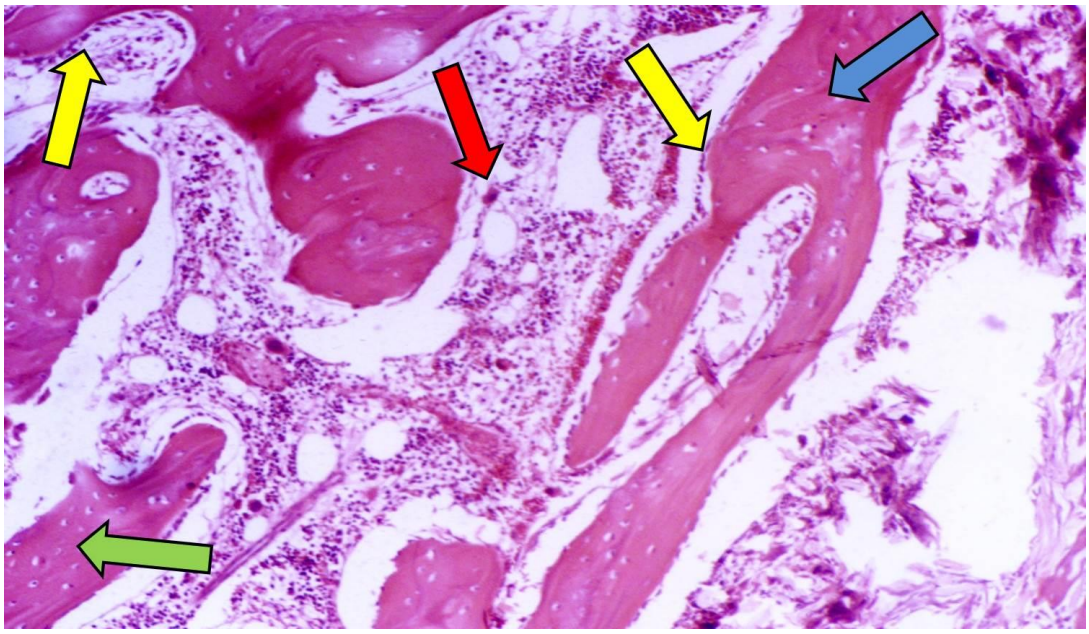


Figure 42:Micrograph of bone defect at 30th post-surgery days in control group shows woven bone(blue arrow) structure formed in the edge of the bone defect area lined by osteoblast (ob) producing osteoid matrix(yellow arrows), osteocytes (os), and osteoclast (cl)(red arrow). inflammatory cells, red blood cells capsulated the bone lamellae (green arrow) H&E40X.

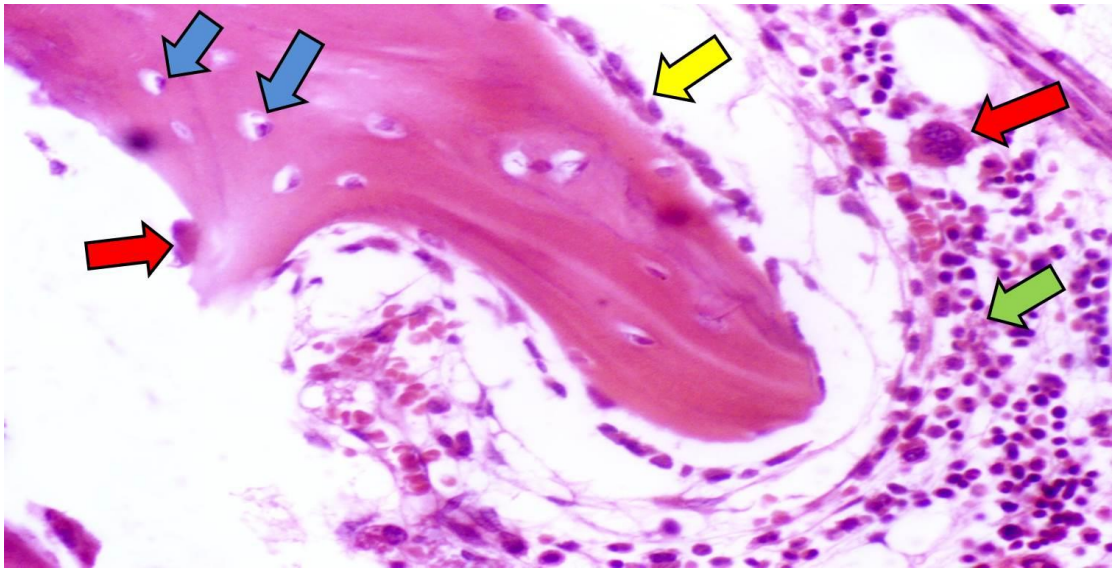


Figure 43:Micrograph of bone defect at 30th post-surgery days in the control group shows osteoblast lined the new bone formation (yellow arrow),osteocyte (blue arrows) osteoclast(red arrows) with sever infiltration of inflammatory cells and RBCs around the bone lamellae(green arrow) H&E100X.

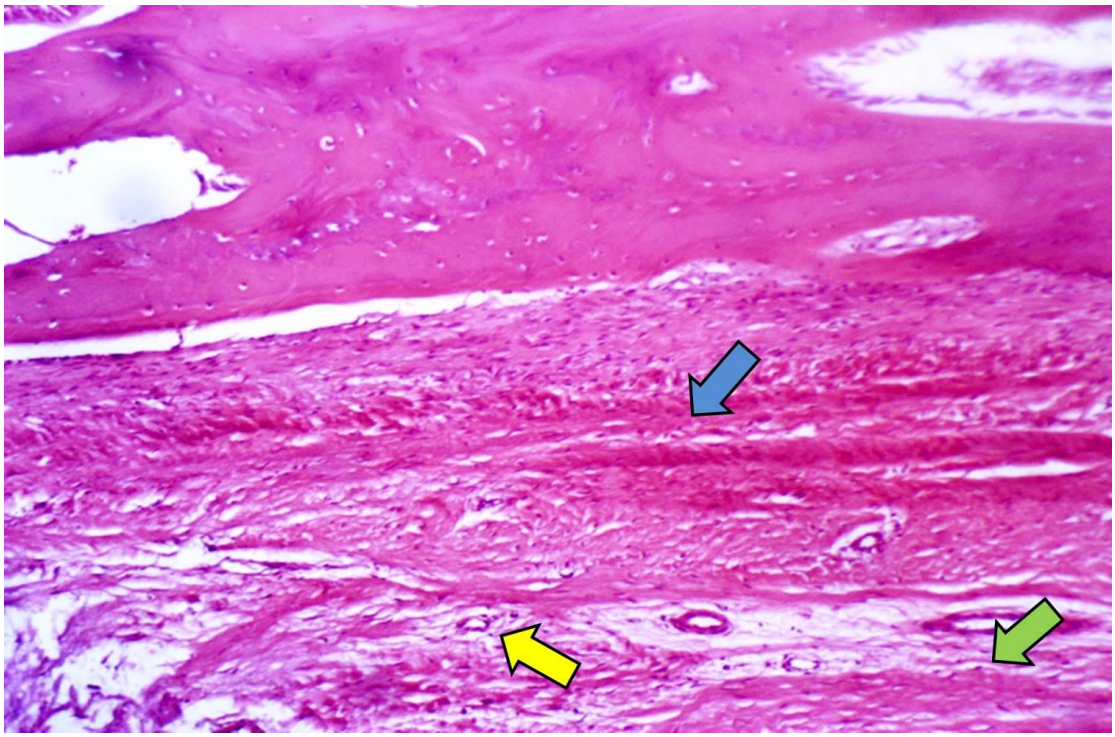


Figure 44:Micrograph of bone defect at 30th postsurgery days in the xenograft group shows formation of woven bone (green arrows) which encapsulated with fibrous tissues (blue arrow)with inflammatory reaction(yellow arrow) H&E40X.

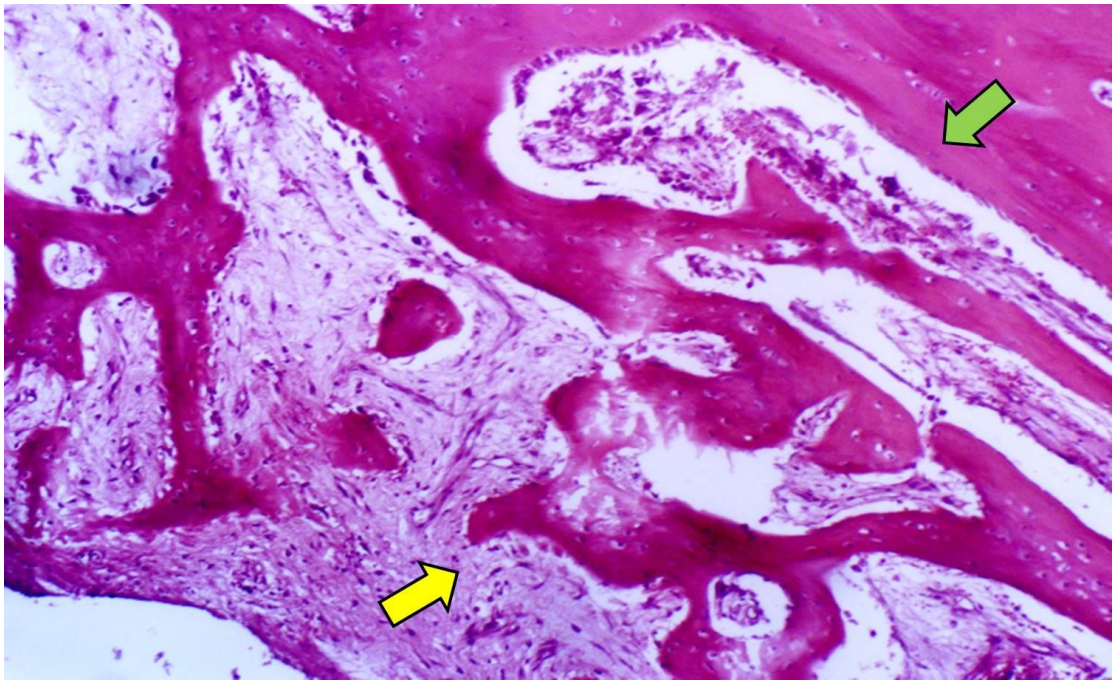


Figure45:Micrograph of bone defect at 30th post-surgery days in the hyaluronic acid group shows mature connective tissue(yellow arrow) surrounding the mature bone lamellae(green arrow) H&E100X.

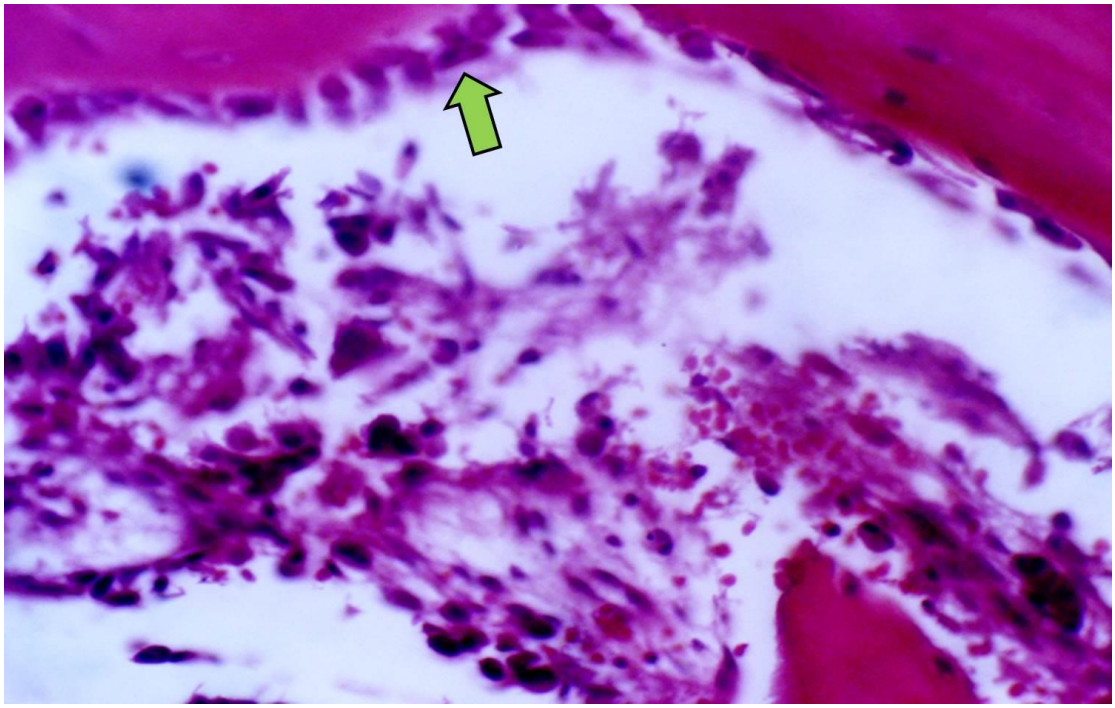


Figure 46:Higher magnification of bone defect at 30th post-surgery days in hyaluronic acid group shows the presence of a large population of organized osteoblast surrounding the new bone formation(green arrow) H&E400X.

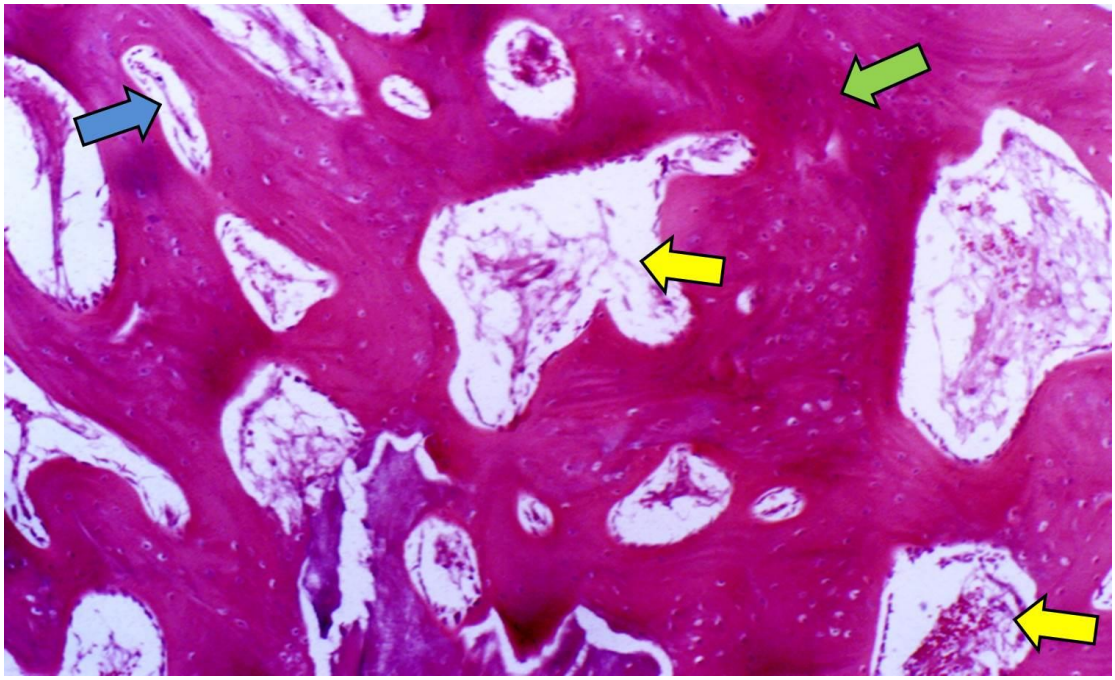


Figure47:Micrograph of bone defect at 30th post-surgery days in hydroxyapatite Nano gel group shows formation of mature compact bone(green arrow) with the development of haversian canals(blue arrow) and adequate amount of bone marrow (yellow arrows) H&E100X.

Table7:Shows characterization of the groups using the healing score system (histological parameters) at 30th post-surgery days.

Histological bone healing parameters	Control group	The xenograft group	Hyaluronic acid group	Hydroxyapatite Nano gel group
Surface bone formation	1	1	2	2
Vascularization	1	1	1	2
Osteoblasts	2	2	1	3
Osteocytes	1	1	2	2
Osteoclast	0	0	0	0
Immature bone	1	1	1	1
Mature bone	1	1	2	3
Bone trabeculae	1	1	2	3
Haversian canals	0	0	1	2
Inflammation	2	1	0	0
Granulation tissue	0	1	0	0
Neo-formation of blood vessels	0	1	1	1

4.6.1.3.At day sixty post surgery

Following 60 days of operation,the histological inspection within the defect sites of the control group displayed the formation of mature bone and bone marrow with an increase in the number of osteocyte and fewer number of osteoclast (Fig. 48).

While the xenograft group showed formation of compact bone with large numbers of osteocytes surrounded by large quantities of bone marrow formation without inflammatory reaction (Fig.49).

On the other hands,the hyaluronic acid treated group exhibited mature compact bone lamellae formation lined with a number of osteoblast and a well-developed bone marrow with the formation of haversian canal (Fig.50).

While for the hydroxyapatite Nano gel group,the histopathological inspection showed that the bone morphology was mainly consist of large volume of mature compact bone tissue creation besides the formation of haversian canal and the presence of numbers of osteocytes (Fig.51).

The results of histopathological scoring at sixty days post-surgery in all groups according to the (Table 8).

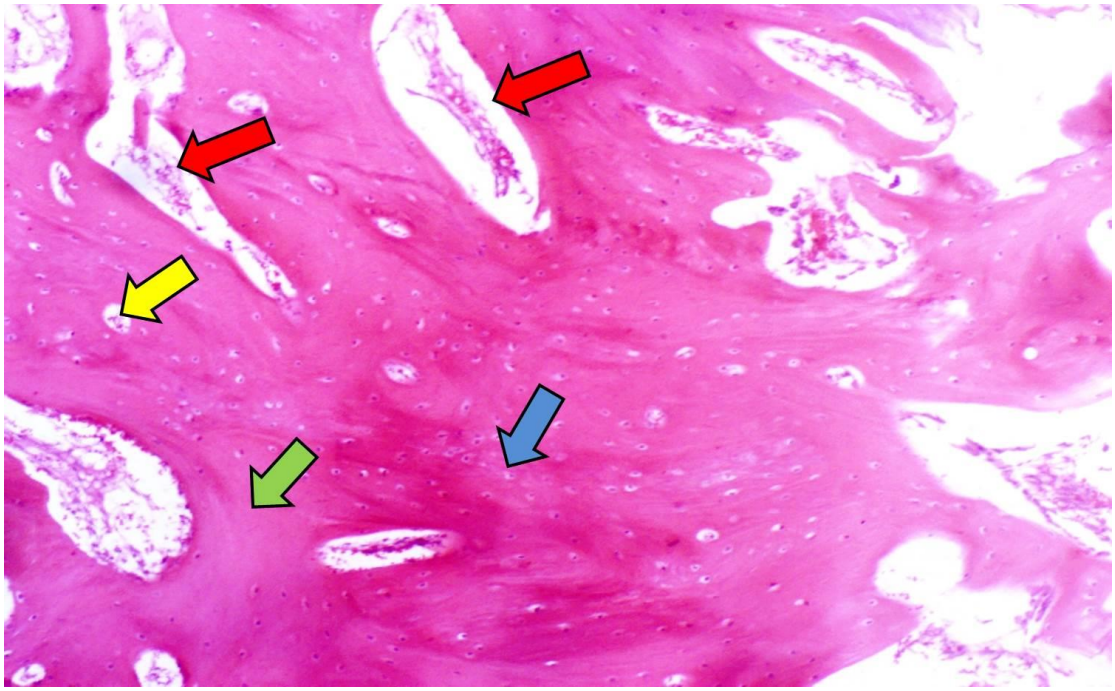


Figure 48:Micrograph of bone defect at 60th post surgery days in the control group shows mature bone(green arrow) and bone marrow formation(red arrows) and increase the number of osteocyte(blue arrow) and fewer number of osteoclast(yellow arrow) at the defect site H&E40X.

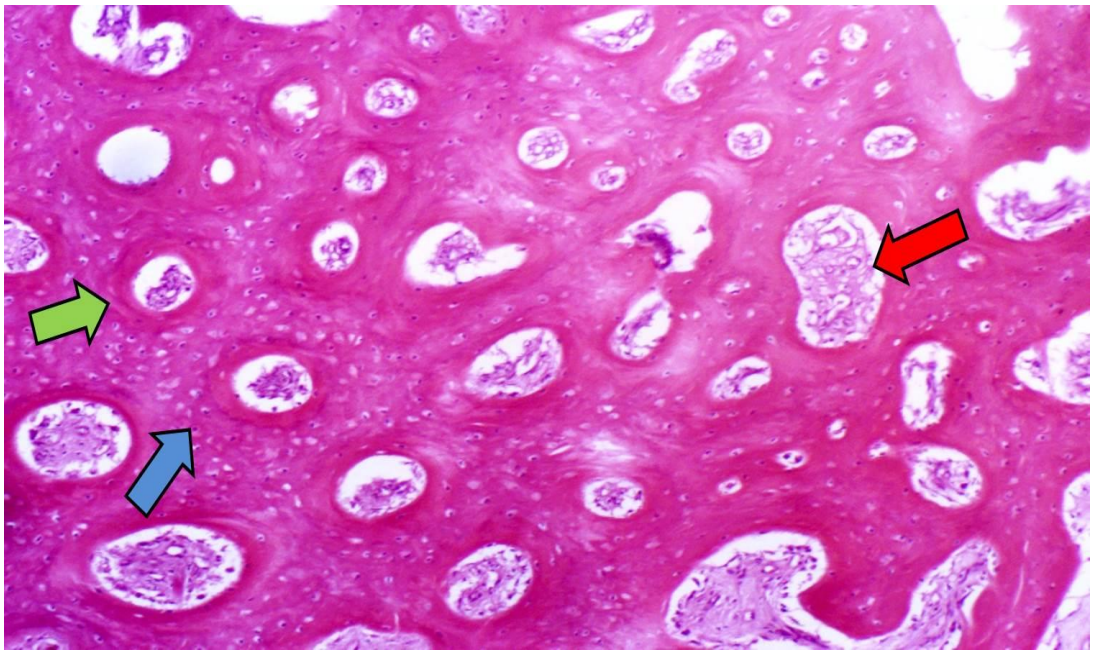


Figure 49:Micrograph of bone defect at 60th postsurgery days in the xenograft group shows the presence of compact bone(green arrow) with high number of osteocyte(blue arrow) and both surrounded by large quantities of bone marrow formation(red arrow) H&E40X.

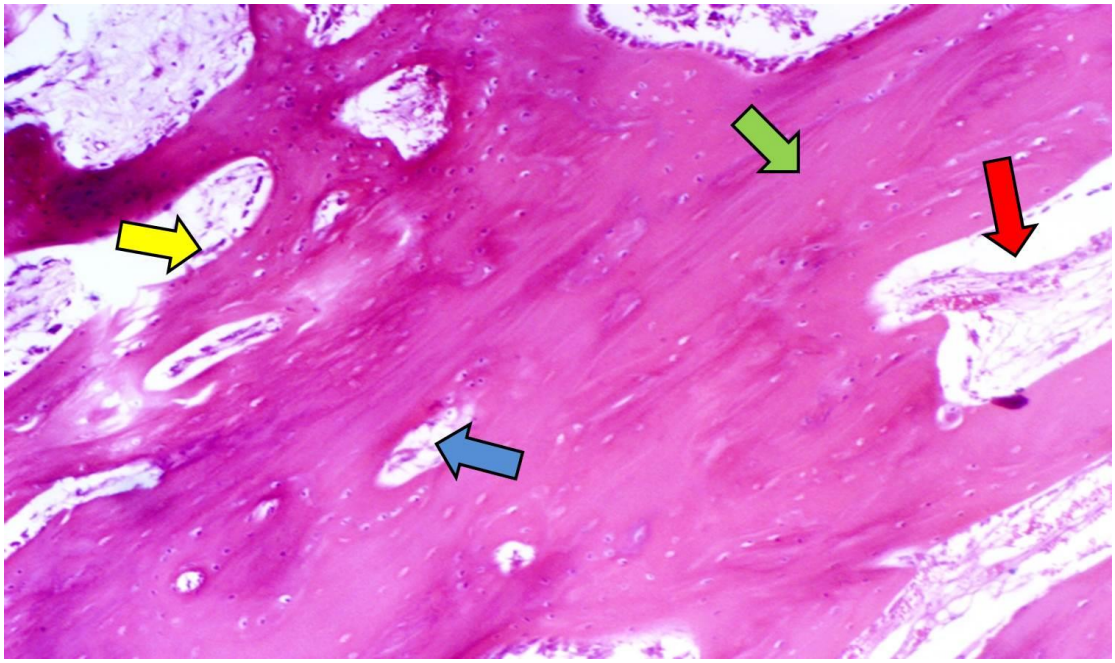


Figure50:Micrograph of bone defect at 60th post-surgery days in hyaluronic acid group shows the presence of mature compact bone(green arrow) with formation of haversian canal(blue arrow) with well formed bone marrow(red arrow) and osteoblast (yellow arrow) H&E100X.

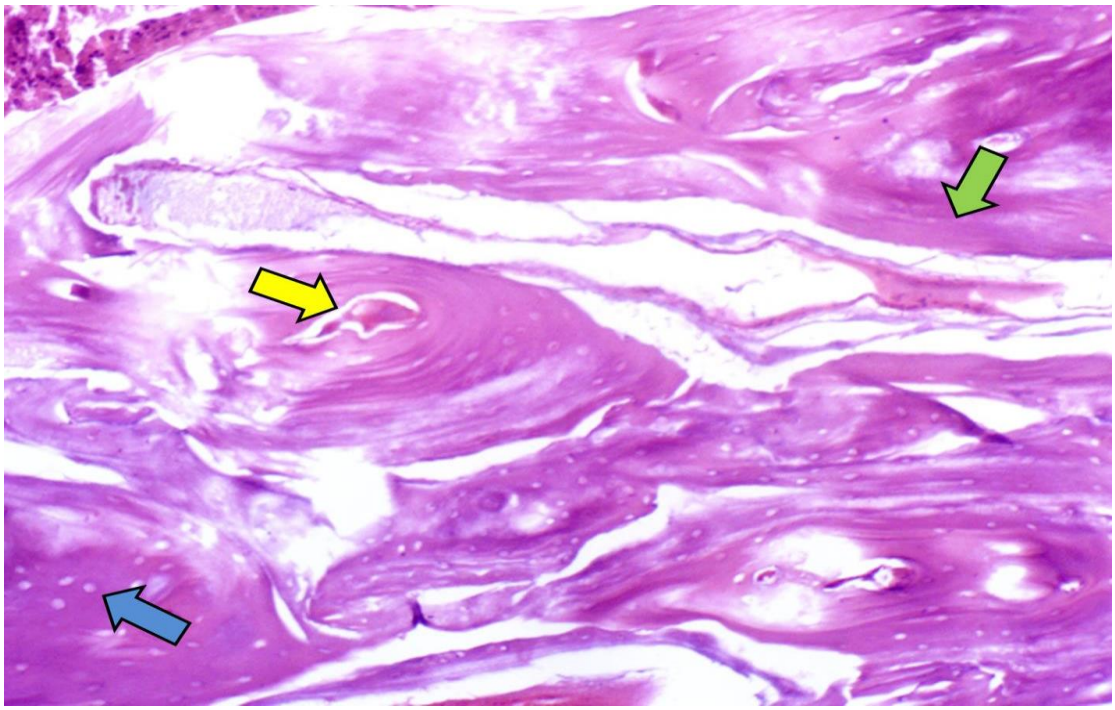


Figure51:Micrograph of bone defect at 60th post surgery days in the hydroxyapatite Nano gel group shows the presence of mature compact bone(green arrow) and haversian canal(yellow arrow) and numbers of osteocytes(blue arrow) also observed H&E100X.

Table 8: Shows characterisation of the groups using the healing score system (histological parameters) at 60th post-surgery days

Histological bone healing parameters	The control group	Xenograft group	Hyaluronic acid group	Hydroxyapatie Nano gel group
Surface bone formation	0	1	3	3
Vascularization	1	3	2	1
Osteoblasts	1	1	2	1
Osteocytes	2	3	3	3
Osteoclast	1	1	1	1
Immature bone	1	2	1	0
Mature bone	1	1	2	3
Bone trabeculae	1	3	2	3
Haversian canals	1	3	2	3
Inflammation	0	0	0	0
Granulation tissue	0	0	0	0
Neo-formation of blood vessels	1	1	1	1

4.6.2.Masson's Trichrome stain histochemical stain

This stain when applied to the mature normal bone,it revealed two main reactions,a blue one that is mainly localized to bone tissue and the distribution of collagen fibers and a second red reaction for the lamellar new bone formation.

As regard for the control group, the histological results for all specimens of different periods showed an increased in the calcified bone via the appearance of the blue reaction at day 60 post operation which was more obvious than for days 14 and 30 post operation (Fig. 52) .

While for the xenograft groups,the blue reactivity at day 14 days post operation revealed the distribution of the present collagen fibers (Fig.53). on the other hands, following 60 days post operation the blue reactivity was more pronounced.

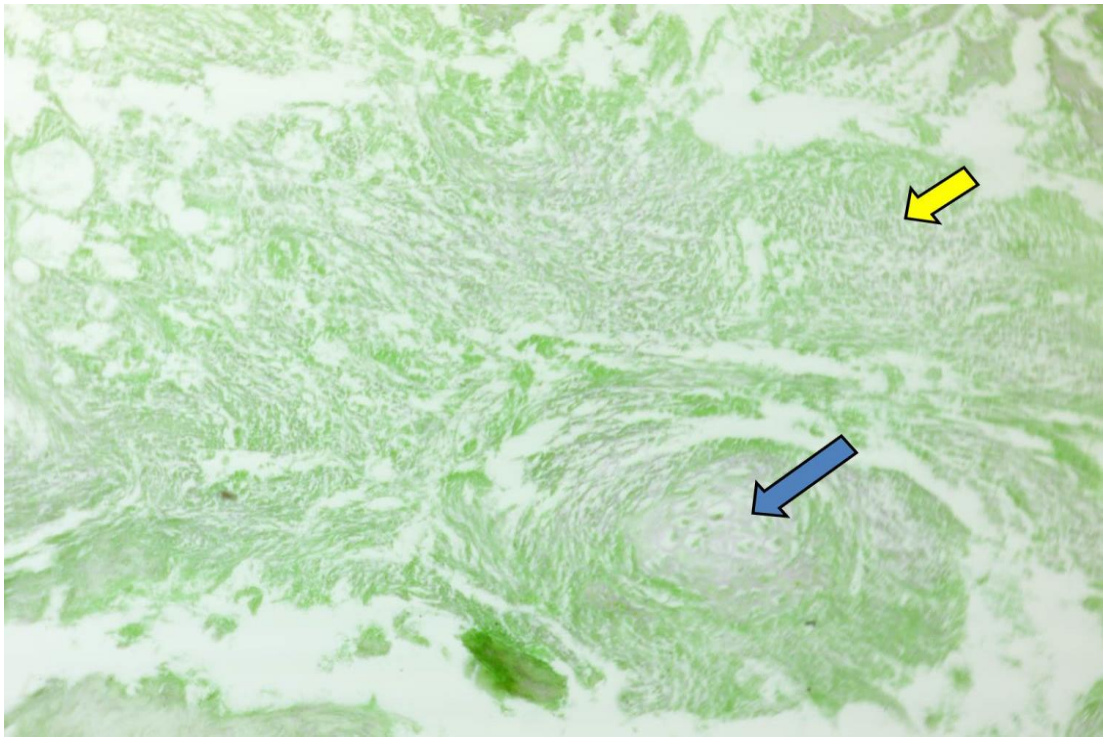


Figure 52:Masson's trichrom staining of bone defect at 14 days post-surery in the control group shows fibrous connective tissue(yellow arrow) and bone lamellae formation(blue arrow) (10X).

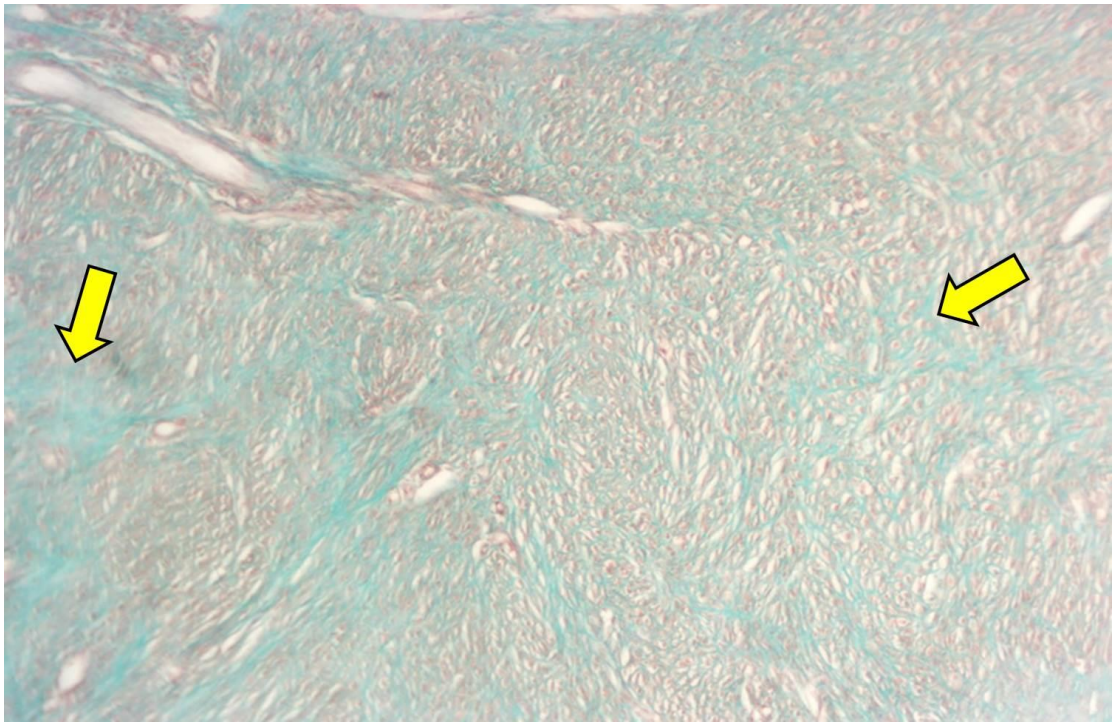


Figure 53:Masson's trichrome staining of bone defect at 14 days post-surgery in the xenograft group shows immature connective tissue(yellow arrows) (10X).

4.6.3.Alcian blue (PH2,5)PAS stain

Normally,the completely formed bone tissue is exhibiting a high reaction for hydroxyapatite Nano gel that is represented by the presence of a blue color which means positive to alcian blue stain.The hydroxyapatite Nano gel showed a highly positive reaction by obvious blue color and rise in bone tissue in comparison with the other groups. Alcian blue staining offered a high positive reaction due to increase in the mineral deposition in bone tissue,while distribution of PAS positive reactivity was weakly noticed due to the decrease acidophilic activity(Fig. 54).While for the control group there was a slight reaction (Fig.55) .

As for in the hyaluronic acid group the reactivity at 60 days is more pronounced than the groups at 14 and 30 days post-surgery (Fig. 56) As regarded of groups of animals treated with hydroxyapatite Nano gel at 14,30 days post-surgery revealed an increase in calcified bone as evident by blue reaction when compared with the other groups, while in hydroxyapatite Nano gel group of 60 days post-surgery indicating thick and organized blue of highly formed mature compact bone with numbers of osteocytes.

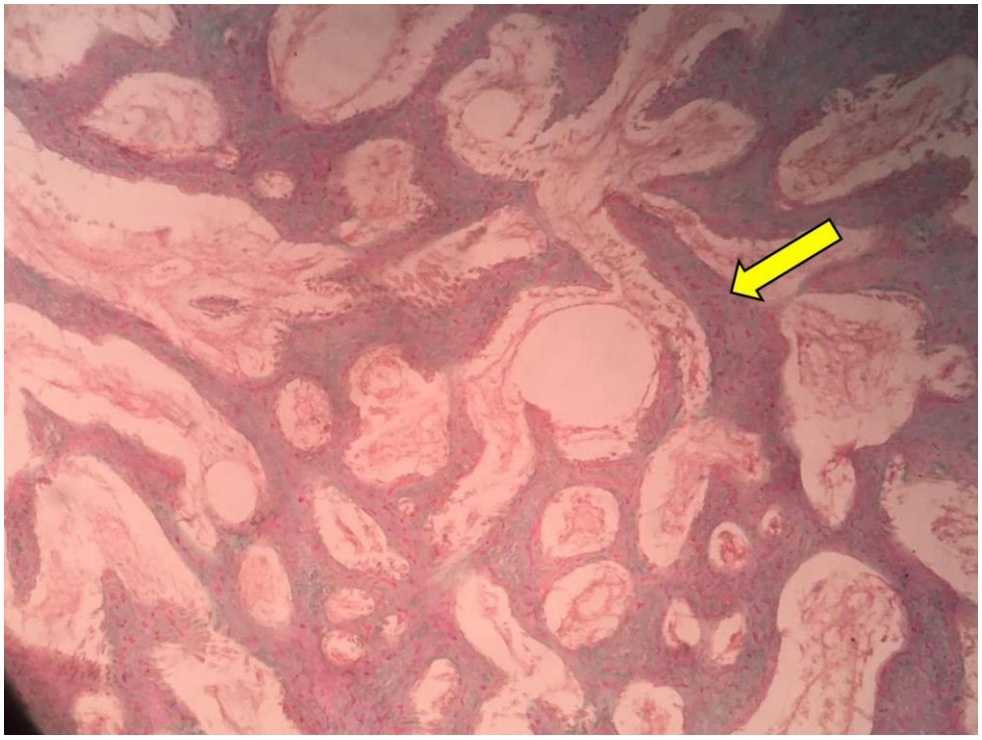


Figure 54:Alcian blue 2.5 PH PAS 30 days post-surgery in hydroxyapatite Nano gel group shows increased distribution of NMP sever blue color of highly formed mature bone(yellow arrow) (40X).

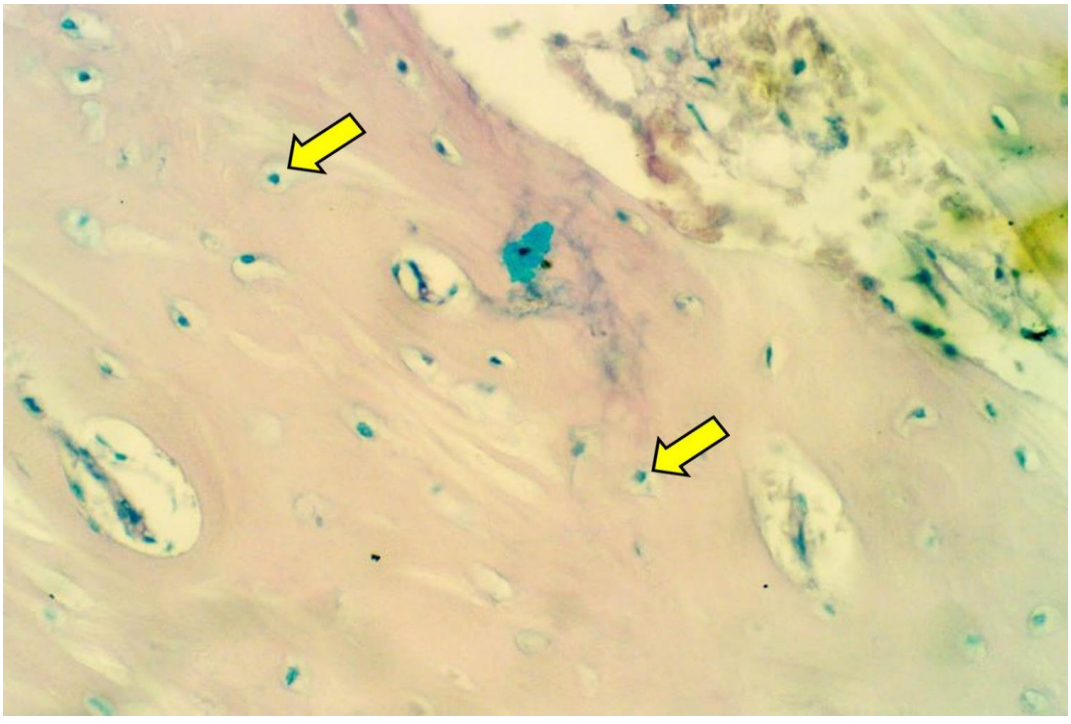


Figure 55:Alcian blue 2.5 PH PAS at 60 days post-surgery in the control group shows numbers of osteocytes(yellow arrows) slight distribution of NMP blue color (100X).

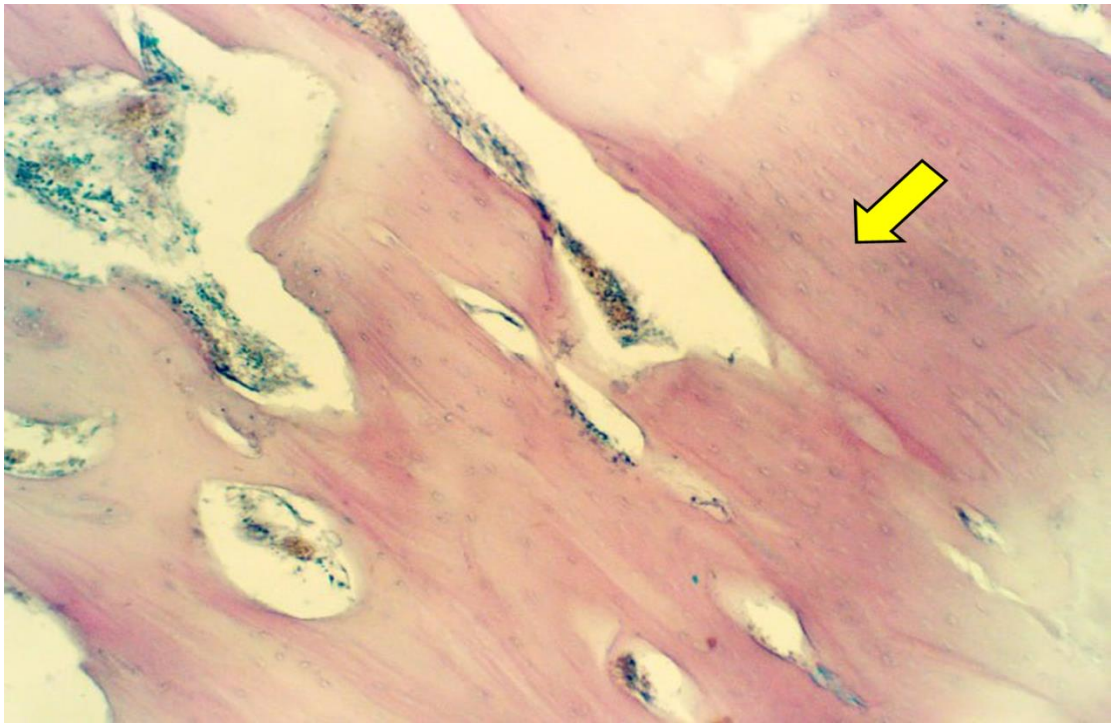


Figure 56:Alcian blue 2.5 PH PAS at 60 days post-surgery in hyaluronic acid group shows mature compact bone (yellow arrow) with moderate distribution of NMP blue color in the formed bone (40X).

4.6.4.Osteoblast count

The results of osteoblast count exhibited increases in the number of osteoblast for all operative animals in hyaluronic acid group at sixty days post-surgery and revealed significant differences ($P \leq 0.05$) among hyaluronic acid and other groups.In the hyaluronic acid groups the highest rates were at day 60 as (38.33 ± 2.3) as compared with hydroxyapatite Nano gel, xenograft, control groups and as (12.25 ± 1.1),(11.66±0.3),(10.8±1.4) respectively (Table 9).

Table 9:shows numbers of osteoblast in all groups.

	14 day	30 day	60 day
Control	0±0 $^{a\,A}$	18±1.2 ^{aB}	10.8±1.4 ^{aC}
Xenoghraft	0±0 ^{aA}	15±1.1 ^{aB}	11.66±0.3 ^{aC}
HA	10±0.9 ^{bA}	17.33±1.8 ^{aB}	38.33±2.3 ^{bC}
HAp	34±1.3 ^{cA}	30.75±3.7 ^{bA}	12.25±1.1 ^{aB}

Small different letters among groups mean there is significant difference at $p \leq 0.05$
Capital different letters among periods mean there is significant difference at $p \leq 0.05$

Chapter Five

Discussion

5.1.Size of defect, implant and composition

Orthopedic surgery is a challenging and quickly advanced branch in veterinary medical practice. Regaining the normal function rapidly with the least problems is a substantial demanding requirement for an orthopedic field as body movement founded mainly on the musculoskeletal system (Perren, 2002).

Cortical bone grafts have structural integrity that mainly subjected to reinstate the function shape, promote osteogenesis and supplied combination among a frame work and two fragments of bone(El-Keiey,2009).

Cortical bone xenografts are usually subjected to repair bone defect.They designed to reduce antigenicity before implantation to minimize rejection (Horváth *et al*.,2013). Chemical treatment, boiling, irradiation, freezing, and freeze-drying of the bone implants were suggested methods for lowering antigenicity (Baharuddin *et al*.,2005). Prior to autoclave sterilization, the implants in the current investigation underwent chemical treatment utilizing the pepsin enzyme and chloroform with ethanol to eliminate organic debris. According to Hofman *et al*. 2003, autoclaving is a dependable way of preparation that doesn't a`1ffect xenografts' ability to osteointegrate properties.

Regarding the immunogenicity of the xenografts, prior research shown that immunogenic reactions such hyper-acute or chronic rejection may manifest when the xenograft is transplanted into the recipient bed. Increased fracture rates, early bone resorption, and non-union were some of the symptoms of transplant rejection (Hammer,1994).The a

forementioned indications of implant or xenograft rejection were not evident in the current investigation, instead, a complete acceptance into the host bed was noticed.

In dogs, the tibial bone defect is approximately 13.81% of the whole bone defects(Muhamad *et al*.,2020).In the present experiment, the size of defect was (2.5 × 0.7cm) with complete excision of periosteum.This size of tibial defects is coincides with another worker who considered 1.5 cm and even more as critical defect (Marei *et al*., 2018).

The dimension of the formed bone tissue defect is considered critical because they do not repair spontaneously through the animals life. In the present study,2 months ovine lamb ribs were used as candidates for xenografting procedure due to its availability and the diameter of the ovine lamb rib diaphysis was approximately identical to the dimension the tibial bone in dogs which agreed with (El-Keiey,2009) who used caprine xenograft bone of femur that was approximately identical to the dimensions the femoral bone in dogs.

Acceleration of the bone tissue regeneration process is an important factor for the surgical restoration for many orthopedic affections. Implantation by using different types of graft can be used either to conduit critical bone tissue defects or to provide the continuousness of a hind limb bones. These implants are indicated for filling of the space in between the graft and border of defects, and to improve bony union in many situations as delay union or a bone tumor (Millis and Martinez ,2003).

A number of implants including bone scaffolds has been tested for repairing the critical bone defect. Their perfect properties must include biocompatible chemical nature to prevent improper tissue response,

Superior resistance to corrosion in the physiological environment, adequate strength, good resistance to wear, and finally, an elastic modulus equivalent to bone to reduce bone resorption around the implant are all requirements(Schmidt *et al*., 2001).

Any substance that could be used to replace bone must be fully biocompatible, able to act as a surface for host cells to anchor to, have sufficient porosity to allows osteoconduction, and gradually resorb and be substituted by new bone tissue (creeping-substitution) (Athanasiou *et al*.,2009).

In previous study the workers used bovine bone morphogenetic proteins (BMP) successfully in canine ulnar non- union model that hasten the healing process (Nilsson *et al*.,1986). In this study we used deproteinized lamb rib xenografts which almost fulfilled all the requirement of bone repairing, as it act as a scaffold that restoring the bone as close to the natural furniture of defective tissue, besides it worked as a bridge to produce beneficial outcome (Wang *et al*., 2020).

Success in bone tissue repair is largely dependent on the body's ability to function biologically and mechanically, the presence of pro-osteogenic cells, growth factors (osteoinduction), implants that permit bone tissue formation (osteoconduction),and sufficient vascularization for an efficient nutrient delivery(Portal *et al*.,2012).

5.2.Animals model

The animal models play a significant role to exam the orthopedic grafts prior to their practice with humans. In spite of the difficulty of dog handling as an experimental model, it has many advantages over other animal including its easier ability to learn and adapt to new environment, cheaper prices and their perfect tolerance of different climatic conditions

and livestock(Pearce *et al*., 2007). For all these mentioned reasons, as well as their availability, the low feed cost and similarity to those of human as well as true skeletal maturity, however, the most significant factor in choosing dogs as an experimental modals were their morphological, physiological and biomechanical similarity to human beings and their better experimental manipulation for various diagnostic and functional tests as compared to rodents (Betancourt *et al*.,2015;Easa *et al*.,2021).

5.3.Fixation methods

In our trail external gypsona was used for transit external fixation which permitted for greater amount of periosteal callus formation and prevented unnecessary micromovement and wound dehiscent during first few days following surgery that all agreed with (Sousa *et al.*,2015).

5.4.Clinical investigations

Clinical examination exhibited no outward signs of inflammatory reaction along the period of experiment nor rejection of implants for all experimental groups.The signs including hematoma, seroma, and loss of appetite were not evident post operatively, however the local pain signs were developed within days post operation then gradually subsided. The signs of lameness were noticed during the first two days post-surgery especially with animals of control and xenograft groups.The subjective estimation reveals superior clinical healing of hydroxyapatite Nano gel group which could be due to the ability of that substance to improve healing process through stimulation cell control of VGEF growth factor(Bayani *et al*., 2017).

Postoperatively, the grade of lameness exhibited continuing enhancement toward the usual weight bearing during the study period. The grade of

lameness used to this study was in conformity with the procedure advanced by(Arias *et al*.,2013). In all groups the degree of lameness postoperatively was zero, while the highest degree of lameness was noticed at 7 days post-surgery for both control and xenograft group. The dogs could not bear their weight by the operative limb, then the lameness rate gradually reduced until 14 days postoperatively. That score was same for both control and xenograft groups.The lameness of the treated limbs in control and the xenograft group may be due to inflammation and local pain at the site of bone defect hence weight bearing was graded as high at this period.This outcome accepted with (Glyde and Arnett,2006)who studied the reduction of weight bearing at the operated limb post-surgery and noticed that lameness was highly correlated with severe inflammatory reaction and pain signs, however these clinical signs started to retard gradually with returning to perform the normal function, besides animal stands normally with perfect weight bearing without lameness in all animals at 60 days postoperatively.This findings were in agree with the finding of(Zebon,2020)who scaled weight bearing scores in both standing and walking following using bone substitutes and found that the lameness was gradually decreased in treated groups on 6th week and disappeared at 7th week. As well as, (Das, 2012) also mentioned that the normal weight bearing on all legs at break and running was showed on day 30 postoperative with return to the normal value on day 60 postoperative. Similar finding was documented with (kumar *et al*., 2019)who mentioned that all dogs which were diagnosed as femur and tibia fractures showed different degrees of lameness during and post surgical treatment.

Ahmed *et al*.,2015 in their work with hydroxyapatite/βtri-calcium phosphate (HAp/β-TCP),noticed marked improvement in the mean

degree of lameness towered the end of the first week postoperatively with partial weight bearing while iliac crest autograft (ICBG) still revealing of lameness with pulling out of the leg during station and walking with dragging of the hind limb. On the 2nd postoperative week dogs of HAp treated group showed intermittent lameness during trotting.

In the current study, early weight bearing in hydroxyapatite Nano gel and hyaluronic acid treated groups were noticed suggesting that both of them acted as a biomaterial that helped in pain relief lessen intensity of inflammation and accelerated of bone defect healing process of bone defect. These findings also documented with (Laurencin *et al*.,2006) who tested using variety of ceramic based graft substitute materials such as HAp for repairing bone defects and reported their effectiveness for long time fracture healing.In another study(Chandrashekar and Saxena, 2009) noted that, the new combination of synthetic hydroxyapatite and b-tricalcium phosphate were biocompatible that improved healing outcomes through increase in the clinical attachment level and amount of bone defect filling defects when compared with other calcium phosphate ceramic materials.

El-keiey,2009 also mentioned that using of autoclaved cortical xenograft for treatment dog bone defects led to ability for well weight bearing for the operated limbs within 4 weeks post-surgery as a compare to using fresh cortical autografts.

The results of our current study for using hydroxyapatite Nano gel and hyaluronic acid revealed great success with weight bearing ability as comparable with control and xenograft groups. Within these both treated groups, there was primary bone formation between edge of bone and implant that then completed with bridging of bone tissue that was so clear

histopathologically and radiographically indicating complete weight bearing without lameness with excellent limb function.Thus, weight bearing was graded as excellent. These observations may be belonged to the effect of hydroxyapatite Nano gel and hyaluronic acid which consider to be as excellent osteoconductive on bone healing and might be due to the ability of these biomaterials to decrease pain and inflammatory reaction and to enhanced healing of bone defect and bone formation. These outcomes are in agree with(Chandrashekar and Saxena 2009)who mentioned that the mean degree of lameness decreased in animals treated with calcium hydroxyapatite/tricalcium phosphate compared to those who did not treated on the 4th week. Besides, they referred that this combination was biocompatible and resorbable in 6 months with osteoconductive activity which enhanced bone healing process. Similar findings were also noticed with (Tunay *et al*., 2002) He proposed that the use of intraarticular HA reduces inflammation and catabolic degeneration in synovial tissues and cartilage by using 2 mg/kg of HA twice daily over the course of every other month in the rehabilitation of empirically produced osteoarthritis. While, (Zhai *et al*.,2020) mentioning that HA products or composite implants using HA had demonstrated remarkable promise for enhancing bone mineralization and osteogenesis.

5.5.Alkaline phosphates

Serum alkaline phosphatase levels within HA and HAp groups increased at seven and fourteen days postoperatively in comparison with the control and untreated xenograft groups.This result might be explained through the increased osteoblastic activity when osteoblast produces huge amounts of ALP that get included in mineralization process and bone defect formation as a result of adding the hyaluronic acid and hydroxyapatite Nano gel as a biomaterials, these findings are in

accordance with those of (Kuo and Prestwich, 2011) who reported that the hyaluronic acid increase ALP action and encourage of osteoblastic cell differentiation and proliferation.Thanoon,2019 also showed that the best activity of osteoblast cells and bone formation was in the sixth week in autologous bone marrow (BM) and platelets rich fibrin (PRF) groups. Similar findings are agree with (Zebon,2020;) who noticed the increased levels of serum alkaline phosphatase up to 20th day postoperative due to increased osteoblastic activity in Nano, micro and control groups respectively. Besides, (Leung *et al*., 1993; Johnson and Watson ,2000) mentioned that the ALP, which is involved in the creation and calcification of bone cells, is secreted in enormous amounts by osteoblasts. Studies have shown that the ALP action in serum is closely associated to the repair of fractures.An even more precise indicator of bone growth is thought to be the bone isoenzyme of ALP (BALP). Phaneendra *et al*.,2016 studied the serum alkaline phosphatase values and found that their levels significantly increased from preoperative day to fourteen day then started to decline until reaching normal value by day 21 because of the maximal contributions from the periosteum of destroyed bone, which is a strong source of serum alkaline phosphatase, increases chondroblastic proliferation to produce bone formation during shattered bone regeneration.Allen *et al*.,1998 mentioned that the AHp are formed mainly of phosphate and calcium and placed in the organic materials.

In the present study the rate serum alkaline phosphatase gradually decreased to return near the normal value in both HA and HAp groups at day 60 postoperatively.This result might be indicative for cessation of osteoblastic activity, receding of the values towards its base value due to ossification and consolidation of bone grafting and quiescence at site of

bone defect(Mahendra *et al*.,2007;Phaneendra *et al*., ;2016 Kumar *et al*.,2018).This higher activity of alkaline phosphatase on 7th,14th days and its gradual decrease was also supported by the clinical, radiography and histopathological findings.This agree with (Kumar *et al*., 2018)who mentioned gradual increase in ALP level up to 14th day after surgery followed by decrease up to day 60 post-operative in dog, they concluded that increased chondroblatic proliferation to cause bone formation during healing of fracture with maximum contributions from the periosteum of fractured bone, which was a rich source of ALP in serum.

Chu *et al*.,2018 showed that the skeletal tissue mineralization process is linked to the ALP, a primary marker of osteogenic differentiation.While (Zhou *et al*.,2019) concluded that mineralization was encouraged by an (ALP) enzyme dialysis technique that can stimulate the creation and unchanging supply of calcium phosphate Nanostructures. Marwaha *et al*.,2010 in his work showed that bone creation markers like (ALP) can work as a perfect, accurate, dependable, reproducible, doctor -patient friendly and cost active technique to measure the process of fracture repairing accurately.

5.6.Insulin like growth factors(IGF)

The role of IGF in bone regeneration is so crucial. It can be formed in bone matrix, osteoblast and chondrocyte.There are two types of it, type IGF-I and II, IGF-I is very important that involve formation of bone matrix(Fowlkes *et al*.,2006).

In the present study, the increased in serum insulin like growth factors IGF-I at day 7 post-surgery within control, xenograft, hyaluronic acid and hydroxyapatite Nano gel groups may indicate increasing the activity of osteoblastic in which the osteoblast and bone matrix secretes

large quantities of Insulin-like growth factors (IGF) which play important role in proliferation of osteoblast, bone matrix synthesis and bone resorption that get involved in process of mineralization and bone defect formation especially with hyaluronic acid and hydroxyapitate Nano gel groups in comparison with control and the xenograft group and this outcome agree with those of(Schmidmaier *et al*., 2003; Salgado *et al*., 2004;Tsiridis *et al*., 2007)who reported that the IGF-1 played critical roles in many serious biological activities including development of the skeletal system, general growth development, cell proliferation and regulation, differentiation of most tissue, metabolism in the animals body and treatment of injuries in various tissues. Besides fracture repairing by encouraging creation of bone matrix like collagen type 1 and decrease collagenase synthesis.IGF-1 also encourages chemotaxis, osteoblasts action proliferations, and has the highest influence on bone creation when it is used in mixture with transforming growth factor-β (TGF-β). Canalis,2009 additionally reported that the general functions of IGF-I are survival and inhibiting apoptosis, stimulating cell proliferation, regulation of osteoblastic purpose. As well as, IGF-I encourages the differentiation and proliferation of osteoblastic precursor cells(Zoidis *et al*.,2011). Meanwhile insulin like growth factor-I and its receptors are extensively expressed in mesenchymal cells ,periosteal cells, osteoblasts, proliferating chondrocytes,it has been suggested that it plays a corresponding role between numerous cell kinds through destructive bone repairing case of developing fracture calluses(Nakasaki *et al*.,2008).

Increases IGF-I values in serum throughout growing result in improvement of all bone phenotypic characters that may play a protective role later on through elderly. Such increases also emphasize the involvement of IGF-I in serum to both cortical mass and bone density and

this agree with(Yakar *et al*.,2010).In this way decrease of IGF-I values in serum caused a alteration of body weight and reduced of cortical bone tissue mineral thickness(Kawai and Rosen,2010).

In the present study, the rate of serum IGF-I level gradually decreased at day 14 post-surgery in all groups and continued decreasing and subsided especially in HA group after 60 days post-surgery and other groups respectively and this result might be indicative for cessation of osteoblastic activity with decreasing of secretion IGF-I, while receding of the values towards its base value could be to ossification and consolidation of bone grafting and quiescence at site of bone defect. This higher activity of IGF-I on day and its gradual decrease was also reinforced and supported by the other findings of clinical, biochemical, radiographical and histopathological examination and these finding are in agreement with results of (Yun *et al*.,2012).

5.7.Radiographic investigations

Radiology is an important diagnostic procedure mainly used to evaluate the repaired defects of flat and long bones which are treated with several bone substitutes including DBM as well as to assess both experimental and clinical models of various bone defects.Taking radiographs at unvarying intermissions throughout the whole repairing method deliver a suitable procedure for successive evaluation of the bone tissue defect repairing (Üngör, 2012).

The perfect bone implant should have osteoinductive, osteoconductive, radiolucent and resorbable properties to allow the radiological assessment of bone repair and formation (Babis and Suocacos ,2005).

The radiographic evaluation in the present study was carried out at14, 30 and 60th days post-surgery to evaluate the grade and state of apposition

and arrangement, angulation, device (cerclage wire) action (the development of bone defect repairing) and construction (bone and soft tissue).This assessment was in agreement with (Saravanan *et al*.,2014)who showed that radiographical assessment for experimentally formed comminuted diaphyseal femoral fractures fixed by bone electroplating should be done on the instant post-surgery day at 15, 30, 45 and 60 days post surgery.

In the present study the radiographical findings at day 14 post operation showed no significant variances among the control and others groups, except the presence of rapid response of bone defect healing in hyaluronic acid and hydroxyapatite Nano gel groups respectively. However, in the control group, there was a lucent line enclosed the fixated bone implant connected with soft tissue swelling neighboring the site without definite periosteal response nor new bone formation. Same findings were observed in the xenograft group and this could be due to not adding both of hyaluronic acid and hydroxyapatite on the site of bone defect as a bioactive filling materials so acceleration of bone tissue formation was not properly occurred in addition to the presence of inflammatory reactions that prevent new bone formation. This results agree with (Wirata *et al*.,2018) who noticed that callus formation of rabbit femur was not developed on control and treated group with mineralized and demineralized powder bone graft at day 14 post-surgery due to the inflammatory process within the first day following operation that prevent early callus formation.These outcomes also agreed with (Pilitsis *et al*., 2002)who reported that inflammatory phase of bone healing process occurred during the first two weeks post –surgery and it was marked by profuse bleeding that developed into hematoma and vascularization that cannot be visualized by radiograph. Bigham *et*

al.,2008 also revealed lack of any radiographical significant differences of rabbit radius defect fixed by cercelage wire between xenogenic DBM groups and fresh autogenous cortical graft at the 14th days posts-surgery. They noticeda that the radial bone defect of rabbit repaired with grafted xenogenic DBM of bovine has healed satisfactorily.The same resulted from cortical autogenous grafting, albeit no problems were noted and healing proceeded more quickly.These results demonstrate that the grafted xenogenic DBM of bovine possesses osteoinductive activity similar to an autogenous cortical bone implant (via the release of certain BMPs).Cortical autograft, however, was discovered to have stronger osteogenic qualities and less osteoinductive activities.This results partially agreed with (Shafiei *et al*.,2009) who found that bone formation occurred in 25% in control groups with fresh cortical allogenous bone implant and fresh cortical autogenous bone implant of rabbit at 14th day post-surgery.Besides the statistical analysis did not support any significant variance on this period as well as there was some bone tissue combination in some animals of fresh cortical autogenous bone graft, while there was no evidence of union in fresh cortical allogenous bone graft at 14th day post-surgery and the repairing was not present in both groups at fourteen days post-surgery.

Both groups of hyaluronic acid and hydroxyapitate Nano gel showed an improvement in bone formation and acceleration of bone defect repairing at fourteen days following surgery.These results showed obviously that using of bioactive materials like HA and hydroxyapitate Nano gel can accelerate the migration and development of osteoblast across the edge of defect (Ding *et a*l., 2017;kaneko *et al*., 2020).In contrast the healing of bone tissue was unclear for control and xenograft groups as a result of not using bioactive materials.Thanoon, 2019 also

concluded that the adding of PRF and bone marrow as biomaterials on radial fracture in dogs had led to rapid healing response because of the richness of bone marrow and platelets rich fibrin with growth factors and stem cells that have rapid inflammatory response as well as their crucial role at early stage for bone healing improvement comparable with control groups after two weeks post-surgery.

However, in hyaluronic acid there were several changes along the 14 days post-surgery included the presence of a lucent line around the fixed bone segments,early periosteal reaction and no new bone formation. These outcomes is in agreement with outcomes of (Oakes *et al*.,2003) who founded minimal periosteal, endochondral ossification,with no callus formation following repairing rat femoral defect with HA.Maus *etal*.,2008 also reported similar findings in a sheep model at that time. The final results are also correlated with those of (Stern *et al*.,1992; Aslain *et al*.,2006; Kim *et al*.,2016) who demonstrated the important of osteoconductive and osteoinductive properties of hyaluronic acid for significant enhancement the migration and proliferation of MSC through bone regeneration can promote morphogenesis and tissue healing when administered alone or in combination. HA was therefore hypothesized to have a molecular mechanism of action that can enhance the osteogenic capacity of bone transplant materials.HA contributes in bone morphogenesis and is a key player in the early stages of the osteogenic activity by changing the effects of cytokines and various growth factors when it communicates with bone. Furthermore, HA works closely with osteogenic substrates like calcitonin and bone morphogenic protein to stimulate the creation of bone tissue. Likewise, it interacts to proteins necessary for healing process of wound such as fibrin,fibrinogen, collagen and fibronectin.Our results are in agree with all these facts.

Other studies exhibited that the use of HA significantly improvement the healing procedure,where HA was connected with greater initial radiological bone tissue healing as compare with the bone implanting alone group (Rhodes *etal*.,2011).Similar finding with (Kim *etal*.,2016) who demonstrated that hyaluronic acid might stimulate bone tissue creation and improve the process of wound healing after adding to new removal sockets that had been previously diseased.On the other side, (Arpağ *et al*.2018) who studied the high molecular weight HA and xenografts were used to fill rabbit calvarial bone defects, researchers looked into how well the bones could heal.They discovered that this method did not seem to significantly alter the trabecular bone structure compared to using xenografts alone.

In group treated with hydroxyapitate Nano gel the radiographic investigation exhibited serious changes including thickening and cortical irregularity with periosteal tissue reaction without new bone formation.The positively healing process results indicated the beneficial effect of hydroxyapatite bone substitute compound and proved its excellent biocompatible and osteoconductive properties.These findings are agree with (Yadegari *et al*.,2015)who used eggshell powder in repairing tibial bone defect in dogs.Shafiei-Sarvestani *et al*.,2012 mentioned that using high concentration of xenogenic platelets with hydroxyapitate had improved bone healing process in rabbits.

At day thirty post-surgery the radiographical investigations indicated the good progress of healing process of bone defect.The radiographic images showed superior healing in hydroxyapatite Nano gel and HA groups as compare to control and xenograft groups.Within control group, there were an interrupted, hazy lucent line, clear periosteal reaction and mild bone formation.These finding are agreed with(Faldini *et al*.,2009 ;Roberts

and Rosenbaum,2012).Their conclusion mentioned that autogenous implant had an ideal properties that integrated more rapidly and totally into the host bone, in addition to its osteogenic properties and the excellent mechanical support and the structural integrity with less amount of osteoprogenitor cells. Atiyah,2018 in his work on rabbits radial defects also showed that the radiological findings indicated mild periosteal reaction commences from both ends in the control group after four weeks post-operation. Jahagirdar and Scammell,2009;Zebon,2020 both mentioned that callus formation is a sign of the healing process.

In xenograft and HA groups, the radiographical findings were same as of the control group except the presence of incomplete new bone formation and ongoing healing process. Xenobone implant have been suggested as a bone substitutes for allobone implant and autobone implant by numerous workers. Most studies used bovine bone (Mahyudin *et al.*,2017) and pork origin (Bracey *et al*,.2018).while the present study used lamb rib bone as an alternative bone substitute in the repairing of a critical bone tissue defects with no evidence of inflammatory reaction and self-mutilation following its use. Our study is disagree with (Oryan *et al*.,2014)who said that the using xenograft compound is associated with tissue incompatibility, intense and hyper acute inflammatory reaction, however no inflammatory reaction or any serious complications of graft rejection were exhibited as with(Mahyundin *et al*.,2017).

HA is a constituent of extracellular matrix (ECM) signed during numerous macromolecules and particularly through regeneration and bone tissue repairing.It plays a main role in chemotaxis ,cell adhesion, proliferation and differentiation. Besides,HA of a specific molecular weight once using in vitro has an osteoconductive properties, that improve and stimulate healing process and it has anti-inflammatory

characteristcs during prevention of tissue damage and this result is on the same line of (Ayanoğlu *et al*.,2015) work who concluded that application of hyalonect along with using alone bone graft during 3weeks post-surgery and showed superior healing process than the control group in this period.This coincides with (Wirata *et al*.,2018)who showed that callus formation was developed massively on control and treated group at 4 weeks post-surgery. Abid and Mukhtar,2019 also stated the regional administration of collagen membranes and HA and prepared a better role to the bone reconstruction method in the tibial bone of rabbits than using the bone tissue implant substitutions (Osteon II Collagen) only after 4weeks post-surgery.

While in hydroxyapatite Nano gel group the main radiographical finding were that the lucent line defect was completely subsided and it was very thin and hazy,complete new bone formation and superior healing process. These outcomes associated with using of hydroxyapatite Nano gel because of its osteoconduction and osteogenesis characters which may lead to migration of osteoblast to the site of operation to stimulate new bone tissue formation to act as filling materials for improvement grafting process and healing.These results as same as (Zebon, 2020) who observed that the using crab shell–derived CaCO3 Nanoparticles and natural polymers in the scaffolds has helped in the creation of new bone tissue like a filling materials of bone tissue gaps. Easa *et al*.,2021 also recorded that the platelet-rich fibrin is a gifted biomaterial to accelerate bone repairing of critical sized bone tissue defects especially when combined with xenograft bone substitute in periradicular surgery in dogs because the PRF acts as an appropriate scaffold with a strong fibrin structure that optimally supports the transplanted and migration of mesenchymal cells and contribute in the gradual release of growth

factors over a long period between 7 to 28 days post-surgery(Tsai *et al*.,2009).As well as,(Yassine *et al*.,2017) recorded that the using hydroxyapitate with bone marrow stimulate a new bone tissue and periosteal reaction formation. The mild periosteal reaction was observed in radiographic investigation of hydroxyapitate group this results coincides with(Nazht *et al*.,2018)who study effect of laser therapy with low level on the ovine xenobone graft of ribs in the management the limb bone breaks of rabbits, its radiographical resulting exhibited initial periosteal response with new bone tissue creation at the seven days post-surgery, while the other group showed increase in opacity and size in the first few weeks post-surgery to build the bridge of bone and this agree with(Kazem-Shakouri *et al*.,2010)who mentioned that laser irradiation like biomaterials promotes callus formation and mineral deposition around the bony device by increasing osteoblastic activity, with improvement in biomechanical properties of the bone healing process.

The radiographical findings at day sixty post- surgery showed the progress of the healing process that was associated with the formation of new bone.The outcome was superior with both hydroxyapatite Nano gel and HA groups as a compare with control and xenograft alone.These data may be due to using of these biomaterial as filling materials which contribute positively in enhancement and improvement of the bone defect healing process, new bone formation, reduce period of bone union and restoring of normal mechanical function of bone that made augmentation of bone greater with using hydroxyapatite Nano gel with high viscosity 33% in the spreading of the Nano substance and consider as minimal invasive methods for bone augmentation and healing process (kaneko *et al*.,2020).

However, in control group there was late chronic periosteal reaction and new bone formation, near complete healing and rapid response of bone defect healing at that period and this result is in agree with(Nandi *et al*.,2010; Calori, *et al*.,2011) who showed that the autografts regarded as the golden standard of bone transplantation in repairing post-traumatic conditions such as fracture and bone defect as a result of their complete histocompatibility and due to its capacity to conduct, induce and generate bone. In our study the control group exhibited complete healing and this result occur because there were little space between the implant and the edge of bone that led to primary healing intention that was same as with (Shafiei *et al*.,2009) who noticed bone tissue creation 100% and healing in group of autogenous.It was showed that the radiological evaluation findings of new autogenic cortical bone were superior statistically after month as compare with new allogenic cortical bone implant.These results in agree with(Zebon,2020) who noticed a slight increase in the soft tissue density with radiolucent of the gap and the periosteal reaction at 8 weeks post-operation in the control group.EL-Keiey,2009 mentioned that the new cortical autograft, remodeling of the periosteal callus began at two month after surgery and was finished at 26 weeks (Control).In contrast, in xenobone graft of dogs, remodeling started at four month and was finished at nine month. This is congruent with the findings of (Anderson *et al*.,1995),who said that in the case of autobone graft in dogs, the periosteal callus undergo gradual remodeling at 10 weeks and was finished at 14 weeks after surgery. However, despite beneficial results in the control group,the time to achieve total healing was still slow.

In hyaluronic acid treated group,there was almost a complete healing manifested by chronic periosteal reaction.Previous study(Stern *et al*.,1992; Sasaki and Watanabe,1995; Hahn *et al*., 2004)reported that the

mechanism of the primary physicochemical features of HA, which act as a favorable substrate for osteoprogenitor cell differentiation and migration as well as protein binding are the processes by which it promotes bone formation.In addition, HA may prevent bacterial contamination of the surgical area and lessen post-operative inflammation related to bone repair.

Because the HA was promoting bone repair through chemotaxis differentiation and proliferation of Msc,HA has the ability to be osteoconductive.The present study demonstrated an inter - dependence between the experimental border defects and bone tissue that permitted bone tissue repairing to occur. HA sanatura (non-sulfated light molecular weight glycosaminoglycan) is a crucial component of extracellular matrix and donates to cell proliferation and migration, particularly from the periphery to the graft this in agree with(Goel *et al*.,2013;Abid and Mukhtar,2019) Though, in our study, bone implant play an important role as a scaffolding in repairing of bone tissue nearby the typical fixtures of bone tissue defect and helped as a link to accelerate the repairing and to attain a good clinical result (Wang *et al*.,2020). In addition HA helped as a cooperative filling biomaterial enhancing mineralization and osteogenesis of bone tissue defect rather than helping as scaffoldings (Zhai *et al*.,2020).

In 2nd treated group of hydroxyapitate Nano gel, there were progress of the healing process within the fixated bone segment appeared as a zone of new bone formations around the bone graft,complete healing with greater radiopacity. As well as, the quality of bone union and healing were significantly improved.The improved hastened mineralization of bone could be due to the role of host network blood vessels that acted as a passage easy way to migrate the osteoblastic cells to the site of

operation also the osteoconductive properties of hydroxyapatite Nano gel that worked as a filling materials (Venkatesan *et al*., 2012; Zebon *et al*., 2020). Simillar findings also noticed with (Zhou, and Lee,2011) who administration the powder of hydroxyapitate to fill radial bone fractures in human being.While(Yassine *et al*.,2017) suggested that the minimal osteogenic differentiation of Msc and osteoblasts at the deficient region, which resulted in inadequate new bone production, could be the cause of the low radiological scores in terms of decreasing the bone tissue defect. These results verified that biomaterials of calcium phosphate lack osteoinductive properties. Additionally, the few osteoblasts' restricted capacity to manufacture and mineralize a significant amount of bone matrix to fill the bone tissue defect could be the cause of the low radiographic density scores observed in the control group as well as due to the slight osteogenic differentiation (Zebon,2020) who stated that within Nano group,the radiography showed that both endosteal and periosteal reaction was increased in radiopaque area with bridging of callus between both segments of the tibia bone defect and the scaffold itself had converted into a radiopaque structure. These data proved that the Nano group had the superior healing process response.The early healing of Nano group might be due to osteoconductive properties of Nano scaffold, which enhanced the process of fracture healing(Atiyah,2018) who noticed good combination between the hydroxyapitate implant and the bone tissue defect ends that permitted good bone tissue creation to happen mostly from the peripheral to the central border of bone defect. Also these results are in agree with(Thanoon,2019)who reported that the adding of PRF and bone marrow as biomaterials on radial fracture in dogs resulted in rapid healing response comparison with control groups after eight weeks postoperatively. Also (Al Nashar and Daoud,2019)who studied and

evaluated the activity of hen eggshell graft as a natural bioactive materials within experimentally induced mandibular defects in rabbits and founded that all groups exhibited normal process of wound healing with no foreign body response.

In 4th treated group, the healing process was superior than other groups because the hydroxyapatite bonded to the bony tissue and increased the activity of osteoblast, accelerated the local growth factors. All these events improved the healing bone process.These results also reported by (Yuan *et al*., 2002) who mentioned that calcium phosphate ceramics are hopeful synthetic bone replacement materials.They showed good biocompatibility with bone due to their chemical similarities with bone mineral that accelerated bone formation.These findings also in coincides with (Baliga *et al*., 1998) who reported that powder of hen's eggshell is not an osteoinductive biomaterial,but it improved bone repairing from margins of the bone defect. Dupoirieux *et al*.,2001 have also showed that grafts of ostrich eggshell had no osteoinductive action in the thigh muscles of rats and established that long-term trainings should be performed to explain the potential role of ostrich eggshell particle's in maxillofacial procedures.

Our study showed that the deproteinized lamb bone graft satisfied exactly all desired characteristcs for bone tissue regeneration.This outcome in agreement with(Ibrahim *et al*.,2019)who used advanced scaffoldings combination from Nanoparticles ,dextran, hydroxyapatite and gelatin for bone rebuilding and repairing.They mentioned that the biological evaluation of the these scaffoldings has improved the rate of osteoblast proliferation and enhanced the function of osteoblast as established by the significant increase in concentration of ALP.

Using xenograft supported with hydroxyapatite Nano gel has been advocated successfully and superiorly in comparison with other groups. The radiological examination exhibited that the quality of bone union and the healing were significantly improved.

5.8.Gross examination

Clinically in all surgical defects sites the grafts were fitted in location, accepted and well tolerated by the animals with no severe inflammation of the neighboring soft tissue. Repairing was accomplished in all dogs with no evidence of implant rejection in every group this outcome is corroborated with (Holmes *et al*.,1986).

Bone tissue defect repair depends on osteoblasts with a suitable blood supply in the areas.The creation of greatly organized bone tissue any where needs a hard surface and mechanically constant depending on which the freshly bone tissue formation can be located (Sverzut *et al*.,2008).

There are numerous bone implant replacements involving autografts allografts, xenografts, ceramics and certain metallic implants that have been used to encourage bone tissue reconstruction (Inoue *et al*.,1997).

Our results for the site of critical sized tibial bone defect grafts revealed that the healing was well in hyaluronic acid and hydroxyapitate Nano gel groups as compare with control and xenograft groups during the 60 days post-surgery.These outcome belongs to the application of (1ml hyaluronic acid and hydroxyapitate Nano gel with high viscosity 33%) as a bioactive materials which accelerated the formation of new bone tissue. However, the results indicated that HA and hydroxyapitate Nano gel can improve the speed of the bone healing process.

In the control group the macroscopical investigation exhibited that the bone had a healthy appearance without any inflammatory reaction during sixty days post-surgery.This could be explained through the using of autograft which is considered as the golden standard in bone implanting (McLaughlin and Rousch,1998). In addition using of autobone grafts diminished the danger of infectious disease diffusion, while osteoinductive, osteoconductive and osteogenic characteristics of the implant were ideal. Furthermore, there was no immune response after implantation of autograft.All these enhanced its capability to integrate into its new area and this agree with (Pokorny *et al.*,2003). Similar findings also seen by (Santos *et al*.,2020) who reported that the macroscopical construction and regeneration was identical in control and matrix groups, but the rate of healing process was quicker in control group.They believed that this variance because of the withholding of autogenous corticocancellous implant at the area of bone defect in the control group because of regenerative properties of these tissue bone segments.They were concluded the using of DBM animals showed an initial repair of bone tissue during thirty days of grafting and bone bridge creation at sixty days post surgery.These outcome disagree with (Zebon,2020) who reported that nonunion and bone defect were seen clearly with amount of soft tissue formation. Similar finding reported with (Atiyah,2018)who mentioned that large radius bone defect in rabbits cannot be healed spontaneously until the bone defect filled with graft or a suitable bone tissue substitute.

In xenograft group, the gross appearance of the defect edges showed the presence of bone creation in the middle part of the bone tissue defect and these could be attributed to xenografts using that consider as an

alternative to autogenous grafts that has osteoconductive and biocompatible characters (Calasans-Maia *et al*.,2009).

In hyaluronic acid group there was an increase in the bone filling at the borders and middle part of the bone tissue defect. Also, formation of bone bridges was found that could be due to the application of HA which considered as bacteriostatic, anti-inflammatory, osteoconductive and osteoinductive material. As well as, hyaluronic acid decreased and delayed the development of granulation tissue and revascularization around the implants and played important role in healing process of bone defect and this agree with(Stern *et al.*,1992;Sasaki andWatanabe,1995) who reported that the HA stimulates bone formation however, it could protect the site of bone defect by reducing post-surgical inflammation and bacterial contamination associated with bone healing process .

In the present study the hydroxyapatite Nano gel group showed complete healing and the bone appeared healthy with no inflammatory reactions, besides a good incorporation between the implant and the induced bone defect at sixty days post-surgery.These outcome data may be related to the application of hydroxyapatite Nano gel of 33% high viscosity.Also, they showed good biocompatibility with bone and connective tissue with less inflammatory reaction due to their chemical similarities with bone mineral.These macroscopical findings were homogenous with other clinical and radiographical examinations and these outcome are in agree with(Shafiei-Sarvestani *et al*.,2012) who studied the application of hydroxyapatite-hPRP, and coral-hPRP on healing of bone tissue in rabbits and reported that the scores of union at grossly level were associated thoroughly with the score of the radiologic union on 56 day post – surgery.Besides, they noticed that the defect area contained various

quantities of new bone tissue that was filled with a combination of cartilage and bone in all cases.

5.9.Histopathological examination

In the current study, the results of the histopathological findings revealed a changes at the area of bone tissue defect healing of the experimental animals in all groups. However, the histopathological scores showed differences between the fourth groups in which there was increased in surface bone formation, numbers of osteoblast and osteocytes, vascularization ,mature bone ,bone trabeculae and haversian canal in hydroxyapatite Nano gel and hyaluronic acid groups at 14,30,60 days post-surgery respectively as a compare with the control and xenograft groups ,while the inflammatory reaction and granulation tissue were lesser in hydroxyapatite Nano gel and hyaluronic acid groups at 14,30, days post-surgery respectively as a compare with the control and xenograft groups and this attributed to role of bioactive materials like hydroxyapatite Nano gel and hyaluronic acid which they have osteoconductive , biocompatible and osteointegration properties as well as they act a filling materials of bone defects and cavities and play important role in acceleration of bone defect healing and improvement of bone tissue formation and this results correlated with others studies which used hydroxyapatite and hyaluronic acid in improvement of bone defect healing(Sempuku *et al*.,1996;Necas *et al*.,2008;Nguyen and Lee,2014;Li *et al*.,2016) who they suggested that this scaffolds is biocompatible and they supports cell interaction and tissue development in addition to having a great value of porosity, this bone substitutes represent a extensive surface of communications with the original tissue and accelerates migration of cells on the inside and surface the matrix and cell differentiation and proliferation.

In the current study the histopathologic investigation at the tibial bone defects sites for all four groups showed perfect healing manifestations without any inflammatory reactions or abnormalities in animals of different groups especially at sixty days post-surgery. Although they have been noticed earlier.The new bone formed was superior and earlier in the hydroxyapatite Nano gel group earlier than other groups including control, xenograft and hyaluronic acid respectively.

At the day 14 post-surgery the results showed decreased amount of bone lamellae formation in the control group that indicated a signs for delay bone formation due to the use of cortical autograft besides not adding both biomaterials (hyaluronic acid and hydroxyapatite) on the site of bone defect that agreed with(Zebon,2020)who observed that the bone formation was not complete and there was no osteogenesis in the center of defect in the control groups as compare with other treatment groups. Moreover the results of the histological finding in the xenograft group at day 14 exhibited the presence of inflammatory cells with granulation tissue due to the secretion of inflammatory cytokines like transforming growth factor beta (TGFB), fibroblast growth factor (FGF-2),vascular endothelial growth factor(VEGF) and angiopoietin(Neels *et al*., 2004). Meanwhile the giant cell were present at the site of bone defect as a kind of response to wound healing process. Giant cells considered as osteoclast like cells that both cells are derived from the same precursor cell(Anderson ,2000).The observed histopathological events could be explained through not adding the biomaterials to the site of bone defect, besides the contamination of xenograft at the site of bone defect as well as its lack of osteoconductive elements that all did not enhance bone tissue formation that was in same line as (Develioglu *et al*., 2009).One of the main problems of using xenobone graft is the incompatibility of

tissues that lead to strong and hyperacute inflammatory reaction(Oryan *et al*.,2014). In hyaluronic acid treated group,the site of bone defects showed mild connective tissue among the new bone lamellae with large quantity of new bone tissue creation at day 14 post-surgery as compared with control and xenograft groups.This indicate that application of hyaluronic acid directly on the bone operative site had improved the healing process. HA contributed in the early osteogenic events, bone morphogenesis, besides controlling the effects of numerous growth factors and cytokines(Rhodes *et al*.,2011).Within the high viscosity 33% hydroxyapatite Nano gel treated group, it was noticed the increased amount of bone formation that reflects the accelerated bone formation and effective of bioactive materials(Torricelli *et al*.,2002).The histological finding exhibited that the bone deficiencies were filled with new reconstructed bone lamellae with different amount, density and thickness with the presence of woven bone trabeculae , bone marrow and osteoblast without inflammatory reaction.The histological imaging indicated a superior and earlier bone healing quality with higher area of new bone tissue creation at the area of tibial bone tissue defect and this facilitate osteointegration and restore the normal functional(Alexander *et al*., 2011).The hydroxyapatite Nano biomaterial are widely used due to their excellent biodegradability, osteointegration and non-toxicity properties. The hydroxyapatite Nano biomaterial works on the osteoblast which is responsible for bone creating cells and osteoclast cells which are responsible for bone resorption (Kim *et al*., 2008; Xu *et al*., 2012; Dhivya *et al*., 2015; Li *et al*., 2016;Zhou *et al;* 2019 ;Jia *et al*., 2021). Calasans-Maia *et al*.,2009 also reported the histopathological finding between the bone tissue and the bioactive material and noticed a minor inflammatory reaction after seven days, which was absent in 14 days.

At day thirty post-surgery the histological investigation indicated the progressing healing process of bone defect.The histological image revealed superior healing in both hydroxyapatite Nano gel and HA groups as compare with control and xenograft group.In the control group the results exhibited the presence of woven bone formation encapsulated by connective tissues due to the excellent structural support and osteogenesis of cortical autograft that promotes to conduct, induce and generate bone tissue that make it used commonly as a standard option in comparison with others substitutes of bone implants (Brinker *et al*.,1997; Mauffrey *et al*.,2015).

In the xenograft group the histological finding showed the presence of mature connective tissues surrounding woven bone formation with inflammatory response. All these may be due to lack of living cells within xenograft and its lower osteoconductive, osteogenesis and osteoinductive properties. As well as, it was not combined with using bioactive materials that accelerates bone formation. In this study, the xenograft was weaker than the control, hyaluronic acid and hydroxyapatite groups and this agreed with (Yu *et al*.,2010) and coincides with (Easa *et al*.,2021)who concluded that the xenograft bone substitute accelerates repairing of critical sized bone defects especially when combined with platelet-rich fibrin and they mentioned that these two combinations (PRF with bone grafts) are advantageous as they reinforce the grafting material at the defective area and permits the release of growth factors that enhance the healing process of bone tissues.While (Mahyudin *et al*.,2017) reported the lack of inflammatory the reaction, self-mutilation or any problems that representing rejection of bone graft or infection of surgical area at the site of bone defect in xenograft group.

In the hyaluronic acid treated group,the site of bone defects showed mature connective tissues with mature bone lamellae formation lined with organized osteoblast.These outcomes could be due to the of application of hyaluronic acid that increased bone formation and accelerated wound healing that agreed with other studies done by(Aslan *et al*., 2006; kim *et al*.,2016; Arpağ *et al*.,2018) who found that hyaluronic acid increases bone formation and accelerates wound healing.As well as,(Aslan *et al*.,2006; Abid and Mukhtar,2019)confirmed that osteon II collagen with HA has a histpathologically superior capacity of bone healing than the others materials.

In the hydroxyapatite Nano gel treated group, the histological finding at thirty days post-surgery showed the formation of mature compact bone with development of haversian canal due to the use of hydroxyapatite Nano gel that being chemically and structurally close to the natural bone, besides its excellent biocompatibility, osteoconductive characteristcs, and resemblance to the inorganic substances of animal and human bones. It also showed no inflammatory reaction and well developed lameller bone containing a haversian canal with space of bone marrow.These results suggested a mesenchymal recruitment process of neighboring tissues and their following formation of bone tissue creating cells(Hruschka *et al*., 2017).Hydroxyapatite has a crystalline form of calcium and phosphate which is identical to the components of bone tissue substance.In vivo, it has osteoconductive and angiogenetic properties (Appleford *et al*.,2009). Furthermore, using of hydroxyapatite ceramics prevents many complications associated with autobone graft, like transmission of infectious diseases and morbidity at the harvest site because process of ultra-heating used for its training(Yassine *et al*.,2017).One of our findings is that hydroxyapatite Nano gel couldn't be recognize nor seen after its

application because of its total combination into the newly formed bone tissue (Oonishi *et al*.,1997). Our outcome data is in same line of (Stubbs *et al* .,2004) work who estimated the use of HAp in tibial bone defects of rabbit and notice that bone healing happened at the same time of our current study particularly that of 6 weeks after grafting of bioactive materials besides, repair of cortical bone could not be recognize radiologically.

The histological findings at day 60 post- surgery showed progress in healing process associated with new bone formation.These outcome was superior in hydroxyapatite Nano gel and HA groups as compare to control and xenograft alone this may be due to application of these biomaterial as a filling materials which contributed positively in enhancement and improvement of the bone defect healing process.The histological imaging in the control group exhibited formation of mature bone and bone marrow because of using of autograft that is still considered as the ‘‘gold standard’’ of bone tissue implant substance in all sides the surgery of orthopedic, bone reconstruction and nonunion. Similar finding were documented with (Bauer and Muschler ,2000; Shafiei *et al*., 2009) whom all noticed the formation of trabecular bone and bone marrow with using autograft. Sadegh *et al*.,2014 also mentioned that autograft is the best implant in its histopathological evaluation.They found that it had proper intensive thickened trabecular bone without any inflammatory reaction that made the bone defect regenerated properly at 8 week post-surgery.

In the xenograft group the histological investigations showed formation of compact bone with large numbers of distributed osteocytes and large quantities of bone marrow formation.These manifestation are largely associated with decreasing the inflammatory reaction due to using

xenograft that has biocompatable and osteocondutive properties which can accelerate bone healing process (Reynolds *et al*.,2010).These results also agree with (Nazht *et al*., 2018) who reported lamellar bone formation and an empty lacuna of the bony device with filling of haversian canal with osteocyte and blood vessels, while the control group showed thin less mineralized trabecula bone with many cavities, an empty lacuna in the bony device.Also the results of our study agreed with (EL-Keiey,2009) who reported that the fresh cortical autograft exhibited the host-graft contacts and found that cartilagenous tissue covered the proximal site, while the distal site was filled with highly vascularized cellular fibrous tissue. In contrast, the two fracture gaps in the autoclaved cortical xenograft were filled with fibrocellular tissue.

While the site of bone defect within hyaluronic acid treated group showed mature compact bone lamellae formation, well-developed of bone marrow with formation of haversian canal.These findings could be a direct result for hyaluronic acid application that played an essential role in acceleration the healing of bone tissue defect and this agreed with others recent studies(Stevens,2008;Rhodes *et al*.,2011)who mentioned that HA supports in regeneration for both bone and soft tissue, decreases development of granulation tissue, increases cell proliferation, migration and differentiation at the surgical area and finally improves organization of extracellular matrix. Furthermore, it stimulates new vascularization surrounding subcutaneous implants and when it became in close by connection with bone tissue, hyaluronic acid likewise induces bone identical to substrates of osteogenic like BMP and calcitonin Moreover, it connects crucial proteins such as, fibrin, fibronectin and fibrinogen collagen for wound healing.On the other side, HA has osteoconductive possible that enhancement regeneration of bone tissue by means of

proliferation ,chemotaxis and succeeding differentiation of Msc(Bansal *et al*.,2010).

The hydroxyl apatite Nano gel treated group histological manifestation at day sixty post-surgery revealed that bone morphology was mainly consist of large amount of formed mature compact bone with formation of haversian canal and numbers of osteocytes.This outcome is in agree with(Ahmed *et al*.,2015) who mentioned that application of both calcium hydroxyapatite/β tricalcium phosphate at 8 week post-surgery caused bridging of the fracture site with large cartilaginous areas as a outcome of the promoting role of both tricalcium phosphate/ calcium hydroxyapatite for the bone healing process. Another support for these results came from the work of(Ghosh *et al*., 2008; Chandrashekar and Saxena ,2009) who found that hydroxyapatite alone has moderate to low solubility within the body with besides its porous crystalline material properties both worked together to provides good osteoconductivity and resorbability.As well as, (Servin-Trujillo *et al*.,2011) observed a cartilaginous center within the cortical bone with the absence of inflammatory cells.

The osteogenesis in the hydroxyapatite Nano gel biomaterial has the highest lamellas density indicating that the bone growth producing factors caused osteogenesis stimulation (Xu *et al*.,2012; Zhou *et al*.,2018) this osteogenesis in the hydroxyapatite Nano gel came from acting as a scaffold in the bone tissue space, inducing reconstruction, improving cell differentiation into osteoblasts and finally scaffold attachment to the bone tissue and this agree with(Shafiei-Sarvestani *et al*.,2012)who reported that application of human platelets rich plasma with hydroxyapatite caused superior and faster bone creation than others group after two months after damage in rabbits.Simillar findings are documented with (Bigham-

Sadegh *et al*.,2020)who noticed the presence of trabecular bone in the defect at 8 weeks post-surgery in all groups besides, osteogenesis was faster when used combination of the royal jelly and hydroxyapatite compared to using hydroxyapatite alone. Huber *et al*.,2006 reported the Nanocrystalline hydroxyapatite paste effect on bone tissue defect and found the presence healing of bone tissue and ramifications of trabecular bone among the particles of implants with the presence of osteoid directly onto the replacement materials with resorption and fast osseous integration that made them conclude that the Nanocrystalline hydroxyapatite paste was safe, with osteoconductive properties that is suitable for filling cavities and bone tissue defects.

The fast mineralization of bone and acceleration in the healing process of hydroxyapatite Nano gel treated group could be due to the role of host blood vessels which transport the osteoblastic to the area of bone deficiency easily and this may be related to the osteoconductive properties of hydroxyapatite Nano gel (Venkatesan *et al*.,2012; Zebon *et al*.,2020).

Not so far from the histological findings of this experiment, the results of histochemical exhibited that the hydroxyapatite Nano gel group was rich with neutral mucopolysaccharides(NMP) reaction as compare with other groups and this outcome agree with (Torricelli *et al*.,2002) who stated that the mucopolysaccharides(NMP) increased at the area of new bone creation.Moreover,the histochemical results of the current study exhibited the existence of neutral mucopolysaccharides(NMP) in collage matrix, normally the completed designed bone matrix showed a highly response for neutral mucopolysaccharides(NMP) appears as a blue color and this means positive reaction to alcian blue stain that in turn indicate the presence of complete bone matrix formation (Rd,1976;Pearse,1985).

In this study the increased quantity of osteoid tissue in the hydroxyapatite Nano gel group represent the enhanced of remodeling, bone tissue formation and the effectiveness of this treatment. Furthermore the existence of osteoid substances enclosing great of margins of trabecular bone in all group is a signal for continuous repairing especially at day sixty post-surgery(Loveridge,1999).

Chapter Six

6.1. Conclusions

In the current study, the conclusion could be as follows:

1.The deproteinized lamb rib xenograft had ability to replace the tibial bone defect without any signs of immune rejection.

2.The lamb rib xenograft easily harvested, prepared and compatible firmly with the recipient host.

3.The using of 1% hyaluronic acid gel to reinforce the grafting process had a beneficial effect on the healing process of bone and acts as a good filling materials to seal the space between the edge of the defect and the implant.

4.The using of the 33% hydroxyapatite Nano gel high viscosity provided mechanical stability of bone implant and good biological performance and this bone substitute represented superior filling bioactive materials.

5.The biochemical, serological, histochemical ,radiological, macroscopical and histopathological investigations emphasized that the 33% hydroxyapatite Nano gel high viscosity was superior bioactive bone substitute in acceleration and improvement of bone healing process.

6.These two substances had bioconduction and bioinduction properties.

6.2. Recommendations

1- Using of deproteinized sternum xenograft of bovine as natural bone substitutes for reconstruction of others long bone defects and calvarias bone in different animals .

2- Using of deproteinized sternum xenograft of a new born lamb supported with other bone substitute as prepared egg shell or coral and used with low level laser therapy and hydroxyapatite Nano particles combination with mesenchymal stem cells, PRP.PRF or bone marrow on the improvement of the tibial bone defects augmentation and bone defects healing in a dog model.

3- Studying the effect of other growth factors like fibroblast growth factor and vascular endothelial growth factor associated with deproteinized ribs xenograft of a new born lamb supported with hydroxyapatite on accelerate bone defects healing in different animals .

4. Studying of the mixed effect of bioactive material as hyaluronic acid and the hydroxyapatite on acceleration of bone defects healing process.

5.Studying the effect of these biomaterials for longer time.

6.3. References

Abid, W. K.; and Mukhtar, Y. H. A.(2019). Repair of surgical bone defects grafted with hydroxylapatite+ β-TCP combined with hyaluronic acid and collagen membrane in rabbits:A histological study. Journal of Taibah University medical sciences.14(1):14-24.

Ahmed, A. G.;Awartani, F. A.; Niazy, A. A.; Jansen, J. A. and Alghamdi, H. S. (2020).A combination of biphasic calcium phosphate (Maxresorb®) and hyaluronic acid gel (Hyadent®) for repairing osseous defects in a rat model. Applied Sciences.10(5):1651.

Ahmed,N.; Mahmoud,T.; Shafik,S. and El Dibany, R. (2015).A comparative study between porous titanium granules and Nanocrystalline hydroxyapatite in healing of mandibular defects in dogs.Alexandria Dental Journal.40(1):126-132.

Akram, M.; Ahmed, R.; Shakir, I.; Ibrahim, W. A. W. and Hussain, R. (2014). Extracting hydroxyapatite and its precursors from natural resources.Journal of Materials Science.49(4): 1461-1475.

Al Nashar, A. and Daoud, A. (2019). Evaluation of the activity of hen eggshell graft in experimentally induced mandibular defects in rabbits: pilot study. International Journal of Research in Medical Sciences.7(4):1133-1135.

Albrektsson,T. and Johansson, C. .(2001).Osteoinduction, osteoconduction and osseointegration. Eur. Spine J.10(1):96-101.

Alexander, K.A.; Chang, M.K.; Maylin, E.R.; Kohler, T.; Muller, R.,Wu, A.C.; Van Rooijen, N,;Sweet, M.J.; Hume, D.A.; Raggatt, L.J.and Pettit, A.R.(2011).Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model. J Bone Miner Res.26(1):1517-32.

Alkhraisat, M. H.; Rueda, C.; Jerez, L. B.; Mariño, F. T.; Torres, J.; Gbureck, U. and Cabarcos, E. L. (2010). Effect of silica gel on the cohesion, properties and biological performance of brushite cement. Acta Biomaterialia.6(1):257-265.

Allen, M. J.; Hoffmann, W. E.; Richardson, D. C. and Breur, G. J. (1998). Serum markers of bone metabolism in dogs. American journal of veterinary research.59(3):250-254.

Alsousou, J.; Thompson, M.; Hulley, P.; Noble, A. and Willett, K. (2009).The biology of platelet-rich plasma and its application in trauma and orthopaedic surgery: a review of the literature.The Journal of bone and joint surgery.91(8):987-996.

Alves Cardoso, D.; Van Den Beucken, J. J. J. P.; Both, L. L. H.,; Bender, J.; Jansen, J. A. and Leeuwenburgh, S. C. G. (2014).Gelation and biocompatibility of injectable Alginate–Calcium phosphate gels for bone regeneration. Journal of Biomedical Materials Research Part A.102(3):808-817.

Andersen, J. R.;Detlie, T. and Griffiths, H. J. (1995).The radiology of bone allografts. Radiologic Clinics of North America. 33(2): 391-400.

Anderson, J. M. (2000).Multinucleated giant cells.Current opinion in hematology,7(1), 40-47.

Ansary, A.(2003).Musculoskeletal Biomechanics.Injury. 34(1): 559-559.

Appleford, M. R.; Oh, S.; Oh, N. and Ong, J. L. (2009).In vivo study on hydroxyapatite scaffolds with trabecular architecture for bone repair. Journal of Biomedical Materials Research Part A:89(4): 1019-1027.

Arias, S. A.; Blanco, J. R. T.; Doretto, J. V.; Vieira, G. L. T.; Oliveira, H. P. and Rezende, C. M. F. (2013).Modified cementless total coxofemoral prosthesis: development, implantation and clinical evaluation.Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 65(16) 1660-1672.

Arpağ, O. F.; Damlar, I.; Altan, A.; Tatli, U. and Günay, A. (2018).To what extent does hyaluronic acid affect healing of xenografts?A histomorphometric study in a rabbit model. Journal of Applied Oral Science. 26.

Aslan, M.; Şimşek, G. and Dayi, E. (2006).The effect of hyaluronic acid-supplemented bone graft in bone healing: experimental study in rabbits. Journal of biomaterials applications.20(3): 209-220.

Athanasiou, V. T.; Papachristou, D. J.; Panagopoulos, A.; Saridis, A.; Scopa, C. D. and Megas, P. (2009).Histological comparison of autograft, allograft-DBM, xenograft, and synthetic grafts in a trabecular bone defect: an experimental study in rabbits.Medical Science Monitor.16(1): 24-31.

Atiyah,A.G. (2018).Use of Eggshell Hydroxyapatite Implant in Repair of Radial Bone Defects in Rabbits.MSc thesis.University of Baghdad, Baghdad, Iraq.

Attenello, N. H. and Maas, C. S. (2015).Injectable fillers:review of material and properties.Facial Plastic Surgery.31(01):29-34.

Ayanoğlu, S.; Esenyel, C. Z.; Adanır, O.; Dedeoğlu, S.; Imren, Y. and Esen, T. (2015).Effects of hyaluronic acid (Hyalonect) on callus formation in rabbits. Acta Orthop Traumatol Turc.49(3):319-25.

Azis, Y.; Jamarun, N.; Zultiniar, Z.;Arief, S. and Nur, H.(2015). Synthesis of hydroxyapatite by hydrothermal method from cockle shell (Anadara granosa).J Chem Pharm Res.7(1):798-804.

Babis, G. C. and Soucacos, P. N. (2005).Bone scaffolds:the role of mechanical stability and instrumentation.Injury.36(4): 38-44.

Babu, S.; Sandiford, N. A. and Vrahas, M. (2015).Use of teriparatide to improve fracture healing: what is the evidence?.World Journal of Orthopedics.6(6):457-461.

Bae, E. B.; Kim, H. J.; Ahn, J. J.; Bae, H. Y.; Kim, H. J. and Huh, J. B. (2019).Comparison of bone regeneration between porcine-derived and bovine-derived xenografts in rat calvarial defects:A non-inferiority study.Materials.12(20): 3412.

Baharuddin, N. A.; Kamin, S. and Samsuddin, A. R. (2005).The Use Of Demineralized Freeze-dried Bovine Bone Xenograft In Reducing Post-surgical Periodontal Recession.Annals of Dentistry University of Malaya.12(1): 37-40.

Baker, C.E.; Moore-Lotridge, S.N.;Hysong, A.A.;Posey, S.L.; Robinette, J.P.; Blum, D.M.; Benvenuti, M.A.; Cole, H.A.; Egawa, S.; Okawa, A. and Saito, M.(2018).Bone fracture acute phase response—a unifying theory of fracture repair: clinical and scientific implications.Clinical reviews in bone and mineral metabolism.16(4):142-158.

Balasundaram, G.; Sato, M.; and Webster, T. J. (2006).Using hydroxyapatite Nanoparticles and decreased crystallinity to promote osteoblast adhesion similar to functionalizing with RGD. Biomaterials.27(14): 2798-2805.

Baldwin, P.; Li, D. J.; Auston, D. A.; Mir, H. S.; Yoon, R. S. and Koval, K. J. (2019).Autograft, allograft, and bone graft substitutes: clinical evidence and indications for use in the setting of orthopaedic trauma surgery. Journal of orthopaedic trauma.33(4):203-213.

Baliga, M.;Davies, P. and Dupoirieux, L. (1998).Powdered eggshell in the repair of cystic cavities of the jaw. Preliminary study. Revue de Stomatologie et de Chirurgie Maxillo-faciale.99(1): 86-88.

Bansal, J.; Kedige, S. D. and Anand, S. (2010).Hyaluronic acid: A promising mediator for periodontal regeneration. Indian journal of dental research.21(4): 575-578.

Barak, M. M.; Lieberman, D. E. and Hublin, J. J. (2013).Of mice, rats and men: Trabecular bone architecture in mammals scales to body mass with negative allometry. Journal of structural biology. 183(2):123-131.

Bauer Thomas, W., and Muschler George, F. (2000).Bone graft materials. Clinical Orthopaedics and Related Research.371(1):10-27.

Bayani, M.; Torabi, S.; Shahnaz, A. and Pourali, M. (2017).Main properties of Nanocrystalline hydroxyapatite as a bone graft material in treatment of periodontal defects. A review of literature. Biotechnology & Biotechnological Equipment.31(2): 215-220.

Bellido, T.; Plotkin, L.I. and Bruzzaniti, A. (2019).Bone cells. In: Burr, D.B. ,Allen, M.R.(eds)Basic and applied bone biology. 2rd edition. Cambridge .Elsevier Inc. -Academic Press. 37--55.

Bernabé, P. F. E.; Melo, L. G. N.; Cintra, L. T. A.;Gomes- Filho, J. E.; Dezan Jr, E. and Nagata, M. J. H. (2012). Bone healing in critical- size defects treated with either bone graft, membrane, or a combination of both materials: a histological and histometric study in rat tibiae.Clinical Oral Implants Research.23(3): 384-388.

Betancourt, N. T.; García-Contreras, L. and Sánchez, T. A. C. (2015). Propolis in dogs: Clinical experiences and perspectives (A Brief Review).Open Journal of Veterinary Medicine.5(01):11-17.

Beuerlein, M. J. and McKee, M. D. (2010).Calcium sulfates: what is the evidence?. Journal of orthopaedic trauma.24(1):46-51.

Bhardwaj, N. and Kundu, S. C. (2010).Electrospinning: a fascinating fiber fabrication technique. Biotechnology advances.28(3):325-347.

Bhatt, R. A. and Rozental, T. D. (2012).Bone graft substitutes.Hand clinics.28(4):457-468.

Bibbo, C. and Patel, D. V. (2006).The effect of demineralized bone matrix-calcium sulfate with vancomycin on calcaneal fracture healing and infection rates: a prospective study.Foot and ankle international.27(7):487-493.

Bigham, A. S.; Dehghani, S. N.; Shafiei, Z. and Torabi Nezhad, S. (2008).Xenogenic demineralized bone matrix and fresh autogenous cortical bone effects on experimental bone healing: radiological, histopathological and biomechanical evaluation.Journal of Orthopaedics and Traumatology.9(2):73-80.

Bigham-Sadegh, A.; Torkestani, H. S.; Sharifi, S. and Shirian, S. (2020). Effects of concurrent use of royal jelly with hydroxyapatite on bone healing in rabbit model:radiological and histopathological evaluation.Heliyon.6(7):1-6.

Blitch, E. and Ricotta, P. (1996) Introduction to bone grafting. J. Foot Ankle Surg.35(1):458–462.

Bonefeld, K. and Møller, S. (2011).Insulin- like growth factor- I and the liver. Liver International.31(7): 911-919.

Boulaiz, H.; Alvarez, P.J.; Ramirez, A.; Marchal, J.A.;Prados, J.; Rodríguez-Serrano, F.; Perán, M.; Melguizo, C. and Aranega, A.(2011).Nanomedicine: application areas and development prospects. International journal of molecular sciences.12(5): 3303-3321.

Boyce, T.; Edwards, J. and Scarborough, N. (1999). Allograft bone: the influence of processing on safety and performance.Orthopedic Clinics.30(4): 571-581.

Boyde, A.; Corsi, A.; Quarto, R.; Cancedda, R. and Bianco, P. (1999). Osteoconduction in large macroporous hydroxyapatite ceramic implants:evidence for a complementary integration and disintegration mechanism.Bone. 24(6): 579-589.

Bracey, D.N.; Seyler, T.M.; Jinnah, A.H.; Lively, M.O.; Willey, J.S.; Smith, T.L.; Van Dyke, M.E. and Whitlock, P.W.(2018).A decellularized porcine xenograft-derived bone scaffold for clinical use as a bone graft substitute: a critical evaluation of processing and structure. Journal of functional biomaterials.9(3):45.

Brannigan, K. and Griffin, M. (2016).Suppl-3, M2: An Update into the Application of Nanotechnology in Bone Healing.The Open Orthopaedics Journal.10(1): 808-823.

Brinker, W. O.; Piermattei, D. L. and Flo, G. L.(1990).Delayed union and non union.In: Handbook of small Animal Orthopedics and fractures treatment.2rd edition. Philadelphia.W.B.Saunders. 71.

Brinker, W.O.; Piermattei, D.L.and Flo, G.L. (1997).Bone grafting. In:Small animal orthopedics and fracture repair.Florida.WB Saunders Company.147–153.

Buckwalter, J. A. and Grodzinsky, A. J. (1999). Loading of healing bone, fibrous tissue, and muscle: implications for orthopaedic practice. JAAOS-Journal of the American Academy of Orthopaedic Surgeons.7(5): 291-299.

Budavari, S.; O'Neil, M. J.; Smith, A.; Heckelman, P. E., and Kinneary, J. F. (2001).The Merk Index. An Encyclopedia of Chemicals, Drugs and Biologicals. 13rd edition. white house station. Merck and Co. Inc., NJ, 1097, 1946.

Calasans-Maia, M. D.; Ascoli, F. O.; Novellino, A. T. N. A.;Rossi, A. M. and Granjeiro, J. M. (2009).Comparative histological evaluation of tibil bone repair in rabbits treated with xenografts. Acta Ortopédica Brasileira.17(1):340-343.

Calori, G. M.; Mazza, E.; Colombo, M. and Ripamonti, C. (2011).The use of bone-graft substitutes in large bone defects: any specific needs?.Injury.42(1):56-63.

Cammisa Jr, F.P.;Lowery, G.; Garfin, S.R.; Geisler, F.H.;Klara, P.M.; McGuire, R.A.; Sassard, W.R.; Stubbs, H. and Block, J.E.(2004). Two-year fusion rate equivalency between Grafton® DBM gel and autograft in posterolateral spine fusion: a prospective controlled trial employing a side-by-side comparison in the same patient. Spine.29(6):660-666.

Campana, V.; Milano, G.I.U.S.E.P.P.E.;Pagano, E.; Barba, M.; Cicione, C.;Salonna, G.;Lattanzi, W. and Logroscino, G.(2014).Bone substitutes in orthopaedic surgery: from basic science to clinical practice.Journal of Materials Science:Materials in Medicine. 25(10) : 2445-2461.

Castillo, R. F.;Ubelaker, D. H. and Djorojevic, M. (2012).Age estimation through histological study of trabecular volume and cortical bone width of the iliac crest.Journal of the forensic science society. 52(3):177-180.

Castro-Ceseña, A. B.; Sánchez-Saavedra, M. P.; Novitskaya, E. E.; Chen, P. Y.; Hirata, G. A. and McKittrick, J. (2013).Kinetic characterization of the deproteinization of trabecular and cortical bovine femur bones. Materials Science and Engineering: C, 33(8): 4958-4964.

Chakravarthi, V. P. and Balaji, N. (2010).Applications of Nanotechnology in veterinary medicine. Veterinary World.3(10): 477-480.

Chan, D. S.;Garland, J.;Infante, A.;Sanders, R. W. and Sagi, H. C. (2014).Wound complications associated with bone morphogenetic protein-2 in orthopaedic trauma surgery. Journal of Orthopaedic Trauma.28(10):599-604.

Chandrashekar, K. T. and Saxena, C. (2009).Biograft-HT® as a bone graft material in the treatment of periodontal vertical defects and its clinical and radiological evaluation:Clinical study.Journal of Indian Society of Periodontology.13(3):138-144.

Chen, Y.(2001). Orthopaedic application of gene therapy. J Orthop Sci. 6(1):199-207.

Cheng, B. H.; Chu, T. M. G.;Chang, C.; Kang, H. Y. and Huang, K. E. (2013).Testosterone delivered with a scaffold is as effective as bone morphologic protein-2 in promoting the repair of critical-size segmental defect of femoral bone in mice.PLoS One. 8(8): e70234.

Chetty, A.; Wepener, I.; Marei, M. K.; Kamary, Y. E. and Moussa, R. M. (2012). Hydroxyapatite: synthesis, properties and applications. Nov Sci Publ. 91-132.

Chiarello, E.; Cadossi, M.;Tedesco, G.; Capra, P.;Calamelli, C.; Shehu, A. and Giannini, S. (2013).Autograft, allograft and bone substitutes in reconstructive orthopedic surgery. Aging clinical and experimental research.25(1): 101-103.

Christou, C.; Oliver, R. A.; Pelletier, M. H. and Walsh, W. R. (2014). Ovine model for critical-size tibial segmental defects.Comparative medicine.64(5):377-385.

Chu, L.; Jiang, G.; Hu, X. L.; James, T. D.; He, X. P.; Li, Y. and Tang, T. (2018).Biodegradable macroporous scaffold with Nano-crystal surface microstructure for highly effective osteogenesis and vascularization. Journal of Materials Chemistry B.6(11):1658-1667.

Claes, L.; Eckert-Hübner, K. and Augat, P. (2003). The fracture gap size influences the local vascularization and tissue differentiation in callus healing. Langenbeck's archives of surgery.388(5): 316-322.

Claes, L.;Recknagel, S. and Ignatius, A. (2012).Fracture healing under healthy and inflammatory conditions. Nature Reviews Rheumatology.8(3): 133-143.

Collin- Osdoby, P.; Rothe, L., Bekker, S.; Anderson, F.; Huang, Y. and Osdoby, P. (2002).Basic fibroblast growth factor stimulates osteoclast recruitment, development, and bone pit resorption in association with angiogenesis in vivo on the chick chorioallantoic membrane and activates isolated avian osteoclast resorption in vitro. Journal of bone and mineral research.17(10): 1859-1871.

Coutu, D. L.; François, M. and Galipeau, J. (2011). Inhibition of cellular senescence by developmentally regulated FGF receptors in mesenchymal stem cells. Blood, The Journal of the American Society of Hematology.117(25): 6801-6812.

Cypher, T. J. and Grossman, J. P. (1996).Biological principles of bone graft healing. The Journal of foot and ankle surgery.35(5): 413-417.

Dallari, D.; Savarino, L.; Stagni, C.; Cenni, E.; Cenacchi, A.; Fornasari, P.M.; Albisinni, U.;Rimondi, E.; Baldini, N. and Giunti, A.(2007). Enhanced tibial osteotomy healing with use of bone grafts supplemented with platelet gel or platelet gel and bone marrow stromal cells.JBJS.

Das, B. C. (2012).Surgical Management of Unstable Diaphyseal Tibial Fracture with Conventional Dynamic Compression Plating (Dcp) in Dogs. International Research Journal of Applied Life Sciences. 1(2):51-58.

Deev, R. V.;Drobyshev, A. Y.; Bozo, I. Y. and Isaev, A. A. (2015). Ordinary and activated bone grafts: applied classification and the main features.BioMed Research International. 2015.

Develioglu, H.; Saraydin, S. U. and Kartal, U. (2009).The bone-healing effect of a xenograft in a rat calvarial defect model. Dental materials journal,28(4): 396-400.

Dhivya, S.; Saravanan, S.; Sastry, T. P. and Selvamurugan, N. (2015). Nanohydroxyapatite-reinforced chitosan composite hydrogel for bone tissue repair in vitro and in vivo.Journal of Nanobiotechnology.13(1): 1-13.

Dimitriou, R.; Jones, E.; McGonagle, D. and Giannoudis, P. V. (2011). Bone regeneration:current concepts and future directions.BMC medicine. 9(1): 1-10.

Ding, Z.; Han, H.;Fan, Z.;Lu, H.; Sang, Y.; Yao, Y.; Cheng, Q.; Lu, Q. and Kaplan, D.L.(2017). Nanoscale silk–hydroxyapatite hydrogels for injectable bone biomaterials.ACS applied materials and interfaces.9(20):16913-16921.

Dinopoulos, H.; Dimitriou, R. and Giannoudis, P. V. (2012).Bone graft substitutes: What are the options?.The Surgeon.10(4): 230-239.

Dogan, E.Z.G.İ.; Dursun, E.R.H.A.N.; Tosun, E.M.R.E.; Bilgic, E.L.İ.F.; Akman, A.C.; Orhan, K.; Celik, H.H.; Korkusuz, P.E.T.E.K. and Caglayan, F.E.R.İ.H.A.(2017).Evaluation of hyaluronic matrix efficacy in sinus augmentation: a randomized-controlled histomorphometric and micro–computed tomography analysis. International journal of oral and maxillofacial surgery.46(7): 931-937.

Dorozhkin, S. V. (2007).Bioceramics based on calcium orthophosphates. Glass and Ceramics.64(11):442-447.

Draenert, G. F. and Delius, M.(2007).The mechanically stable steam sterilization of bone grafts. Biomaterials.28(8):1531-1538.

Duan,W.,and Lopez, M. J. (2019).Bone Grafts and Bone Replacements.In: Auer, J.A., Stick, J.A.**,** Kümmerle, J.M.and Prange,T.5rd edition.Equine Surgery.Elsevier.1314-1326.

Dupoirieux, L.; Pourquier, D.; Picot, M. C. and Neves, M.(2001). Comparative study of three different membranes for guided bone regeneration of rat cranial defects. International journal of oral and maxillofacial surgery.30(1): 58-62.

Dyce, K.M.;Sack ,W.O. and Wensing, C.L.G (2010).The skeleton of the hind limb. In: Dyce, K.M.;Sack ,W.O. and Wensing, C.L.G(eds.). Textbook of Veterinary anatomy.4rd edition. St. Louis, Missour. Saunders/ Elsevier.490-500.

Easa,M.S.;Alkhawas,M.B;Gobran,H.G.;Islam,T.M.(2021). Evaluation of the Efficacy of PRF as a Bone Substitute Material Alternative to Xenograft in Critical-Sized Bone Defects in Periradicular Surgery: an Animal Study.4(3).8-17.

Ebenezer, V.; Balakrishnan, K. and Christopher, P. (2015).Comparitive Study of Immediate Implant Placment Using Autografts and Nanoparticles. Biomedical and Pharmacology Journal.8 (1):445-449.

Egol, K.A.; Nauth, A.;Lee, M.; Pape, H.C.;Watson, J.T. and Borrelli J.r. J.(2015).Bone grafting: sourcing, timing, strategies, and alternatives.Journal of orthopaedic trauma, 29(1):10-14.

Einhorn, T. A., and Gerstenfeld, L. C. (2015).Fracture healing: mechanisms and interventions.Nature Reviews Rheumatology. 11(1):45-54.

El-Keiey, M.; Gadallah, S. and Amer, M. S. (2009). Experimental studies on cortical bone xenografts: clinical and radiographical assessment.Veterinary Medical Journal Giza.57(4): 543-561.

Ercin, E., Hurmeydan, O. M., & Karahan, M. (2017).Bone Anatomy and the Biologic.Bio-orthopaedics: A New Approach.34(1):437.

Evans, H.E. and De Lahunta, A. (2013).The skeleton .In :Evans, H.E. and De Lahunta, A.(eds).Miller,s anatomy of the dog .4rd edition. Missouri.Elsevier.80-151.

Fakhari, A. and Berkland, C. (2013). Applications and emerging trends of hyaluronic acid in tissue engineering, as a dermal filler and in osteoarthritis treatment. Acta biomaterialia.9(7): 7081-7092.

Faldini, C.; Pagkrati, S.; Nanni, M.;Menachem, S. and Giannini, S.(2009).Aseptic forearm nonunions treated by plate and opposite fibular autograft strut. Clinical Orthopaedics and Related Research.467(8):2125-2134.

Finkemeier, C. G. (2002).Bone-grafting and bone-graft substitutes. Journal bone joint surgery.84(3): 454-464.

Fowlkes, J.L.;Thrailkill, K.M.; Liu, L.; Wahl, E.C.; Bunn, R.C.; Cockrell, G.E.; Perrien, D.S.;Aronson, J. and Lumpkin Jr, C.K.(2006). Effects of systemic and local administration of recombinant human IGF- I (rhIGF- I) on de novo bone formation in an aged mouse model. Journal of Bone and Mineral Research.21(9) :1359-1366.

Friedlaender, G. E. (1987).Bone grafts.The basic science rationale for clinical applications.JBJS. 69(5):786-790.

Gao, T. J.;Tuominen, T. K.;Lindholm, T. S.;Kommonen, B. and Lindholm, T. C. (1997).Morphological and biomechanical difference in healing in segmental tibial defects implanted with Biocoral® or tricalcium phosphate cylinders.Biomaterials. 18(3):219-223.

Gauthier, O.; Bouler, J. M.; Weiss, P.; Bosco, J.; Daculsi, G. and Aguado, E. (1999). Kinetic study of bone ingrowth and ceramic resorption associated with the implantation of different injectable calcium-phosphate bone substitutes.Journal of Biomedical Materials Research.47(1):28-35.

Gerstenfeld, L. C.;Cullinane, D. M.; Barnes, G. L.;Graves, D. T. and Einhorn, T. A. (2003).Fracture healing as a post- natal developmental process: molecular, spatial, and temporal aspects of its regulation. Journal of cellular biochemistry.88(5): 873-884.

Ghassemi, T.;Shahroodi, A.;Ebrahimzadeh, M. H.;Mousavian, A.; Movaffagh, J. and Moradi, A. (2018).Current concepts in scaffolding for bone tissue engineering. Archives of bone and joint surgery.6(2): 90-99.

Ghiasi, M. S.;Chen, J.;Vaziri, A.;Rodriguez, E. K. and Nazarian, A. (2017).Bone fracture healing in mechanobiological modeling: A review of principles and methods. Bone reports. 6(1):87-100.

Ghimire, S.;Miramini, S.;Edwards, G.;Rotne, R.; Xu, J.;Ebeling, P. and Zhang, L. (2021).The investigation of bone fracture healing under intramembranous and endochondral ossification.Bone Reports. 14(1):1-11.

Ghosh, S. K., Nandi, S. K., Kundu, B., Datta, S., De, D. K., Roy, S. K., and Basu, D. (2008).In vivo response of porous hydroxyapatite and β- tricalcium phosphate prepared by aqueous solution combustion method and comparison with bioglass scaffolds. Journal of Biomedical Materials Research.86(1):217-227.

Giannoudis, P. V. and Einhorn, T. A. (2009).Bone morphogenetic proteins in musculoskeletal medicine.Injury.40(1): 1-3.

Giannoudis, P. V.; Einhorn, T. A. and Marsh, D. (2007). Fracture healing: the diamond concept.Injury. 38(1): 3-6.

Ginebra, M. P.;Espanol, M., Maazouz, Y.;Bergez, V. and Pastorino, D.(2018).Bioceramics and bone healing.EFORT open reviews.3(5):173-183.

Glyde, M. and Arnett, R.(2006).Tibial fractures in the dog and cat: options for management. Irish Veterinary Journal.59(5): 290-295.

Göçmen, G.;Atalı, O.;Aktop, S.;Sipahi, A. and Gönül, O.(2016). Hyaluronic acid versus ultrasonic resorbable pin fixation for space maintenance in non-grafted sinus lifting. Journal of Oral and Maxillofacial Surgery.74(3):497-504.

Goel, S. C.;Singh, D.;Rastogi, A.;Kumaraswamy, V.;Gupta, A. and Sharma, N.(2013).Role of tricalcium phosphate implant in bridging the large osteoperiosteal gaps in rabbits. Indian J of Exp.Biol. 51(5):375-380.

Goel, S.;England, C. G.; Chen, F. and Cai, W. (2017).Positron emission tomography and Nanotechnology:A dynamic duo for cancer theranostics. Advanced drug delivery reviews.113(1):157-176.

Goldberg, S. R.;Georgiou, J.; Glogauer, M. and Grynpas, M. D.(2012). A 3D scanning confocal imaging method measures pit volume and captures the role of Rac in osteoclast function.Bone.51(1),145-152.

Goldberg, V. M., and Akhavan, S. (2005).Biology of bone grafts,in:J.R.Liberman ,G.E.Friedlaender(EDs.), Bone regeneration and repair.Springer. 57-65.

Golub, E. E. and Boesze-Battaglia, K.(2007).The role of alkaline phosphatase in mineralization.Current opinion in Orthopaedics. 18(5):444-448.

Gosain, A. K.; Song, L.; Yu, P.; Mehrara, B. J.; Maeda, C. Y.; Gold, L. I. and Longaker, M. T. (2000).Osteogenesis in cranial defects: reassessment of the concept of critical size and the expression of TGF-β isoforms. Plastic and reconstructive surgery.106(2): 360-371.

Greene, S. A. and Thurmon, J. C. (1988).Xylazine–a review of its pharmacology and use in veterinary medicine. Journal of veterinary pharmacology and therapeutics.11(4): 295-313.

Greenwald, A. S.; Boden, S. D.; Goldberg, V. M.; Khan, Y., Laurencin, C. T. and Rosier, R. N.(2001).Bone-graft substitutes: facts, fictions, and applications. Jbjs.83(2):S98-103.

Gugala, Z.;Lindsey, R. W. and Gogolewski, S. (2007).New approaches in the treatment of critical- size segmental defects in long bones. In Macromolecular symposia Weinheim:Wiley-vch verlag.253(1): 147-161.

Haben Fesseha, M. (2020). Bone grafting, its principle and application: A review.Osteol Rheumatol Open J. 1(1):43-50.

Habraken, W.;Habibovic, P.; Epple M. and Bohner, M.(2016).Calcium phosphates in biomedical applications: materials for the future? Mater Today.19(1):69:87.

Hadjidakis, D. J. and Androulakis, I. I.(2006). Bone remodeling. Ann. N. Y. Acad. Sci. 1092(1):385–96.

Hahn, S. K.; Jelacic, S.;Maier, R. V.; Stayton, P. S. and Hoffman, A. S. (2004).Anti-inflammatory drug delivery from hyaluronic acid hydrogels. Journal of Biomaterials Science, Polymer Edition. 15(9):1111-1119.

Hammer, C. R. (1994).Nature's obstacles to xenotransplantation. Transplantation Reviews.8(4): 174-184.

Handel, I. G. (2013).Statistics for veterinary and animal science.The Veterinary Record.173(23): 584.

Hasan, A.;Paul, A.;Vrana, N.E.;Zhao, X.;Memic, A.;Hwang, Y.S.; Dokmeci, M.R. and Khademhosseini, A.(2014).Microfluidic techniques for development of 3D vascularized tissue. Biomaterials, 35(26):7308-7325.

Hatakeyama, W.;Taira, M.;Takefuji, K.;Kihara, H. and Kondo, H.(2013).Bone-regeneration Trial of rat critical-size calvarial defects using Nano-apatite/collagen composites.Nano Biomedicine.5 (2):95-103.

Haugen, H. J.; Lyngstadaas, S. P.; Rossi, F. and Perale, G. (2019). Bone grafts:which is the ideal biomaterial?.Journal of Clinical Periodontology.46(1): 92-102.

Heo, S. H.; Na, C. S. and Kim, N. S. (2011). Evaluation of equine cortical bone transplantation in a canine fracture model.Veterinarni Medicina. 56(3):110-118.

Heppenstall, R. B. (1980).Bone grafting. In Hepenstall, R. B. (ed). Fracture treatment and healing. Philadelphia. W. B. Saunders Co.97.Schroeder, E. F. (1938).Intramedullary pegging of femoral fractures with beef bone. J. Am. Med. Assoc. 93:109.

Hofmann, C.; Schädel-Höpfner, M.; Berns, T.; Sitter, H. and Gotzen, L. (2003).Influence of processing and sterilization on the mechanical properties of pins made from bovine cortical bone.Der Unfallchirurg.106(6):478-482.

Hollinger, J. O.; Hart, C. E.; Hirsch, S. N.; Lynch, S. and Friedlaender, G. E. (2008).Recombinant human platelet-derived growth factor: biology and clinical applications.JBJS.90(1):48-54.

Holmes, R.E.; Buchloz, R.W., Mooney, V.(1986).Porous hydroxyapatite as abone-graft substitute in metaphyseal defects.A histometric study.JBone Joint Surg Am.68(1):904-11.

Holstein, J.H.;Karabin- Kehl, B.;Scheuer, C.; Garcia, P.;Histing, T.; Meier, C.; Benninger, E.; Menger, M.D. and Pohlemann, T.(

2013).Endostatin inhibits callus remodeling during fracture healing in mice.Journal of Orthopaedic Research.31(10):1579-1584.

Horváth, A.;Stavropoulos, A.;Windisch, P.; Lukács, L.; Gera, I. and Sculean, A. (2013).Histological evaluation of human intrabony periodontal defects treated with an unsintered Nanocrystalline hydroxyapatite paste.Clinical oral investigations.17(2):423-430.

Hruschka, V.;Tangl, S.;Ryabenkova, Y.;Heimel, P.;Barnewitz, D.; Möbus, G.; Keibl, C.;Ferguson, J.; Quadros, P.; Miller, C. and Goodchild, R.(2017).Comparison of Nanoparticular hydroxyapatite pastes of different particle content and size in a novel scapula defect model. Scientific reports.7(1): 1-11.

Hu, Y. Y.; Liu, W. and Lu, Y. P. (1991).Recombined Xenograft of Cancellous Bone and Bone Morphogenetic Protein (BMP).The Iowa Orthopaedic Journal.11(1): 54-58.

Huang, R. L.;Kobayashi, E.; Liu, K. and Li, Q.(2016).Bone Graft Prefabrication Following the.Vivo Bioreactor Principle. EBioMedicine.12(1): 43- 54.

Huber, F. X.; Belyaev, O.; Hillmeier, J.; Kock, H. J.; Huber, C.; Meeder, P. J. and Berger, I. (2006).First histological observations on the incorporation of a novel Nanocrystalline hydroxyapatite paste OSTIM® in human cancellous bone. BMC Musculoskeletal Disorders. 7(1):1-14.

Hung, N. N. (2012).Basic knowledge of bone grafting. Zorzi A. Bone Grafting. Croatia: InTech,Chapters.21(1):11-38.

Hutchinson, I. V. (2001). The immunobiology of transplant rejection and acceptance. In Transplantation Surgery. Springer.London.55-72.

Ibrahim, S.;Mahmood, S.;Abdul Razak, I. S. B.; Yusof, L. M.; Mahmood, Z. K.; Gimba, F. I. and Zakaria, M. Z. A. B. (2019). Characterization and In Vitro evaluation of a novel coated Nanocomposite porous 3D scaffold for bone repair. Iraqi Journal of Veterinary Sciences.33(1):157-173.

Igashira- Kamiyama, A.;Fukushima, A. and Konno, T. (2013). Disproportionation of achiral nickel (II) centers into two kinds of chiral nickel (II) centers caused by an achiral diimine ligand. Chemistry–A European Journal.19(49):16532-16536.

Inoue, K.; Ohgushi, H.; Yoshikawa, T.; Okumura, M.; Sempuku, T.; Tamai, S. and Dohi, Y. (1997).The effect of aging on bone formation in porous hydroxyapatite: biochemical and histological analysis. Journal of Bone and Mineral Research.12(6):989-994.

Iwamoto, T.; Terada, T.; Kogai, Y.; Okada, M.; Fujii, S. and Furuzono, T. (2012). Development of microspheres covered with hydroxyapatite Nanocrystals as cell scaffold for angiogenesis. Functional Materials Letters.5(2):614-616.

Jahagirdar, R.; and Scammell, B. E. (2009).Principles of fracture healing and disorders of bone union. Surgery (Oxford). 27(2):63-69.

Jenis, L. G., Banco, R. J., and Kwon, B. (2006). A prospective study of Autologous Growth Factors (AGF) in lumbar interbody fusion. The spine journal.6(1):14-20.

Jia, S.;Liu, Y.; Ma, Z.; Liu, C.; Chai, J.; Li, Z.; Song, W. and Hu, K. (2021).A novel vertical aligned mesoporous silica coated Nanohydroxyapatite particle as efficient dexamethasone carrier for potential application in osteogenesis.Biomedical Materials.16(3): p.035030.

Johnson, A. L. (1991).Principles of bone grafting. In Seminars in Veterinary Medicine and Surgery (Small Animal). 6(1):90-99.

Johnson, K.A. and Watson, A.D.J.(2000).Skeletal diseases. In:Ettinger, S.J., Feldman,E.C.(eds).Textbook of Veterinary Internal Medicine. Diseases of the Dog and Cat.5rd edition.Philadelphia.WB Saunders1887–1916.

Joshi, D. O.; Tank, P. H.; Mahida, H. K.; Dhami, M. A.;Vedpathak, H. S.; and Karle, A. S. (2010).Bone grafting: An overview.Veterinary World. 3(4): 198-200.

Kaito, T.; Myoui, A.; Takaoka, K.; Saito, N.; Nishikawa, M.; Tamai, N.; Ohgushi, H. and Yoshikawa, H.(2005).Potentiation of the activity of bone morphogenetic protein-2 in bone regeneration by a PLA–PEG/hydroxyapatite composite.Biomaterials. 26(1):73-79.

Kalfas, I. H. (2001).Principles of bone healing.Neurosurgical focus.*10*(4):1-4.

Kaneko, A.; Marukawa, E. and Harada, H. (2020).Hydroxyapatite Nanoparticles as injectable bone substitute material in a vertical bone augmentation model. In Vivo.34(3):1053-1061.

Kattimani, V. S.; Kondaka, S. and Lingamaneni, K. P.(2016). Hydroxyapatite–-Past, present, and future in bone regeneration. Bone and Tissue Regeneration Insights.7(1):BTRI-S36138.

Katz, J. M.; Nataraj, C.; Jaw, R.; Deigl, E. and Bursac, P. (2009). Demineralized bone matrix as an osteoinductive biomaterial and in vitro predictors of its biological potential. Journal of Biomedical Materials Research Part B: Applied Biomaterials: 89(1):127-134.

Kawai, M. and Rosen, C. J. (2010).The IGF- I regulatory system and its impact on skeletal and energy homeostasis. Journal of cellular biochemistry.111(1):14-19.

Kazem Shakouri, S.; Soleimanpour, J.; Salekzamani, Y. and Oskuie, M. R. (2010).Effect of low-level laser therapy on the fracture healing process. Lasers in medical science.25(1):73-77.

Khan, S. N.;Cammisa Jr, F. P.; Sandhu, H. S.; Diwan, A. D.; Girardi, F. P.and Lane, J. M. (2005). The biology of bone grafting. Journal of the American Academy of Orthopaedic Surgeons.13(1):77-86.

Khan, S. N.; Tomin, E. and Lane, J. M.(2000).Clinical applications of bone graft substitutes. Orthopedic Clinics.31(3):389-398.

Kim, J. J.; Song, H. Y.; Ben Amara, H.; Kyung- Rim, K. and Koo, K. T. (2016).Hyaluronic acid improves bone formation in extraction sockets with chronic pathology: a pilot study in dogs. Journal of periodontology.87(7): 790-795.

Kim, S. S.;Gwak, S. J. and Kim, B. S. (2008).Orthotopic bone formation by implantation of apatite- coated poly (lactide- co- glycolide)/hydroxyapatite composite particulates and bone morphogenetic protein- 2.Journal of Biomedical Materials Research Part A:87(1), 245-253.

Kini, U. and Nandeesh, B. N.(2012). Physiology of bone formation, remodeling, and metabolism. In:Fogelman,I., Gnanasegaran,G. and Hans V.D.W.(eds.).Radionuclide and hybrid bone imaging.Berlin: Springer berlin Heidelberg.29-57.

Komnenou, A.;Karayannopoulou, M.;Polizopoulou, Z. S.; Constantinidis, T. C. and Dessiris, A. (2005).Correlation of serum alkaline phosphatase activity with the healing process of long bone fractures in dogs.Veterinary Clinical Pathology.34(1):35-38.

Kon, E.;Muraglia, A.; Corsi, A.; Bianco, P.;Marcacci, M.;Martin, I.; Boyde, A.;Ruspantini, I.; Chistolini, P.; Rocca, M. and Giardino, R.(2000).Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical- size defects of sheep long bones.Journal of Biomedical Materials Research.49(3): 328-337.

Kumar, K. M.; Prasad, V. D.; Lakshmi, N. D. and Raju, N. K. B. (2018). Evaluation of biochemical parameters for assessment of fracture healing in dogs.Phar Innov J. 7(3):577-580.

Kumar, P. S.;Latha, C.; Rao, T. M. and Purushotham, G.(2019).Clinical study on the use of limited contact dynamic compression plate (LC-DCP) for stabilization of long bone fractures in dogs.The Pharma Innovation Journal.8(12):323-329.

Kuo, J. W., and Prestwich, G. D. (2011).Hyaluronic acid. Comprehensive Biomaterials; Ducheyne, P., Ed.; Elsevier Ltd.: Amsterdam,The Netherlands.2(1): 239-259.

Kurien, T.; Pearson, R. G. and Scammell, B. E. (2013).Bone graft substitutes currently available in orthopaedic practice: the evidence for their use.The bone and joint journal.95(5): 583-597.

Lasanianos, N. G.; Kanakaris, N. K. and Giannoudis, P. V. (2010). Current management of long bone large segmental defects. Orthopaedics and Trauma.24(2): 149-163.

Laurencin, C. T. and El-Amin, S. F. (2008).Xenotransplantation in orthopaedic surgery.Journal of the American Academy of Orthopaedic Surgeons.16(1):4-8.

Laurencin, C.; Khan, Y. and El-Amin, S. F. (2006).Bone graft substitutes. Expert review of medical devices.3(1): 49-57.

Lei, P.; Sun, R.; Wang, L.; Zhou, J.; Wan, L.; Zhou, T. and Hu, Y. (2015). A new method for xenogeneic bone graft deproteinization: comparative study of radius defects in a rabbit model. PLoS One. 10(12):0146005.

Leung, K. S.; Fung, K. P.; Sher, A. H.; Li, C. K. and Lee, K. M. (1993). Plasma bone-specific alkaline phosphatase as an indicator of osteoblastic activity. The Journal of bone and joint surgery. British volume.75(2): 288-292.

Li, H.; Zhao, Q.; Li, B.; Kang, J.; Yu, Z.; Li, Y.; Song, X.; Liang, C. and Wang, H.(2016).Fabrication and properties of carbon Nanotube-reinforced hydroxyapatite composites by a double in situ synthesis process.Carbon.101(1):159-167.

Lieberman, J. R.; Daluiski, A. and Einhorn, T. A. (2002).The role of growth factors in the repair of bone: biology and clinical applications.JBJS. 84(6):1032-1044.

Liu, M. and Lv, Y. (2018). Reconstructing bone with natural bone graft: a review of in vivo studies in bone defect animal model. Nanomaterials.8(12): 999.

Loi, F.; Córdova, L. A.;Pajarinen, J.; Lin, T. H.; Yao, Z. and Goodman, S. B. (2016). Inflammation, fracture and bone repair. *Bone*. *86*(1): 119-130.

Loveridge, N.(1999).Bone:more than a stick.J Anim Sci ;77 suppl 2:190-196.

Lucacio, O.; Gheban, D.; Soritau, O. ; Baciut, M.; Câmpian, R. S. and Baciutţ, G. (2015).Comparative assessment of bone regeneration by histometry and a histological scoring system. Revista Română de Medicină de Laborator.23(1):18-23.

Lyu, S.; Huang, C.; Yang, H. and Zhang, X. (2013). Electrospun fibers as a scaffolding platform for bone tissue repair. Journal of Orthopaedic Research.31(9):1382-1389.

Mahendra, A. M.; Ranganath, L. and Vasanth, M. S. (2007).Effect of polymethylmethacrylate in femoral fracture repair on haemato: Biochemical parameters in dogs. Indian veterinary journal.84(6): 587-589.

Mahyudin, F.; Utomo, D. N.; Suroto, H.; Martanto, T. W.; Edward, M. and Gaol, I. L. (2017). Comparative effectiveness of bone grafting using xenograft freeze-dried cortical bovine, allograft freeze-dried cortical New Zealand White rabbit, xenograft hydroxyapatite bovine, and xenograft demineralized bone matrix bovine in bone defect of femoral diaphysis of white rabbit: experimental study in vivo. International Journal of Biomaterials.2017.

Marei, H. F.; Mahmood, K. and Almas, K. (2018).Critical size defects for bone regeneration experiments in the dog mandible: a systematic review. Implant Dentistry.27(1):135-141.

Marenzana, M. and Arnett, T. R. (2013).The key role of the blood supply to bone. Bone research.1(1):203-215.

Marongiu, G.; Dolci, A.; Verona, M. and Capone, A. (2020).The biology and treatment of acute long-bones diaphyseal fractures: Overview of the current options for bone healing enhancement. Bone reports. 12(1): 1-12.

Marsell, R. and Einhorn, T. A.(2010).Emerging bone healing therapies.Journal of orthopaedic trauma.24(1):4-8.

Marwaha, R.K.; Khadgawat, R.; Tandon, N.; Kanwar, R.; Narang, A.; Sastry, A.;Bhadra, K. and Kalaivani, M. (2010).Reference intervals of serum calcium, ionized calcium, phosphate and alkaline

phosphatase in healthy Indian school children and adolescents.Clinical biochemistry.43(15):1216-1219.

Marsell, R. and Einhorn, T.A. (2011).The Biology of Fracture Healing. J. Injury.42(6):551-555.

Martinez, S. A. and Walker, T. (1999). Bone grafts. Veterinary Clinics of North America: Small Animal Practice.29(5):1207-1219.

Mauffrey, C.;Barlow, B. T. and Smith, W.(2015).Management of segmental bone defects.JAAOS-Journal of the American Academy of Orthopaedic Surgeons.23(3):143-153.

Maus, U.; Andereya, S.; Gravius, S.; Siebert, C. H.; Ohnsorge, J. A. and Niedhart, C.(2008).Lack of effect on bone healing of injectable BMP-2 augmented hyaluronic acid. Archives of orthopaedic and trauma surgery.128(12):1461-1466.

McCarthy, I. (2006).The physiology of bone blood flow: a review.The Journal of bone and joint surgery American volume.88 (3):4-9.

McGonagle, D.; English, A.and Jones, E.A.(2007).The relevance of mesenchymal stem cells in vivo for future orthopaedic strategies aimed at fracture repair.Curr Orthop .21(4):262-267.

Mckibbin, B.(1978).The biology of fracture healing in long bones, J Bone Joint Surg Br.60-B(2):150-62.

McLaughlin, R. M. and Rousch, J. K. (1998).Autogenous cancellous and corticocancellous bone grafting.Veterinary medicine.93(1):1071-1074.

Meena, N. S.;Sahni, Y. P.;Thakur, D. and Singh, R. P. (2018). Applications of Nanotechnology in veterinary therapeutics. Journal of Entomology and Zoology Study.6(2):167-175.

Mendonça, G.; Mendonça, D. B.; Aragao, F. J. and Cooper, L. F.(2008). Advancing dental implant surface technology–from micron-to Nanotopography.Biomaterials.29(28): 3822-3835.

Merli, M.; Moscatelli, M.; Mariotti, G.; Pagliaro, U.; Raffaelli, E. and Nieri, M. (2015).Comparing membranes and bone substitutes in a

one-stage procedure for horizontal bone augmentation.A double-blind randomised controlled trial. Eur J Oral Implantol. 8(3): 271-281.

Millis,D.L.andMartinez,S.A.(2003).Bonegrafts.In:Slatter,D.(ed).Textbook of small animal surgery.3rd edition .Philadelphia .Saunders, W.B. 1875-1891.

Mistry, A.S. and Mikos, A. G.(2005).Tissue engineering strategies for bone regeneration.Adv.Biochem.Eng. Biotechnol.94(1):1–2.

Moroni, A.; Pegreffi, F.; Cadossi, M.;Hoang-Kim, A.; Lio, V. and Giannini, S. (2005).Hydroxyapatite-coated external fixation pins. Expert review of medical devices.2(4):465-471.

Motomiya, M.; Ito, M.; Takahata, M.; Kadoya, K.; Irie, K.; Abumi, K. and Minami, A. (2007).Effect of hydroxyapatite porous characteristics on healing outcomes in rabbit posterolateral spinal fusion model. European Spine Journal.16(12):2215-2224.

Mukherjee, S.; Nandi, S. K.; Kundu, B.; Chanda, A. Sen, S. and Das, P. K. (2016).Enhanced bone regeneration with carbon Nanotube reinforced hydroxyapatite in animal model.Journal of the mechanical behavior of biomedical materials.60(1) 243-255.

Myers, H. M. (1991).Calcium phosphates in oral biology and medicine. Calcium Phosphate Biomaterials in Preventive and Restorative Dentistry.Farmington.CT: Karger.154-71.

Nakasaki, M.; Yoshioka, K.; Miyamoto, Y.; Sasaki, T.; Yoshikawa, H. and Itoh, K. (2008). IGF-I secreted by osteoblasts acts as a potent chemotactic factor for osteoblasts.Bone.43(5):869-879.

Nakashima, T.(2013).Regulation of bone homeostasis by bone cells. Clinical calcium.23(2):218-228.

Nandi, S. K.; Roy, S.; Mukherjee, P.; Kundu, B.;De, D. K. and Basu, D. (2010).Orthopaedic applications of bone graft & graft substitutes: a review. Indian Journal of Medical Reseach.132(1):15-30.

Nather, A.; David, V.; Teng, J. W.; Lee, C. W. and Pereira, B. P. (2010). Effect of autologous mesenchymal stem cells on biological healing

of allografts in critical-sized tibial defects simulated in adult rabbits.Annals Academy of Medicine Singapore.39(8): 599.

Nauth, A.; McKee, M. D.;Einhorn, T. A.; Watson, J. T.; Li, R. and Schemitsch, E. H. (2011).Managing bone defects. Journal of orthopaedic trauma.25(8):462-466.

Nazht, H. H.;Omar, R. A. and Ahmed, H. K. (2018).Sheep rib xenograft femoral bone implant in rabbits.Online Journal of Veterinary Research.22(4):290-299.

Necas, J. B.; Bartosikova, L.; Brauner, P. and Kolar, J. J.(2008). Hyaluronic acid (hyaluronan): a review. Veterinarni medicina. 53(8):397-411.

Neels, J. G., Thinnes, T., and Loskutoff, D. J. (2004).Angiogenesis in an in vivo model of adipose tissue development.The Faseb journal. 18(9):983-985.

Nguyen, T. B. L. and Lee, B. T. (2014).A combination of biphasic calcium phosphate scaffold with hyaluronic acid-gelatin hydrogel as a new tool for bone regeneration.Tissue Engineering Part A. 20(13-14): 1993-2004.

Nilsson, O. S.; Urist, M. R.; Dawson, E. G.; Schmalzried, T. P. and Finerman, G. A. (1986).Bone repair induced by bone morphogenetic protein in ulnar defects in dogs. The Journal of Bone and Joint Surgery.British volume.68(4): 635-642.

Noshi, T.; Yoshikawa, T.; Ikeuchi, M.; Dohi, Y.; Ohgushi, H.; Horiuchi, K.; Sugimura, M.; Ichijima, K. and Yonemasu, K.,(2000). Enhancement of the in vivo osteogenic potential of marrow/hydroxyapatite composites by bovine bone morphogenetic protein. Journal of biomedical materials research.52(4):621-630.

Oakes, D. A.; Lee, C. C. and Lieberman, J. R. (2003).An evaluation of human demineralized bone matrices in a rat femoral defect model. Clinical Orthopaedics and Related Research(1976-2007). 413(1): 281-290.

Ogose,A.;Kondo,N.;Umezu,H.;Hotta,T.;Kawashima,H.;Tokunaga,K.;Ito, T.;Kudo, N.;Hoshino,M.,Gu,W.and Endo, N.(2006).Histological assessment in grafts of highly purified beta-tricalcium phosphate (OSferion®) in human bones. Biomaterials.27(8):1542-1549.

Oonishi, H.;Kushitani, S.;Yasukawa, E.; Iwaki, H.; Hench, L.L.; Wilson, J.; Tsuji, E. and Sugihara, T.(1997).Particulate bioglass compared with hydroxyapatite as a bone graft substitute.Clinical orthopaedics and related research.(334): 316-325.

Oryan, A.;Alidadi, S.; Moshiri, A. and Maffulli, N. (2014).Bone regenerative medicine: classic options, novel strategies, and future directions. Journal of orthopaedic surgery and research.9(1):1-27.

Paderni, S.; Terzi, S. and Amendola, L. (2009).Major bone defect treatment with an osteoconductive bone substitute. Musculoskeletal Surgery.93(2):89-96.

Papathanasopoulos, A.and Giannoudis, P.V. (2008).Biological considerations of mesenchymal stem cells and endothelial progenitor cells.Injury.39(2): 21-32.

Pastides, P.;Khan, I.;Jones, M. and Theobald, P. (2012).The biomechanical role of the periosteum in fracture prevention.Injury Extra.43(10):82.

Paterson, T. E.; Halliwell, R. E.; Fields, P. J.; Louw, M. L.; Ball, G., Louw, J. and Pinckney, R. (2014). Canine generalized demodicosis treated with varying doses of a 2.5% moxidectin+ 10% imidacloprid spot-on and oral ivermectin: Parasiticidal effects and long-term treatment outcomes. Veterinary Parasitology. 205(3-4): 687-696.

Pearce, A. I.;Richards, R. G.; Milz, S.; Schneider, E. and Pearce, S. G. (2007). Animal models for implant biomaterial research in bone: a review.Eur Cell Mater.13(1):1-10.

Pearse, A. G. E. (1985). Histochemical: Theoretical and applied.vol.2,4rd edition.Edinburgh, Churchill Livingstone.1055.

Perren, S. M. (2002).Evolution of the internal fixation of long bone fractures: the scientific basis of biological internal fixation: choosing a new balance between stability and biology. The Journal of bone and joint surgery.British volume.84(8):1093-1110.

Peters, C. L.; Hines, J. L.; Bachus, K. N.; Craig, M. A. and Bloebaum, R. D. (2006).Biological effects of calcium sulfate as a bone graft substitute in ovine metaphyseal defects. Journal of Biomedical Materials Research.76(3):456-462.

Phaneendra, M. S. S. V.; Lakshmi, N. D.; Prasad, V. D. and Raju, N. K. B. (2016).Evaluation of biochemical parameters for assessment of fracture healing in dogs. Journal of Livestock Science.7(1):111-113.

Pilitsis, J. G.;Lucas, D. R. and Rengachary, S. R. (2002).Bone healing and spinal fusion.Neurosurgical focus.13(6):1-6.

Pirnazar, P.;Wolinsky, L.;Nachnani, S.;Haake, S.;Pilloni, A. and Bernard, G. W.(1999).Bacteriostatic effects of hyaluronic acid.Journal of periodontology.70(4):370-374.

Pokorny, J. J.; Davids, H. and Moneim, M. S. (2003).Vascularized bone graft for scaphoid nonunion.Techniques in Hand and Upper Extremity Surgery.7(1):32-36.

Portal Núñez, S.; Lozano, D. and Esbrit, P.(2012). Role of angiogenesis on bone formation.

Rabin, S. (2005). Immune response to implants. 2004. Available at: www.emedicine. com/orthoped/topic615.htm. Accessed March 15, 2005.

Rafiee, B.(2009).Imaging and Understanding Fracture Fixation.In: Diagnostic Imaging of Musculoskeletal Diseases. Bonakdarpour A, Reinus WR and Khurana JS, editors. Humana Press, New Jersey, USA.203-239.

Ramesh,N.;Moratti,S.C. and Dias,G.J.(2018).Hydroxyapatite polymer biocomposite for bone regeneration :A review of current tends .J.Biomed master.Res.B.Appl.Biomater.106(1):2046-2057.

Rd, L.(1976).Fullmer,H.M.Histopathologic technic and practical histochemistry.New York, McGraw-Hill.316- 318.

Recker, R. R. and Barger-Lux, J.(1992). In:Coe, F.l.,Favus, M.J. (eds).Embryology, anatomy, and microstructure of bone. Disorders of bone and mineral metabolism.New York.Raven.219-240.

Reddi, A. H.;Wientroub, S.and Muthukumaran, N.(1987).Biologic principles of bone induction.Orthop Clin North Am.18(1): 207-12.

Reichert, J.C.; Saifzadeh, S.; Wullschleger, M.E.; Epari, D.R.; Schütz, M.A.; Duda, G.N.; Schell, H.; van Griensven, M.; Redl, H. and Hutmacher, D.W.(2009).The challenge of establishing preclinical models for segmental bone defect research. Biomaterials.30(12): 2149-2163.

Reynolds,M. A.;Aichelmann-Reidy, M. E. and Branch-Mays, G. L. (2010).Regeneration of periodontal tissue: bone replacement grafts. Dental Clinics.54(1):55-71.

Rho, J. Y.;Kuhn-Spearing, L. and Zioupos, P. (1998).Mechanical properties and the hierarchical structure of bone. Med Eng Phys.20(1):92-102.

Rhodes, N. P.; Hunt, J. A.; Longinotti, C. and Pavesio, A. (2011).In vivo characterization of Hyalonect, a novel biodegradable surgical mesh. Journal of Surgical Research.168(1): 31-38.

Ridiandries, A. Tan, J. and Bursill, CA.(2018).The role of chemokines in wound healing.International journal of molecular sciences. 19(10):1-20.

Roach, H. I. (1994).Why does bone matrix contain non-collagenous proteins?The possible roles of osteocalcin, osteonectin, osteopontin and bone sialoprotein in bone mineralization and resorption.Cell Biol. Int. 18(1):617–628.

Roberts, T. T. and Rosenbaum, A. J.(2012). Bone grafts, bone substitutes and orthobiologics: the bridge between basic science and clinical advancements in fracture healing.Organogenesis.8(4):114-124.

Rucci, N.(2008). Molecular biology of bone remodelling. Clinical cases in mineral and bone metabolism.5(1): 49-56.

Sadegh, A. B.; Basiri, E.; Oryan, A. and Mirshokraei, P. (2014).Wrapped omentum with periosteum concurrent with adipose derived adult stem cells for bone tissue engineering in dog model. Cell and tissue banking.15(1):127-137.

Saito, E.; Saito, A.; Kuboki, Y.; Kimura, M.; Honma, Y.; Takahashi, T. and Kawanami, M. (2012).Periodontal repair following implantation of beta-tricalcium phosphate with different pore structures in class III furcation defects in dogs.Dental materials journal.31(4):681-688.

Salgado, A. J.; Coutinho, O. P. and Reis, R. L. (2004). Bone tissue engineering: state of the art and future trends. Macromolecular bioscience. 4(8): 743-765.

Sampson, S.;Gerhardt, M. and Mandelbaum, B.(2008).Platelet rich plasma injection grafts for musculoskeletal injuries: a review. Current reviews in musculoskeletal medicine.1(3):165-174.

Santos, F.R.D.;Minto, B.W.; Silva, S.W.G.D.; Coelho, L.D.P.; Rossignoli, P.P.; Costa Junior, J.S.;Taba Junior, M. and Dias, L.G.G.G.(2020).Caprine demineralized bone matrix (DBMc) in the repair of non-critical bone defects in rabbit tibias. A new bone xenograft. Acta cirurgica brasileira.35(8):202000801.

Santos, M. H.;Valerio, P.; Goes, A. M.; Leite, M. F.; Heneine, L. G. D. and Mansur, H. S.(2007).Biocompatibility evaluation of hydroxyapatite/collagen Nanocomposites doped with Zn+ 2. Biomedical Materials.2(2):135-141.

Saputri, D.(2018). Gambaran radiograf pada penyakit periodontal. Journal of Syiah Kuala Dentistry Society.3(1):16-21.

Saravanan, B.; Maiti, S. K.; Hoque, M.; Aithal, H. P. and Singh, G. R. (2014). Management of comminuted femoral fracture by different internal fixation techniques in dogs.Indian J Anim Sci. 72(1): 1104-1107.

Sasaki, T. and Watanabe, C. (1995).Stimulation of osteoinduction in bone wound healing by high-molecular hyaluronic acid. Bone.16(1):9-15.

Scheer, J. H. and Adolfsson, L. E.(2009).Tricalcium phosphate bone substitute in corrective osteotomy of the distal radius. Injury. 40(3): 262-267.

Schmidmaier, G.;Wildemann, B.; Gäbelein, T.; Heeger, J.; Kandziora, F.; Haas, N. and Raschke, M. (2003).Synergistic effect of IGF-I and TGF-ß1 on fracture healing in rats Single versus combined application of IGF-I and TGF-ß1. Acta Orthopaedica Scandinavica. 74(5): 604-610.

Schmidt, C.;Ignatius, A. A. and Claes, L. E. (2001).Proliferation and differentiation parameters of human osteoblasts on titanium and steel surfaces. Journal of Biomedical Materials Research.54(2): 209-215.

Seeman, E. and Martin, T.J. (2019). Antiresorptive and anabolic agents in the prevention and reversal of bone fragility.Nature Reviews Rheumatology.15(4):225-236.

Sempuku, T.;Ohgushia, H.; Okumur, M. and Tamai, S.(1996). Osteogenic potential of allogeneic rat marrow cells in porous hydroxyapatite ceramics: a histological study. Journal of orthopaedic research.14(6): 907-913.

Şenyiğit, Z. A.;Karavana, S. Y.; Eraç, B.; Gürsel, Ö.; Limoncu, M. H. and Baloğlu, E.(2014).Evaluation of chitosan based vaginal bioadhesive gel formulations for antifungal drugs.Acta Pharmaceutica.64(2):139-156.

Servin-Trujillo, M. A.; Reyes-Esparza, J. A.; Garrido-Farina, G.; Flores-Gazca, E.; Osuna-Martinez, U. and Rodriguez-Fragoso, L.(2011). The Combined Use of a Graft of Demineralized Bone Matrix along TGF-β1 Leads to an Early Bone Repair in Dogs. Journal of Veterinary Medical Science.73(9):1151-1161.

Shafiei, Z., Bigham, A. S., Dehghani, S. N., & Torabi Nezhad, S.(2009). Fresh cortical autograft versus fresh cortical allograft effects on

experimental bone healing in rabbits: radiological, histopathological and biomechanical evaluation. Cell and tissue banking.10(1):19-26.

Shafiei-Sarvestani, Z., Oryan, A., Bigham, A. S., and Meimandi-Parizi, A. (2012).The effect of hydroxyapatite-hPRP, and coral-hPRP on bone healing in rabbits: radiological, biomechanical, macroscopic and histopathologic evaluation. International Journal of Surgery. 10(2):96-101.

Shapino, F.(2008).Bone development and its relation to fracture repair.The role of mesenchymal osteoblasts and surface osteoblasts. Eur Cell Mater.15(1):53-76.

Shapiro, F.(1988).Cortical bone repair.The relationship of the lacunar-canalicular system and intercellular gap junctions to the repair process.J Bone Joint Surg Am.70(7):1067-81.

Sharifi, D.; Khoushkerdar, H. R.; Abedi, G.; Asghari, A. and Hesaraki, S. (2012).Mechanical properties of radial bone defects treated with autogenous graft covered with hydroxyapatite in rabbit.Acta cirurgica brasileira.27(3):256-259.

Sharma, U.;Pal, D. and Prasad, R. (2014).Alkaline phosphatase: an overview. Indian Journal of Clinical Biochemistry.29(3):269-278.

Sheikh,L.(2010).Synthesis and characterization of stable hydroxyapatite slurry.PhD thesis.North Orissa university.5-10.

Shenoy, R. and Pillai, A.(2017). Biology of fracture healing -an overview. Journal of Trauma and orthopaedic.5(2):48-51.

Sikavitsas, V. I.;Temenoff, J. S. and Mikos, A. G.(2001).Biomaterials and bone mechanotransduction.Biomaterials.22(19):2581-2593.

Silber, J.S.; Anderson, D.G.; Daffner, S.D.; Brislin, B.T.; Leland, J.M.; Hilibrand, A.S.; Vaccaro, A.R. and Albert, T.J.(2003).Donor site morbidity after anterior iliac crest bone harvest for single-level anterior cervical discectomy and fusion. Spine.28(2):134-139.

Sims, N. A. and Walsh, N. C.(2010).GP130 cytokines and bone remodelling in health and disease. BMB reports.43(8): 513-523.

Sinibaldi,K.R. and Roy,R.G.(1998).Bone Grafting principles and techniques.In: Bojrab, M.J.;Ellison,G.W. and Slocum,B (eds).Current technique in small animals surgery. 4rd edition.Maryland.Williams and Wilkins.901-910.

Sonoda, M.; Harwood, F.L.; Amiel, M.E.; Moriya, H.;Temple, M.; Chang, D.G.; Lottman, L.M.; Sah, R.L. and Amiel, D.(2000). The effects of hyaluronan on tissue healing after meniscus injury and repair in a rabbit model. The American journal of sports medicine. 28(1): 90-97.

Sotome, S.; Ae, K.; Okawa, A.; Ishizuki, M.; Morioka, H.;Matsumoto, S.; Nakamura, T.; Abe, S.; Beppu, Y. and Shinomiya, K.(2016). Efficacy and safety of porous hydroxyapatite/type 1 collagen composite implantation for bone regeneration: a randomized controlled study.Journal of Orthopaedic Science.21(3):373-380.

Sousa, C.P.; Dias, I.R.; Lopez-Peña, M.; Camassa, J.A.; Lourenço, P.J.; Judas, F.M.; Gomes, M.E. and Reis, R.L.(2015). Bone turnover markers for early detection of fracture healing disturbances: A review of the scientific literature. Anais da Academia Brasileira de Ciências.87(2) :1049-1061.

Spicer, P. P.; Kretlow, J. D.; Young, S.; Jansen, J. A.; Kasper, F. K. and Mikos, A. G. (2012). Evaluation of bone regeneration using the rat critical size calvarial defect. Nature protocols.7(10): 1918-1929.

Stern, M.; Schmidt, B.; Dodson, T. B.; Stern, R. and Kaban, L. B. (1992). Fetal cleft lip repair in rabbits: histology and role of hyaluronic acid. Journal of oral and maxillofacial surgery.50(3):263-268.

Stevens, M. M. (2008).Biomaterials for bone tissue engineering. Materials today.11(5):18-25.

Stubbs, D.;Deakin, M.;Chapman-Sheath, P.; Bruce, W.;Debes, J.; Gillies, R. M. and Walsh, W. R. (2004).In vivo evaluation of resorbable bone graft substitutes in a rabbit tibial defect model. Biomaterials, 25(20):5037-5044.

Su, N.; Sun, Q.; Li, C.; Lu, X.; Qi, H.; Chen, S.; Yang, J.; Du, X.; Zhao, L.; He, Q. and Jin, M.(2010). Gain-of-function mutation in FGFR3

in mice leads to decreased bone mass by affecting both osteoblastogenesis and osteoclastogenesis. Human molecular genetics. 19(7):1199-1210.

Sutherland, D. and Bostrom, M.(2005).Grafts and Bone Graft Substitutes.In: Liebermen, J.R. and Friedlaender, G.E(eds).Bone and Repair. Biology and Clinical Applications.Berlin/Heidelberg. Humana Press.133-151.

Suvarna, S.K.; Christopher, L.; Bancroft, J.D.(2013).Theory and practice of histological technique.In: 3rd edition., N.Y.. New York. Churchill Livingstone.109-121.

Suzuki,K.; Anada, T.; Miyazaki, T.; Miyatake, N.; Honda, Y.; Kishimoto, K.N.;Hosaka, M.;Imaizumi, H.; Itoi, E. and Suzuki, O.(2014). Effect of addition of hyaluronic acids on the osteoconductivity and biodegradability of synthetic octacalcium phosphate.Acta Biomaterialia.10(1):531-543.

Sverzut, C. E.; Lucas, M. A.; Sverzut, A. T.; Trivellato, A. E.; Beloti, M. M.; Rosa, A. L.; and De Oliveira, P. T. (2008).Bone repair in mandibular body osteotomy after using 2.0 miniplate system–histological and histometric analysis in dogs. International Journal of Experimental Pathology.89(2):91-97.

Taichman, R. S. (2005).Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood. 105(7):2631-2639.

Tang C.H.;Yang, R.S.; Huang, T.H., Liu, S.H.and Fu, W.M.(2004). Enhancement of fibronectin fibrillogenesis and bone formation by basic fibroblast growth factor via protein kinase c-dependent pathway in rat osteoblasts.Mol Pharmacol .66(1):440-449.

Taylor, D.;Hazenberg, J.G. and Lee, T.C.(2007).Living with cracks: damage and repair in human bone. Nature Materials.6(1):263–268..

Thanoon, M.G.(2019).A comparative study of effect of autologous implant of bone marrow and platelets rich fibrin on healing of experimental induced radial fracture in dogs.Ph.D. / Dissertation.Mosul university .Iraq.

Tietz,N.W.(2006).Clinical guide to laboratory tests.4th ed.78-73.

Tolg,C.;McCarthy,J.B.;Yazdani, A. and Turley, E. A.(2014). Hyaluronan and RHAMM in wound repair and the "cancerization" of stromal tissues.BioMed research international.2014.1-18.

Torimitsu,S.;Chiba,S.;Otsuka,K.;Kobayashi,K.;Odo,Y.;Nishida,Y.; Takano,T.;Koizumi,Y.;Makino,Y.;Yajima,D.;Hayakawa,M.;Inokuchi,G.;Motomura,A. and Iwase, H. (2014).Statistical analysis of biomechanical properties of the adult skull and age-related structural changes by sex in a Japanese forensic sample. Forensic science international.234(1):185-e1.

Torricelli,P.;Fini,M.;Giavaresi,G.;Rimondini, L. and Giardino, R. (2002). Characterization of bone defect repair in young and aged rat femur induced by xenogenic demineralized bone matrix.Journal of periodontology.73(9):1003-1009.

Tsai, C.H.; Shen, S. Y.;Zhao, J. H. and Chang, Y. C. (2009).Platelet-rich fibrin modulates cell proliferation of human periodontally related cells in vitro. Journal of Dental Sciences.4(3):130-135.

Tsai,L.C.;Hung, M. W.;Chen, Y. H.; Su, W. C.; Chang, G. G. and Chang, T. C. (2000). Expression and regulation of alkaline phosphatases in human breast cancer MCF- 7 cells. European journal of biochemistry.267(5): 1330-1339.

Tsiridis, E.;Upadhyay, N. and Giannoudis, P. (2007).Molecular aspects of fracture healing: which are the important molecules?.Injury,38(1): 11-25.

Tuğcu-Demiröz, F.; Acartürk, F. and Özkul, A. (2015).Preparation and characterization of bioadhesive controlled-release gels of cidofovir for vaginal delivery.Journal of Biomaterials Science,Polymer Edition.26(17):1237-1255.

Tunay, S.;Bilgili, H.;Yildiz, C.;Yanmiş, İ.;Solakoğlu, C. and Gür, E. (2002).The use of intraarticular hyaluronic acid on the treatment of experimental osteoarthritis: A radiological and histopathological study on a rabbit stifle joint.Turkish Journal of Veterinary and Animal Sciences.26(4):939-947.

Underwood, C.and Van Eps, A. W.(2012).Nanomedicine and veterinary science: The reality and the practicality.The Veterinary Journal. 193(1):12-23.

Üngör, C. (2012).Radiologic evaluation of putty versus powder form of demineralized bone matrix in sinus floor elevation. Journal of Oral Implantology.38(4):337-344.

Urist,M.R.(1989).Bone morphogenetic protein, bone regeneration, heterotopic ossification, and the bone-bone marrow consortium. Bone Min Res.6(1):57-112.

VanPutte,C.L.;Regan,J.L.;Russo,A.F.andRod, R. S.(2013).Reproductive System. Seeley's Anatomy and Physiology.McGraw-Hill.1016.

Venkatesan,J.; Pallela, R.; Bhatnagar, I. and Kim, S. K. (2012). Chitosan–amylopectin/hydroxyapatite and chitosan–chondroitin sulphate/hydroxyapatite composite scaffolds for bone tissue engineering.International journal of biological macromolecules. 51(5):1033-1042.

Verron, E.;Pissonnier, M.L.;Lesoeur, J.; Schnitzler,V.; Fellah, B.H.; Pascal-Moussellard, H.; Pilet, P.; Gauthier, O. and Bouler, J.M.(2014).Vertebroplasty using bisphosphonate-loaded calcium phosphate cement in a standardized vertebral body bone defect in an osteoporotic sheep model. Acta biomaterialia.10(11):4887-4895.

Walsh,W.R.;Morberg, P.; Yu, Y.; Yang, J.L.; Haggard, W.; Sheath, P.C.; Svehla, M. and Bruce, W.J.M.(2003).Response of a calcium sulfate bone graft substitute in a confined cancellous defect. Clinical Orthopaedics and Related Research.406(1):228-236.

Wan, C.;Gilbert, S.R.;Wang, Y.;Cao, X.; Shen, X.;Ramaswamy,G.; Jacobsen, K.A.;Alaql, Z.S., Eberhardt, A.W.; Gerstenfeld, L.C. and Einhorn, T.A.(2008). Activation of the hypoxia-inducible factor-1α pathway accelerates bone regeneration. Proceedings of the National Academy of Sciences.105(2): 686-691.

Wang, C.;Huang, W.;Zhou, Y.; He, L., He; Z., Chen, Z.; He, X.; Tian, S.; Liao, J.; Lu, B. and Wei, Y.(2020).3D printing of bone tissue engineering scaffolds. Bioactive materials.5(1): 82-91.

Wang,J.C.;Alanay, A.; Mark, D.; Kanim, L. E.; Campbell, P. A.;Dawson, E. G. and Lieberman, J. R. (2007). A comparison of commercially available demineralized bone matrix for spinal fusion. European Spine Journal.16(8):1233-1240.

Wang,W.and Yeung,K.W.(2017).Bone grafts and biomaterials substitutes for bone defect repair: A review. Bioactive materials.2(4): 224-247.

Wang,Y. F.;Wang, C. Y.;Wan, P.; Wang, S. G. and Wang, X. M. (2016). Comparison of bone regeneration in alveolar bone of dogs on mineralized collagen grafts with two composition ratios of Nano-hydroxyapatite and collagen.Regenerative Biomaterials.3(1):33-40.

Webster,T.J.and Ahn, E. S. (2006). Nanostructured biomaterials for tissue engineering bone.Tissue Engineering. II(1):275-308.

Weisbrode,S.E.(2007).Bone and Joint.In:McGavin, M.D. and Zachary, J.F. Pathological basis of veterinary disease.4rd edition .Missouri. Mosby Elsevier.1041-1105.

Wilczewska,A.Z.;Niemirowicz,K.;Markiewicz, K. H. and Car, H.(2012). Nanoparticles as drug delivery systems.Pharmacological reports. 64(5):1020-1037.

Williams,D.F. (2014).There is no such thing as a biocompatible material. Biomaterials.35(38):10009-10014.

Wirata, I. W.;Purbantoro, S. D.;Sudimartini, L. M.; Gunawan, I. W. F.;Dharmayudha, A. A. and Pemayun, I. G.(2018).Radiographic Evaluation of Rabbit Femur Implanted Bali Cattle Bone Graft. Jurnal Veteriner September.19(3):439-445.

Woldeamanuel, K. M.; Kurra, F. A. and Roba, Y. T.(2021).A review on Nanotechnology and its application in modern veterinary science. International Journal of Nanomaterials.Nanotechnology and Nanomedicine.7(1):026-031.

Wolfe,M.W.;Salkeld, S. L. and Cook, S. D. (1999). Bone morphogenetic proteins in the treatment of non-unions and bone defects: historical perspective and current knowledge.Univ Pa Orthop J.12(1):1-6.

Wood,K.J. and Goto,R.(2012). Mechanisms of rejection:current perspectives. Transplantation. 93(1):1-0.

Wu,S.;Liu, X.;Yeung, K. W.;Liu, C. and Yang, X.(2014).Biomimetic porous scaffolds for bone tissue engineering. Materials Science and Engineering:80(1)1-36.

Xu,Z.;Liu,C.;Wei,J.andSun,J.(2012).Effects of four types of hydroxyapatite Nanoparticles with different Nanocrystal morphologies and sizes on apoptosis in rat osteoblasts. Journal of Applied Toxicology.32(6): 429-435.

Yadegari, M.;Karimi, M.; Sheishi, P. and Derakhshan, S. (2015).A radiographic survey of eggshell powder effect on tibial bone defect repair tested in dog.Human and Veterinary Medicine.7(4):392-396.

Yakar, S.;Courtland, H. W. and Clemmons, D.(2010).IGF- 1 and bone: new discoveries from mouse models. Journal of Bone and Mineral Research.25(12): 2543-2552.

Yamasaki,N.;Hirao, M.; Nanno, K.; Sugiyasu, K.; Tamai, N.; Hashimoto, N.; Yoshikawa, H. and Myoui, A.(2009).A comparative assessment of synthetic ceramic bone substitutes with different composition and microstructure in rabbit femoral condyle model. Journal of Biomedical Materials Research .91(2):788-798.

Yassine,K.A.;Mokhtar,B.;Houari,H.;Karim, A. and Mohamed, M. (2017). Repair of segmental radial defect with autologous bone marrow aspirate and hydroxyapatite in rabbit radius:A clinical and radiographic evaluation.Veterinary World.10(7):752.

Yu, B.;Han, K.; Ma, H.;Zhang,C.;Su,J.; Zhao, J.; Li, J., Bai, Y. and Tang, H.(2009).Treatment of tibial plateau fractures with high strength injectable calcium sulphate.International orthopaedics.33(4) :1127-1133.

Yu,N.Y.;Schindeler,A.;Little,D.G. and Ruys, A. J. (2010). Biodegradable poly (α- hydroxy acid) polymer scaffolds for bone tissue engineering. Journal of Biomedical Materials Research Part B: 93(1):285-295.

Yuan, H.;Van Den Doel, M.; Li, S.;Van Blitterswijk, C. A.; De Groot, K. and De Bruijn, J. D.(2002).A comparison of the osteoinductive potential of two calcium phosphate ceramics implanted intramuscularly in goats. Journal of Materials Science: Materials in Medicine.13(12): 1271-1275.

Yun,Y.R.; Jang, J.H.;Jeon, E.;Kang, W.; Lee, S.;Won, J.E.; Kim, H.W. and Wall, I.(2012).Administration of growth factors for bone regeneration.Regenerative medicine.7(3): 369-385.

Zebon, S. H. (2020). A Comparative Study of Effectiveness of Micro and Nano composite Scaffold on Healing of Tibial Bone Defect in Dogs. PhD thesis .Iraq 80-84.

Zebon,S.H.;Eesa,M.J.andHussein,B.F.(2020).Efficacyof Nano Composite Porous 3D Scaffold of Crab Shell and Al-Kharit Histological and Radiological for Bone Repair in Vivo.The Iraqi Journal of Veterinary Medicine.44(2):15-24.

Zhai,P.;Peng,X.;Li,B.; Liu, Y.; Sun, H. and Li, X. (2020).The application of hyaluronic acid in bone regeneration. International journal of biological macromolecules.151(1):1224-1239.

Zhang,N.;Ma, L.; Liu, X.; Jiang, X.; Yu, Z.; Zhao, D.; Zhang, L.; Zhang, C. and Huang, F.(2018).In vitro and in vivo evaluation of xenogeneic bone putty with the carrier of hydrogel derived from demineralized bone matrix. Cell and Tissue Banking.19(4):591-601.

Zhou, H.; and Lee, J.(2011).Nanoscale hydroxyapatite particles for bone tissue engineering.Acta biomaterialia.7(7):2769-2781.

Zhou, P.;Wu, J.;Xia, Y.;Yuan, Y.;Zhang H.;Xu, S.;Lin K.(2018).Loading BMP-2 on Nanostructural hydroxyapatite microspheres for rapid bone regeneration.Int.J.Nanomed.13(1):4083-4092.

Zhou,M.;Geng,Y.;Yang,X.B.;J;Pathak,J.L.;Wu,G.(2019).Nanocrystalline Hydroxyapatite Based Adsorbs and Gives Sustained Release of Osteoinductive Growth Factor and Facilitates Bone Regeneration in Mice Ectopic Model.J.Nanomater.2019,1202159.

Zoidis,E.;Ghirlanda-Keller,C.and Schmid, C.(2011).Stimulation of glucose transport in osteoblastic cells by parathyroid hormone and insulin-like growth factor I.Molecular and cellular biochemistry. 348(1):33-42.

Zwingenberger,S.;Nich,C.;Valladares,R.D.;Yao,Z.,Stiehler,M.and Goodman, S. B.(2012).Recommendations and considerations for the use of biologics in orthopedic surgery.BioDrugs.26(4):245-256.

Appendix 1

BIOLABO REAGENTS
www.biolabo.fr

MANUFACTURER:
BIOLABO SA,
02160, Maizy, France

ALKALINE PHOSPHATASE

Colorimetric method

Reagent for quantitative determination of alkaline phosphatase activity [EC 3.1.3.1] in human serum and plasma

REF 80014	**R1** 2 x 56 mL **R2** 1 x 2,5 mL **R3** 1 x 27 mL **R4** 1 x 27 mL

TECHNICAL SUPPORT AND ORDERS
Tel : (33) 03 23 25 15 50
Fax : (33) 03 23 256 256

IVD IN VITRO DIAGNOSTIC USE

CLINICAL SIGNIFICANCE (1)

Alkaline phosphatase (ALP) is found in many tissues, including bone, liver, intestine, kidney, and placenta. Serum ALP measurements are of particular interest in the investigation of two groups of conditions : hepatobiliary diseases (hepatitis, cirrhosis or malignacy) and bone diseases associated with increased osteoblastic activity (child's rickets with D vitamin deficiency, Paget's disease, hyperparathyroidism in the skeleton, metastasic carcinoma).
ALP determined by usual biochemical methods reflects total serum levels and does not distinguish the source of the isoenzyme. Clinicians must thererefore rely on other parameters of liver or other organ function or a more specific determination of ALP to assess its source.

PRINCIPLE (4) (5)

Colorimetric determination of the ALP activity which reaction scheme is as follows :

Phenylphosphate —(Alkaline phosphatase)→ Phenol + Phosphate

Free phenol liberated by hydrolysis of the substrate reacts then with 4-amino-antipyrine in the presence of alkaline potassium ferricyanide to form a red-coloured complexe wich absorbance measured at 510 nm is directly proportional to the ALP activity in the specimen.
Sodium arsenate incorporated in the reagent abolishes further enzyme activity and prevents the dilution of the colour inherent in earlier methods.

REAGENTS

Vial R1 **SUBSTRATE-BUFFER**

Disodium Phenylphosphate	5	mmol/L
Carbonate-bicarbonate buffer pH 10	50	mmol/L
Stabilizer		

Vial R2 **STANDARD**

Phenol corresponding to 20 U King and Kind

Vial R3 **BLOCKING REAGENT** **(Toxic)**

4-Amino-antipyrine	60	mmol/L
Sodium arsenate	240	mmol/L

R 45 : May cause cancer.
R 23/25 : Toxic by inhalation and ingestion.
S 28 : In case of contact with skin, wash promptly with copious amount of water.
S 45 : If you feel unwell, seek medical advice (eventually, show him the label)

Vial R4 **DYE REAGENT**

Potassium ferricyanide	150	mmol/L

SAFETY CAUTIONS

BIOLABO reagents are designated for professional, in vitro diagnostic use.
- Use adequate protections (overall, gloves, glasses).
- Do not pipette by mouth.
- In case of contact with skin or eyes, thoroughly wash affected areas with plenty of water and seek medical advice.
- Reagents contain sodium azide (concentration < 0.1%) which may react with copper and lead plumbing. Flush with plenty water when disposing.
- Material Safety Data Sheet is available upon request.
- Waste disposal : Respect legislation in force in the country.

All specimens should be handled as potentially infectious, in accordance with good laboratory practices using appropriate precautions. Respect legislation in force in the country.

REAGENTS PREPARATION

Reagents are ready to use.

STABILITY AND STORAGE

Store at 2-8°C and away from light.
- Unopened :
  Reagents are stable upon expiry date stated on the label.
- Once reconstituted :
  Reagents are stable at least for 3 months when free from contamination.

SPECIMEN COLLECTION AND HANDLING (2)

Unhemolysed serum or heparinised plasma, immediately refrigerated.

ALP activity is stable in the specimen for :
- 2-3 days at 2-8°C.
- 1 month at –25°C.

INTERFERENCES (3)

Avoid hemolysed serum.
For a more comprehensive review of factors affecting this assay refer to the publication of Young D.S.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Basic medical analysis laboratory equipment.
2. Normal and pathological control sera.

CALIBRATION

- Kit Standard (vial R2)

QUALITY CONTROL

- Assayed control sera referring to the same method.
- External quality control program.

It is recommended to control in the following cases :

- At least once a run.
- At least once within 24 hours.
- When changing vial of reagent.
- After maintenance operations on the instrument.

If control is out of range, apply following actions :

1. Check the temperature and repeat the test with the same control.
2. If control is still out of range, prepare a fresh control serum and repeat the test.
3. If control is still out of range, use a new vial of Standard and repeat the test.
4. If control is still out of range, calibrate with a new vial of reagent.
5. If control is still out of range, please contact BIOLABO technical support or your local Agent.

EXPECTED VALUES (2)

	Kind and King Units /dL	IU/L (37°C)
Birth	5-15	[36-107]
Premature	(1.5 to 2 x adult value)	
1 month	10-30	[71-213]
3 years	10-20	[71-142]
10 years	15-30	[107-213]
Adults	4.5-13	[32-92]

Children : Values may be increased (up to threefold during puberty)

Each laboratory should establish its own normal ranges for the population that it serves.

LINEARITY

The assay is linear up to 40 Kind and King U (285 IU/L).

Above, reduce the volume of assay to :
20 µL and multiply the result (§ CALCUL) by 2.5.
10 µL and multiply the result (§ CALCUL) by 5.
Linearity limit depends on specimen/reagent ratio.

MANUAL PROCEDURE

Let stand reagents and specimens at room temperature.

Prepare tubes as follows :	Reagent blank	Specimen blank	Standard	Assay
Reagent R1	2 mL	2 mL	2 mL	2 mL
Incubate 5 minutes at 37°C.				
Specimen				50 µL
Reagent R2 (Standard)			50 µL	
Let stand **exactly 15 minutes at 37°C.**				
Reagent R3	0,5 mL	0,5 mL	0,5 mL	0,5 mL
Mix well.				
Reagent R4	0,5 mL	0,5 mL	0,5 mL	0,5 mL
Specimen		50 µL		
Demineralised water	50 µL			
Mix. Incubate 10 minutes at room temperature and away from light. Read absorbances of the blank specimen, standard and assay at 510 nm against reagent blank. Coloration is stable for 45 minutes away from light.				

CALCULATION

Calculate the result as follows :

$$\text{ALP activity (Kind and King units/ 100 mL)} = \frac{\text{Abs Assay - Abs Specimen blank}}{\text{Abs Standard}} \times 20$$

Notes :

1) One Kind and King unit is the quantity of enzyme which, on reaction's conditions, liberates 1 mg of phenol in 15 minutes at 37°C.

2) Result (IU/L) = 7,09 x Result (Kind and King Unit/100 mL)

REFERENCES

(1) *TIETZ N.W. Text book of clinical chemistry, 3rd Ed. C.A. Burtis, E.R. Ashwood, W.B. Saunders (1999) p. 676-684 et p.1429-1431.*
(2) *Clinical Guide to Laboratory Test, 4th Ed., N.W. TIETZ (2006) p. 78-83*
(3) *YOUNG D.S., Effect of Drugs on Clinical laboratory Tests, 4th Ed. (1995) P.3-26 à 3-35*
(4) *Kind P. R. N., King E. J, Estimation of plasma Phosphatase by determination of hydrolysed phenol with amino-anti-antipyrine, J. Clin. Path. (1954), 7, p.322-326*
(5) *Belfield A, Goldberg D. M., Revised assay for serum phenylphosphatase activity using 4-amino-antipyrine, Enzyme (1971), 12, p.561-573*

Manufacturer | Use by | IVD | Temperature limitation | REF | See insert | LOT | Store away from light | sufficient for | dilute with

Appendix 2

Dog Insulin-Like Growth Factor 1 (IGF1) ELISA Kit

Catalog Number: MBS706394

For the quantitative determination of dog insulin-like growth factor 1 (IGF1) concentrations in serum, plasma, tissue homogenates.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for IGF1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IGF1 present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for IGF1 is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IGF1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

DETECTION RANGE

31.25 ng/ml-2000 ng/ml.

SENSITIVITY

The minimum detectable dose of dog IGF1 is typically less than 7.8 ng/ml.
The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined the mean O.D value of 20 replicates of the zero standard added by their three standard deviations.

SPECIFICITY

This assay has high sensitivity and excellent specificity for detection of dog IGF1. No significant cross-reactivity or interference between dog IGF1 and analogues was observed.

Note: Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between dog IGF1 and all the analogues, therefore, cross reaction may still exist.

PRECISION

Intra-assay Precision (Precision within an assay): CV%<8%
Three samples of known concentration were tested twenty times on one plate to assess.

Inter-assay Precision (Precision between assays): CV%<10%
Three samples of known concentration were tested in twenty assays to assess.

LIMITATIONS OF THE PROCEDURE

- **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Sample Diluent and repeat the assay.
- Any variation in Sample Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Reagents	Quantity
Assay plate (12 x 8 coated Microwells)	1(96 wells)
Standard (Freeze dried)	2
Biotin-antibody (100 x concentrate)	1 x 120 µl
HRP-avidin (100 x concentrate)	1 x 120 µl
Biotin-antibody Diluent	1 x 15 ml
HRP-avidin Diluent	1 x 15 ml
Sample Diluent	1 x 50 ml
Wash Buffer (25 x concentrate)	1 x 20 ml
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml
Adhesive Strip (For 96 wells)	4
Instruction manual	1

STORAGE

<table>
<tr><td>Unopened kit</td><td colspan="2">Store at 2 - 8°C. Do not use the kit beyond the expiration date.</td></tr>
<tr><td rowspan="9">Opened kit</td><td>Coated assay plate</td><td>May be stored for up to 1 month at 2 - 8°C. Try to keep it in a sealed aluminum foil bag, and avoid the damp.</td></tr>
<tr><td>Standard</td><td rowspan="3">May be stored for up to 1 month at 2 - 8° C. If don’t make recent use, better keep it store at -20°C.</td></tr>
<tr><td>Biotin-antibody</td></tr>
<tr><td>HRP-avidin</td></tr>
<tr><td>Biotin-antibody Diluent</td><td rowspan="6">May be stored for up to 1 month at 2 - 8°C.</td></tr>
<tr><td>HRP-avidin Diluent</td></tr>
<tr><td>Sample Diluent</td></tr>
<tr><td>Wash Buffer</td></tr>
<tr><td>TMB Substrate</td></tr>
<tr><td>Stop Solution</td></tr>
</table>

***Provided this is within the expiration date of the kit.**

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- An incubator which can provide stable incubation conditions up to 37°C±0.5°C.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Absorbent paper for blotting the microtiter plate.
- 100ml and 500ml graduated cylinders.
- Deionized or distilled water.
- Pipettes and pipette tips.
- Test tubes for dilution.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

- **Serum** Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 × g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
- **Plasma** Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 × g at 2 - 8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles. Centrifuge the sample again after thawing before the assay.
- **Tissue Homogenates** 100mg tissue was rinsed with 1X PBS, homogenized in 1 ml of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g, 2 - 8°C. The supernate was removed and assayed immediately. Alternatively, aliquot and store samples at -20°C or -80°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.

Note:

1. The supplier is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Samples to be used within 5 days may be stored at 2-8°C, otherwise samples must be stored at -20°C (≤1month) or -80°C (≤2month) to avoid loss of bioactivity and contamination.
3. Grossly hemolyzed samples are not suitable for use in this assay.
4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
5. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
6. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
7. Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
8. Influenced by the factors including cell viability, cell number and also sampling time, samples from cell culture supernatant may not be detected by the kit.
9. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

REAGENT PREPARATION

Note:

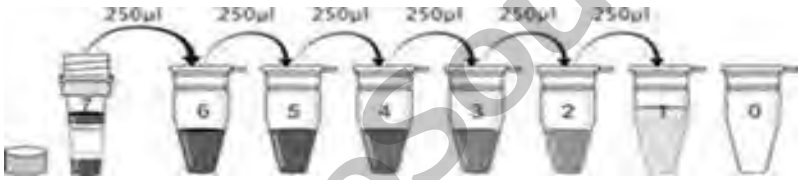
- **Kindly use graduated containers to prepare the reagent. Please don't prepare the reagent directly in the Diluent vials provided in the kit.**
- Bring all reagents to room temperature (18-25°C) before use for 30min.
- Prepare fresh standard for each assay. Use within 4 hours and discard after use.
- Making serial dilution in the wells directly is not permitted.
- Please carefully reconstitute Standards according to the instruction, and avoid foaming and mix gently until the crystals have completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μl for once pipetting.
- Distilled water is recommended to be used to make the preparation for reagents. Contaminated water or container for reagent preparation will influence the detection result.

1. **Biotin-antibody (1x) -** Centrifuge the vial before opening.
   Biotin-antibody requires a 100-fold dilution. A suggested 100-fold dilution is 10 µl of **Biotin-antibody** + 990 µl of **Biotin-antibody Diluent**.

2. **HRP-avidin (1x) -** Centrifuge the vial before opening.
   HRP-avidin requires a 100-fold dilution. A suggested 100-fold dilution is 10 µl of **HRP-avidin** + 990 µl of **HRP-avidin Diluent**.

3. **Wash Buffer(1x)-** If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 ml of Wash Buffer Concentrate (25 x) into deionized or distilled water to prepare 500 ml of Wash Buffer (1 x).

4. **Standard**
   Centrifuge the standard vial at 6000-10000rpm for 30s.
   Reconstitute the **Standard** with 1.0 ml of **Sample Diluent**. Do not substitute other diluents. This reconstitution produces a stock solution of 2000 ng/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.
   Pipette 250 µl of **Sample Diluent** into each tube (S0-S6). Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Standard serves as the high standard (2000 ng/ml). **Sample Diluent** serves as the zero standard (0 ng/ml).



Tube	S7	S6	S5	S4	S3	S2	S1	S0
ng/ml	2000	1000	500	250	125	62.5	31.25	0

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.

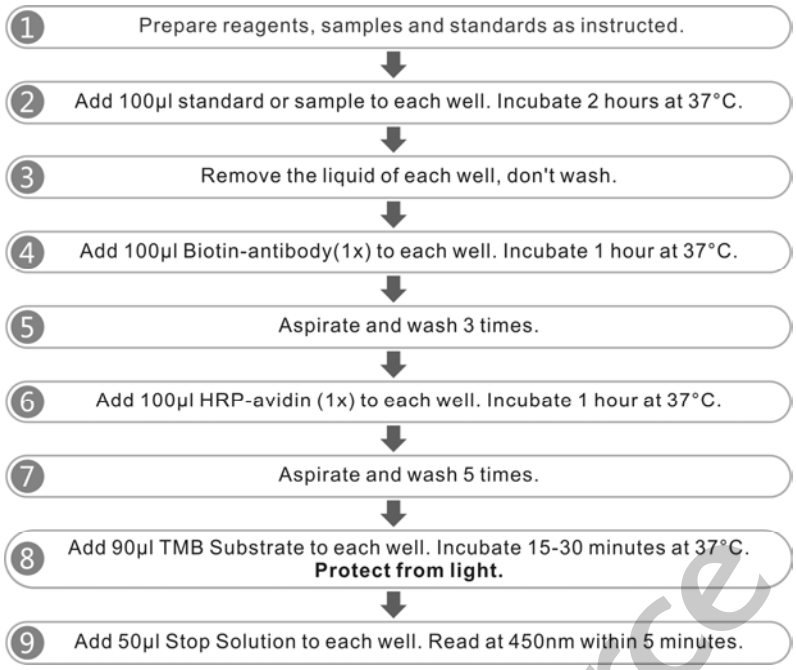
1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. Add 100µl of standard and sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at 37°C. A plate layout is provided to record standards and samples assayed.
4. Remove the liquid of each well, **don't wash.**
5. Add 100µl of **Biotin-antibody (1x)** to each well. Cover with a new adhesive strip. Incubate for 1 hour at 37°C. (**Biotin-antibody (1x)** may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.)
6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

7. Add 100µl of **HRP-avidin (1x)** to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
8. Repeat the aspiration/wash process for five times as in step 6.
9. Add 90µl of **TMB Substrate** to each well. Incubate for 15-30 minutes at 37°C. **Protect from light.**
10. Add 50µl of **Stop Solution** to each well, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Note:

1. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments.
2. Samples or reagents addition: Please use the freshly prepared Standard. Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall as possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.
4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading. When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
5. Controlling of reaction time: Observe the change of color after adding TMB Substrate (e.g. observation once every 10 minutes), TMB Substrate should change from colorless or light blue to gradations of blue. If the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. TMB Substrate is easily contaminated. TMB Substrate should remain colorless or light blue until added to the plate. Please protect it from light.
7. Stop Solution should be added to the plate in the same order as the TMB Substrate. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the TMB Substrate.

ASSAY PROCEDURE SUMMARY



***Please determine whether the sample needs to be diluted or the optimal dilution factor based on preliminary experiment result.**

CALCULATION OF RESULTS

Using the professional soft "Curve Expert" to make a standard curve is recommended, which can be downloaded from our web.

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density.
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the IGF1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.
If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Appendix 3: Hematoxylin and eosin (H&E) stain

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Solutions

Hematoxylin Solution (Harris):

Potassium or ammonium (alum……...…... 10 g

Distilled water ……..……... 500 ml

Heat to dissolve. Add 25 ml of 10% alcoholic hematoxylin solution and heat to boil for 1 minute. Remove from heat and slowly add 1.25 g of mercuric oxide (red). Heat to the solution and until it becomes dark purple color. Cool the solution in cold water bath and add 10 ml of glacial acetic acid (concentrated). Filter before use.

Eosin Y Stock Solution (1%):

Eosin Y ……... 1 g

Distilled water …… 20 ml

95% Ethanol……. 80 ml

Mix to dissolve and store at room temperature.

Eosin Y Working Solution (0.25%):

Eosin Y stock solution …… 25 ml

80% Ethanol …… 75 ml

Glacial acetic acid (concentrated) ……...0.5 ml

Mix well and store at room temperature.

1% Acid Alcohol Solution (for differentiation):

Hydrochloric acid …….. 1 ml

70% ethanol ……... 100 ml

Mix well.

Procedure:

1. Deparaffinize sections, 2 changes of xylene, 10 minutes each.
2. Re-hydrate in 2 changes of absolute alcohol, 5 minutes each.
3. 95% alcohol for 2 minutes and 70% alcohol for 2 minutes.
4. Wash briefly in distilled water.
5. Stain in Harris hematoxylin solution for 8-10 minutes.
6. Wash in running tap water for 15 minutes.
7. Differentiate in 1% acid alcohol for 30 seconds.
8. Wash running tap water for 1 minute.
9. Rinse in 95% alcohol, 10 dips.
10. Counterstain in eosin Y solution for 30 seconds to 1 minute.
11. Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each.
12. Clear in 2 changes of xylene, 5 minutes each.
13. Mount with xylene based mounting medium.

Results: nuclei ….blue

Cytoplasm ….pink

Appendix 4: Preparation of Masson's Trichrome stain:

A. Bouin's solution

Picric acid (saturated) ---------- 75 ml

Formaldehyde (37-40%) ---------- 25 ml

Glacial acetic acid ---------- 5 ml

B. Weigert's iron haematoxylin solution

Stock Solution A:

Hematoxylin ---------- 1 g
95% Alcohol ---------- 100 ml

Stock Solution B:

29% Ferric chloride in water --------- 4 ml
Distilled water ---------- 95 ml
Hydrochloric acid, concentrated ----- 1ml

Weigert's Iron Hematoxylin Working Solution:

Mix equal parts of stock solution A and B. This working solution is stable for 3 months (no good after 4 months)

C. Biebrich scarlet acid fuchsine solution:

1- Biebrich scarlet aqueous solution 1%.....90 ml
2- Acid fuchsine aqueous solution 1%.....10 ml
3- Glacial acetic acid…….……1 ml

D. Phosphotungstic –acid solution:

1- Phosphotungstic acid……..…….…… 5 gm
2- Distilled water……...……...….100 ml

E. Light green solution:

1- Light geen……..……2.5 gm
2- Glacial acetic acid…….……..…..…….……2 ml
3- Distilled water……..…..……..…….100 ml

F. 1% Glacial acetic acid:

1- Glacial acetic acid…….…..…….…...…..1 ml
2- Distilled water……..……...…….100 ml

Procedure:

1. Deparaffinize and rehydrate through 100% alcohol, 95% alcohol 70% alcohol.

2. Wash in distilled water.

3. For Formalin fixed tissue, re-fix in Bouin's solution for 1 hour at 56 C to improve staining quality although this step is not absolutely necessary.

4. Rinse running tap water for 5-10 minutes to remove the yellow color.

3. Stain in Weigert's iron hematoxylin working solution for 10 minutes.

4. Rinse in running warm tap water for 10 minutes.

5. Wash in distilled water.

6. Stain in Biebrich scarlet-acid fuchsin solution for 10-15 minutes. Solution can be saved for future use.

7. Wash in distilled water.

8. Differentiate in phosphomolybdic acid solution for 10-15 minutes or until collagen is not red.

9. Transfer sections directly (without rinse) to aniline blue solution and stain for 5-10 minutes. Rinse briefly in distilled water and differentiate in 1% acetic acid solution for 2-5 minutes.

10. Wash in distilled water.

11. Dehydrate very quickly through 95% ethyl alcohol, absolute ethyl alcohol (these step will wipe off Biebrich scarlet-acid fuchsin staining) and clear in xylene.

12. Mount with resinous mounting medium.

Results:

Collagen ---------- green

Nuclei ---------- black

Muscle, cytoplasm, keratin ---------- red

- **<u>Appendix 5: Combined Aldehyde fuchsin Alcian blue technique:</u>**
- **A. Aldehyde fuchsin solution:**
- Basic fuchsin ……..……..……..……..1 g
- 70% ethanol ……...……...…….……200 ml
- Concentrated hydrochloric acid .....……..……...…2 ml
- Paraldehyde ……...…….……...…. 2 ml
- Dissolve the basic fuchsin in the alcohol and add the paraldehyde and HCL. let stand at room temperature for 2 to 3 days, filter and refrigerate. A fresh preparation should be made every 3 to 6 months. The PH of this solution should be 1.7.
- **B. Alcian blue solution (in 3% acetic acid):**
- Alcian blue ……..…… 1g
- 3% acetic acid solution ……..…100 ml
- **Procedure:**
- 1. Deparaffinize in xylene and hydrate to 70% ethanol
- 2. Stain in Aldehyde fuchsin solution for 20 minutes
- 3. Rinse sections in 70% ethanol
- 4. Rinse briefly is running tap water
- 5. Stain in Alcian blue (pH 2.5) for 30 minutes
- 6. Rinse in running tap water for 2 minutes
- 7. Dehydrate in graded ethanol and clear with xylene
- 8. Cover slip using a miscible mounting medium
- **Results:**
- Proteoglycans and strongly acidic sulphomucins ……...…… deep purple
- weakly acidic sulphomucins …… purple
- Sialomucin and hyaluronic acid …….. Blue

الخلاصة

هدفت هذه الدراسه التحري عن دور كل من المواد الفعاله حيويا (الهلام النانوي الهايدروكسي أبتيت ذات الكثافه العاليه وهلام حامض الهايلورونك) في تحسين عمليات الالتئام عظم الرقعه الغريبه ولأعاده تشكيل عيب عظم الظنبوب في الكلاب .في هذه الدراسه الحاليه تم أستخدام 36 حيوانا من الكلاب البالغه صحيا و بمعدل أوزان وأعمارتراوحت بين (21 ± 0.3كغم), (2.1±0.9 سنه)على التوالي .قسمت حيوانات التجربه عشوائيا الى اربع مجاميع متساويه ,تسع حيوانات لكل مجموعة ,مجموعة السيطره, الرقعه الغريبه, هلام حامض الهايلورونك و الهلام النانوي الهايدروكسي أبتيت. أنجزت العمليه الجراحيه تحت التخدير العام و الظروف التعقيميه المثالية ,خضعت جميع حيوانات التجربه لعمل قطع جراحي طولي في الجلد بمقدار (3-5سم) في الجزء الداني الأنسي لعظم الظنبوب الأيمن للوصول الية وخضعت جميع حيوانات التجربه لأحداث عيب في العظم مستطيل الشكل بأبعاد قياسيه (2.5 × 0.7 سم) بواسطه المنشارالكهربائي .

في مجموعة السيطره تم أعادة غرس نفس الرقعه في العيب المحدث و تم تثبيتها بثبات بأستخدام الخيط الفولاذي التطويقي المعقم بحجم 0-2ملم وبعدها تم غلق العضلات والجلد بالطرق الروتينية .في مجموعه الرقعه الغريبه تم أجراء العمليه الجراحيه بشكل مشابه لمجموعة السيطره بأستثناء أستخدام الرقعه الغريبه بأبعاد قياسيه (2.4 × 0.6 سم) لضلع الحمل خالي البروتين بعمر 2 شهر لأصلاح عيب العظم التجريبي .بينما في المجموعة الثالثة والرابعة أستخدم هلام حامض الهايلورنك مع الهلام النانوي الهايدروكسي ابتيت ذات الكثافه العاليه كمادة مالئه لأعادة تدعيم العيب المراد اصلاحه مع أستخدام ضلع الرقعه الغريبه.

تم مراقبة جميع الحيوانات يوميا ولمدة ستون يوما لتسجيل حاله الحيوان, الظروف الصحية و العلامات السريرية .وتم أجراء الفحوصات الشعاعيه والعيانيه والنسيجية المرضيه والكيمياء النسيجيه والكيمياء الحيويه والمصليه للتحري وخلال الفترات 60,30,14,7,0 يوما بعد العمليه الجراحيه وفق المعايير المطلوبه.

وأوضحت نتائج العلامات السريريه في هذه الدراسه عدم وجود اي علامات مهمه تؤثرعلى صحه الحيوان أضافة الى أن علامات العرج الخفيف ظهرت خلال اليومين الاوليين بعد العمليه الجراحيه في مجموعتي السيطره والرقعه الغريبه ومن ثم أشتدت شدة العرج خلال سبعة ايام

بعد العمليه الجراحيه والتي كانت بنفس المستوى في كلا المجموعتين على التوالي وبعدها تلاشت علامات العرج تدريجيا خلال ستون يوما من العمليه الجراحيه مع رجوع الحيوان لحمل الوزن الطبيعي في كل المجاميع.

بينما أظهرت نتائج الفحوصات الكيمياء الحيويه والمصليه زياده أنزيم الفوسفات القلوي المصلي في اليومين السابع والرابع عشر بعد العمليه الجراحيه في كل مجموعتي حامض الهايلورنك و الهلام النانوي الهايدروكسي أبتيت علئ التوالي وبعدها قل المستوى تدريجيا لحد اليوم الستون بعد العمليه الجراحيه في كل من مجموعتي المعالجه على التوالي .

كما واظهرت النتائـج زياده عامل نمو الشبيه الانسولين في اليــوم السابع بعد العمليه الجراحيه في كل مجاميع التجربة على التوالي وفي اليوم الرابع عشر بعد العمليه الجراحيه فان مستوى عامل النمو شبيه الانسولين قل تدريجيا في كل المجاميع كما وان مستواة استمر بالتناقص والتلاشي خاصة في مجموعة حامض الهايلورونك بعد ستون يوم من العمليه الجراحيه.

اشارت نتائج الفحوصات الشعاعية ان عمليات الالتئام كانت أسرع في كل مجموعتي حامض الهايلورونك والهايدروكسي ابيتايت والتي كانت الاعلى وذلك من خلال وجود التفاعل السمحاقي المزمن وتكوين االعظم الجديد الناضج حول الغرسه المصلحه في اليوم الستون بعد العمليه الجراحيه مقارنة مع مجموعتي السيطرة والرقعة الغريبة والتي اظهرت وجود خط خفيف غمامي حول قطعه العظم الثابته .

أوضحت نتائج الفحوصات العياينة في كل من مجموعتي حامض الهايلورونك والهايدروكسي ابيتيت بأن عمليات الالتئام كانت أفضل من خلال تكوين العظم الجديد ومن دون التفاعل الالتهابي وظهور العظم بشكل صحي مع أمتلاء الفراغات بين الغرسه وحافه العيب بنسيج ضام ليفي ناضج في اليوم الثلاثون والستون بعد العمليه الجراحيه بالمقارنة مع مجموعتي السيطرة والرقعة الغريبة.

أوضحت نتائج الفحوصات النسيجيه المرضية تكوين العظم الصفائحي الصلب الناضج في مجموعة حامض الهايلورونك بينما في مجموعه الهايدروكسي ابيتايت لوحظ تكوين العظم الصلب الناضج مع تكوين قنوات هافرس وعدد من الخلايا العظمية في اليوم الستون بعد العمليه الجراحيه.في حين أوضحت نتائج الفحوصات الكيمياء النسيجيه بأن مجموعه الهلام النانووي الهايدروكسي أبيتيد كانت غنيه بفعاليه المخاط المتعدد السكريات المتعادل.

نستنتج من هذه الدراسة أن أضافة كل من حامض الهالورونك و الهلام النانوي الهايدروكسي ايبيتايت كمواد مالئه فعاله حيويا أعطت تاثيرا مفيدا في خصائص التطابق النسيجي بين المضيف والغرسه الغريبه مع تحسين عمليات التئام العظم في الكلاب .

دراسة مقارنة لتأثيرات أستخدام الرقع المغايره بالإشتراك مع جزئيات الهايدروكسي أبيتيت النانويه وحامض الهايلورونك على شفاء عيب عظم الظنبوب المحدث في الكلاب

اطروحة تقدم بها

فؤاد مؤيد محمد سعيد البارودي

الى

مجلس كلية الطب البيطري في جامعة الموصل

في إختصاص الطب البيطري/ الجراحة البيطرية

وهي جزء من متطلبات شهادة الدكتوراه فلسفة

بأشراف

الأستاذ الدكتور ليث محمود داود القطان الأستاذ الدكتورة هناء خليل اسماعيل

ربيع الثاني 1444 هـ تشرين الثاني 2022 م

جامعة الموصل

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