Histological studies on the use of bovine bone chips and composite as bone graft substitutes in reconstruction of gap defects in canine tibia.

Emara, S. A.[1]; Gadallah, S.M.[2]and Sharshar, A. M. [2]

[1]Department of Cytology & Histology and [2]Department of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Menofiya University, Sadat branch.
saademara@yahoo.com

Abstract: The aim of this work was to evaluate the ability of bovine bone chips (B.ch) and composite (B.co) in repair of artificially induced bone defects in canine tibia, and to examine the ability of bovine bone chips (B.ch) and bovine bone composite (B.co) to induce new bone formation when used to fill critical size gap defect in canine tibia. Twenty four adult apparently healthy Mongrel dogs of both sexes (weight 15-25 kg.). The dogs were used as recipient for (B.ch) and (B.co). Three defects were created in the tibia of the recipient dogs. The first was filled with (B.co), the second left empty to act as control, while the third defect filled with (B.ch). The dogs were divided into 8 groups (3 dogs each) according to the follow up periods (one, two, three, four, six, eight, twelve and sixteen weeks). At the end of each follow up period the animals were euthanized and the operated tibia were harvested and subjected to gross examination. The implantation sites were harvested and subjected to histological examination for assessment of graft incorporation. The histological results revealed the process of bone repair of the created defects.
[Emara, S. A.; Gadallah, S.M. and Sharshar, A. M. **Histological studies on the use of bovine bone chips and composite as bone graft substitutes in reconstruction of gap defects in canine tibia.** *J Am Sci* 2013;9(7):514-525]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 64

Key words: bone grafts, bovine bone chips and composite, histological studies.

1. Introduction

Clinically, treatment of fractured bone due to acute trauma have been great attention of many investigators and surgeons. In spite of presence of different methods of bone graft substitutes; the treatment of the fractured bone needs more new biological substances used for enhance the process of bone repair. Previous studies have demonstrated the viability of xenografts in bone defects of rat femur, **(Torricelli *et al.,* 1999)** with different results depending on the experimental defect used. **(Torricelli *et al.,* 2002**; **Calasans-Maia *et al.,* 2007 and Calasans-Maia *et al.,* 2009)**. Repairing large bone defects continues to be a challenge for orthopedists that use traditional autologous bone grafts because of their osteogenic properties and easier incorporation than alloplastic and xenogenic grafts.**(Finkemeier, 2002).** Gelatin has been used as binder to form composite of bone graft substitute containing deglued bone (DGB) and chitosan **(Saraswathy *et al.,* 2001)**. **Saraswathy *et al.*, (2004**), used gelatin to fill an experimentally preformed defect in dog's femur. Cortical bone grafts are used clinically in the repair of severely comminuted diaphyseal fractures when the mechanical support of cortical bone is necessary for rigid stabilization of the fracture **(Sinibaldi, 1989; Johnson *et al.,* 1996)**. Three basic mechanisms share in bone regeneration, these are osteogenesis, osteoinduction, and osteoconduction **(Lane and Sandhu, 1987)**.The sequential histological changes occurred in cortical autograft over one year in dogs verified the process of resorption, revascularization and replacement which occurred much faster in cortical autografts than allografts **(Schena *et al.,* 1985)**.The aim of this study is to evaluate clinically, and morphohistologically the role of some selected bone substitutes; bovine bone chips (B.ch) and composite (B.co) in repair of experimentally induced bone defects in canine tibia.

2-Material and Methods

Twenty four adult, apparently healthy, Mongrel dogs of both sexes weighing 15 to 25 kg. were used as recipient for bone graft substitutes. The animals were randomly divided into eight groups; (3 dogs each) according to the follow up periods of 16 weeks. The follow up was occurred at the end of 1,2,3,4,6,8,12, and 16 weeks from the operation.

2.1-Preparation of (B.co) and (B.ch):-

Bovine bone composite (B.co):- (bovine bone chips were powdered alone and added to chitosan (**chitosan®: Yaizu, Suisankagku Co., Japan**) and gelatin (**Gelatin powder®: ADWIC, El Nasr),**Then packed in double wrapped plastic roll and sterilized.

Bovine bone chips (B.ch): (cortical bone of Bovine long bones (tibia and radius) was cut into small pieces to form bone chips and dried for 12 to 24 hours, and packed in plastic roll and sterilized. All these materials were prepared and sterilized according to **Arnaud *et al.,* (1999)** and **Saraswathy *et al.*, (2004)**.

2.2-Anaesthesia and Preoperative technique:

All dogs were pre-medicated with I/V injection of mixture of **atropine sulfate 0.05 mg/kg**

(Atropine sulfate ®: 1mg/ml Med. Co., A.R.E.) and **diazepam 1 mg/kg (Neuril ®: 0.5% sol. Memphis Co. for Pharm. &Animal Ind. Cairo A.R.E.).** Anaesthesia was induced immediately through I/V injection of a mixture of **Ketamin 10 mg/kg (Ketalar®: 5% sol. Amoun Co. A.R.E.) and Xylazine 1 mg/kg (Xylaject®: 2% sol. ADWIA Co., A.R.E.).** The anaesthetic depth was maintained with **2.5 % thiopental sodium (Thiopental®: EPICO Co., A.R.E.)** administrated by I/V rout (**Schmidt-Oechtering & Alef, 1995**).The lower region of the hind limb (tibia) was prepared for aseptic surgery followed by routine orthopedic operative draping and gowning procedures. Aprophylactic course of Cefotaxim sodium (***Cefotax®: EPICO, A.R.E***) at dose of 4.5 mg/kg b.w. intravenously every 8 hours was administered for five successive days preoperatively and continued every 12 hours for 3 successive days post-operatively.

2.3-Surgical procedure:

A 10 cm skin incision was made at the medial surface of the right tibia. The incision extended through the periosteum, which was reflected to expose the bone (MacNeill ***et al.,*** 1999). Three 10 mm diameter holes and 1cm apart were created at the proximal third using a sterile 10 mm Ø drill bit under continuous sterile saline irrigation. Each defect extended through only one cortex. The drilled holes were packed using sterile gauze to control hemorrhage from the medullary cavity. The first hole was packed with bone composite (B. co), while, the second one was left empty to serve as negative control. The third hole was packed with bovine bone chips (B.ch) (Figs. a&b). The implantation sites were flushed with sterile saline solution. The surgical wound was closed using polyglactin 910 (Vicryl®) for close approximation of the adjacent muscles to the implanted bone substitutes. The subcutaneous tissue was sutured with Vicryl No. 0 in simple continuous manner and the skin incision was closed as usual.

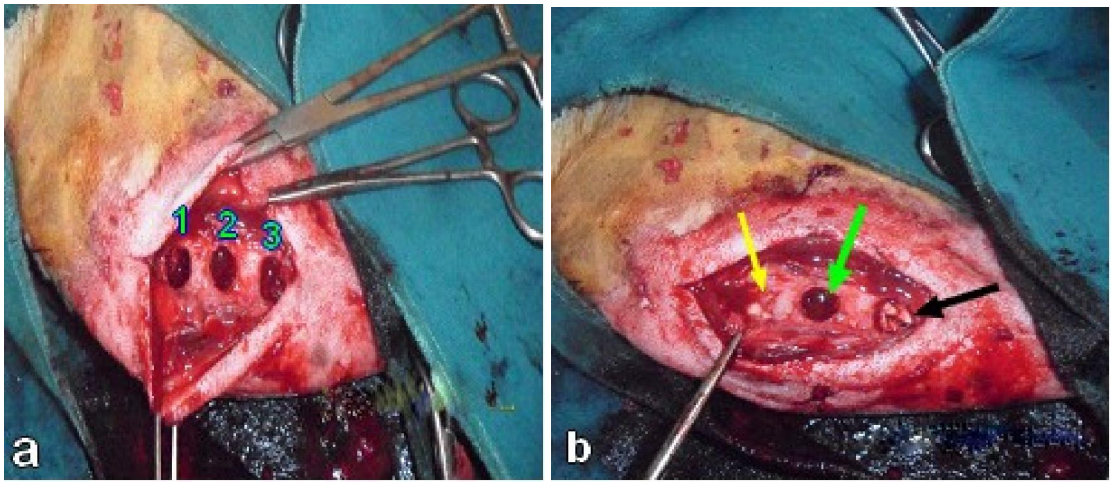


Fig. a: Three induced defects (1,2,3) in the proximal third of the tibia..
Fig. **b:** The defects after filling with the graft materials: first was filled with bone composite (yellow arrow), the second was left empty as control (green arrow) while, the third one was filled with bone chips (black arrow).

2.4-Morphological and Histological studies:

Euthanasia was performed at the end of each (1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 6^{th}, 8^{th}, 12^{th}, 16^{th} week). The operated tibia were harvested for morphological evaluation. Then, small blocks of bone, each one containing the hole defect surrounded with the host bone were taken directly after euthanasia, then immediately fixed in 10% neutral formalin for one week. The samples were decalcified using 10% EDTA di-sodium solution. P.93®: El-Nasr pharmaceutical chemical, Egypt) for one month. **(Shibata *et al.,* 2000)**. Then washed in running tap water for 24 hours, dehydrated in ethyl alcohol,cleared in xylene and embedded in soft paraffin then blocked in hard paraffin wax. Sections of 5-7 µm thick were cut and stained with **Harri's haematoxylin and eosin (Bancroft *et al.,* 1996).** Photomicrographs were taken using binocular digital microscope **(Leica DMLB ®)** with 3.2 mega pixels digital camera (Leica EC3) and program software (**LASEZ. 1.6**).

3-Results

The results of morphological and histological studies were illustrated in tables (1-2-3-4-5-6-7 and 8), moreover figures in the related plates showing the photomicrographs at one week, two weeks, three weeks, four weeks, six weeks, eight weeks, twelve weeks and sixteen weeks observation period.

3.1-Morphological results:

-At one week observation period: the control "empty" bone grafts showed empty tibial gap defects. B.ch gap defects showed easily recognized implanted materials. while, B.co gap defects showed partial resorption of implanted materials. (Plate 1, Fig. a).

-At two weeks observation period: the control gap defects still appeared empty. B.ch gap defects showed easily recognized implanted materials. while, B.co gap defects showed complete resorption of the implanted material. (Plate 1, Fig. b).

-At three weeks observation period: the control gap defects still appeared empty. B.ch gap defects showed easily recognized implanted materials. while, The holes of B.co gap defects were completely filled with granulation tissue.

-At four weeks observation period: the all gap defects of control, B.ch and B.co: showed periosteal reaction at the implantation site manifested by callus formation (Plate 1, Fig. c).

-At six weeks observation period: The periosteal callus was abundant than that of the previous stage in all gap defects of control, B.ch and B.co (Plate 2, Fig.a).

-At eight weeks observation period: All the induced holes of control, B.ch and B.co were nearly disappeared. Signs of remodeling could be detected at this period manifested by beginning of callus resorption. (Plate 2,Fig.b).

-At twelve weeks observation period: All holes of control, B.ch and B.co were completely disappeared**.** Remodeling of the periosteal callus was nearly completed (Plate 2, Fig. c).

-At sixteen weeks observation period: Remodeling was completed in all defects and the operated tibia returned to the normal anatomical features as compared with the tibia of the contra-lateral limb (Plate 2, Fig. d).

3.2-Histological results:

-At one week observation period: the control gap defects showed dilated blood vessels and extravasated red blood cells. Fibroblasts were detected lined the bone marrow cavity. (Plate 3,Fig.a). B.ch gap defects showed abundant and dens amount of B.ch particles at the implantation site, with osteoclastic activities (Plate 3,Figs.b&c), also B.co gap defects showed abundant and dense amount of B.co particles at the implantation site, with osteoclastic activities (Plate 3, Figs. d & e).

-At two weeks observation period: the control gap defects showed dense layer of fibrocellular tissue filled the bony spaces. It marked by large number of fibroblasts arranged in an epithelioid manner (Plate 4,Fig.a). B.ch gap defects showed more dense fibrocellular tissue enclosing B.ch particles in between, with numerous osteoclastic activities. (Plate 4,Fig.b). while, B.co gap defects showed newly formed fibrocellular tissue that was thinner than that of the B.ch defects. Thick trabeculae of woven bone were appeared in between the fibrocellular tissues. (Plate 4,Fig.c).

-At three weeks observation period: the control gap defects showed thin layer of woven bone trabeculae enclosing fibrocellular tissues in between. (Plate 5,Fig.a). B.ch gap defects showed newly formed thin woven bone trabeculae at the surface of B.ch particles. It encloses fibrocellular tissue in between. (Plate 5,Fig.b), while B.co gap defects appeared as that of the previous period beside; localized areas of osteoclastic activities at the surface of the newly formed woven bone (Plate 5,Figs.c&d).

-At four weeks observation period: The control gap defects appeared as that of the previous period except, the newly formed woven bone trabeculae were thicker (Plate 6,Fig.a). B.ch gap defects showed denser amount of newly formed woven bone than that of the previous period. Localized areas of osteoclastic activities at the surface of the woven bone indicating resorption of the woven bone (Plate 6,Fig.b). B.co gap defects showed denser and thicker woven bone trabeculae and fibrocellular tissue than that of the previous period (Plate 6, Fig.c).

-At six weeks observation period: The control gap defects appeared as that of the previous period, with localized areas of osteoclastic activities at the surface of the woven bone trabeculae and several resorption spaces (Plate 7, Fig.a)**.** B.ch gap defects showed denser woven bone trabeculae more than the previous period with localized areas of osteoclastic activities at the surface of the woven bone trabeculae and several resorption spaces (Plate 7, Figs.b&c)**.** B.co gap defects showed localized areas of osteoclastic activities at the surface of the woven bone accompanied by formation of bone matrix (collagen fibers) secreted by newly formed osteocytes at the surface of the resorped woven bone (Plate 7, Fig. d).

-At eight weeks observation period: The control gap defects showed localized areas of osteoclastic activities at the surface of the formed woven bone (Plate 8,Fig.a). B.ch gap defects showed dense layer of woven bone trabeculae, with osteoclastic activities at the surface of the woven bone and scanty amount of collagen bundles by the newly formed osteoblasts (Plate 8,Fig.b). B.co gap defects showed abundant amounts of collagen bundles (Plate 8, Fig.c).

-At twelve weeks observation period: The control gap defects appeared as that of the previous period, beside, thicker layer of woven bone trabeculae was detected (Plate 9, Fig.a). B.ch gap defects showed thin trabeculae of lamellar bone forming Haversian systems on the surface of the old woven bone.with Haversian canals. (Plate 9, Figs.b&c). B.co gap defects showed thicker and interconnected Haversian systems than that

of the B.ch defect. The replacing Haversian systems were thin and dispersed. Remnants of woven bone appeared in between the newly formed Haversian systems (Plate 9, Figs.d & e).

-At sixteen weeks observation period: The control gap defects showed thicker trabeculae of woven bone than that of the previous stage. The formed fibrocellular tissue filled the bony spaces was denser than the previous stage (Plate 10, Fig.a). B.ch gap defects showed increased thickness of the Haversian systems than that of the previous stage. Large areas of woven bone were detected in between the newly formed Haversian systems (Plate 10, Figs.b & c). B.co gap defects showed advancement of bone growth, greater than that in case of B.ch defect. The bone growth includes increase in the thickness of lamellar bone which formed the Haversian system. Remnants of interstitial lamellar bone could be detected in between the newly formed Haversian systems. (Plate 10,Figs. d & e).

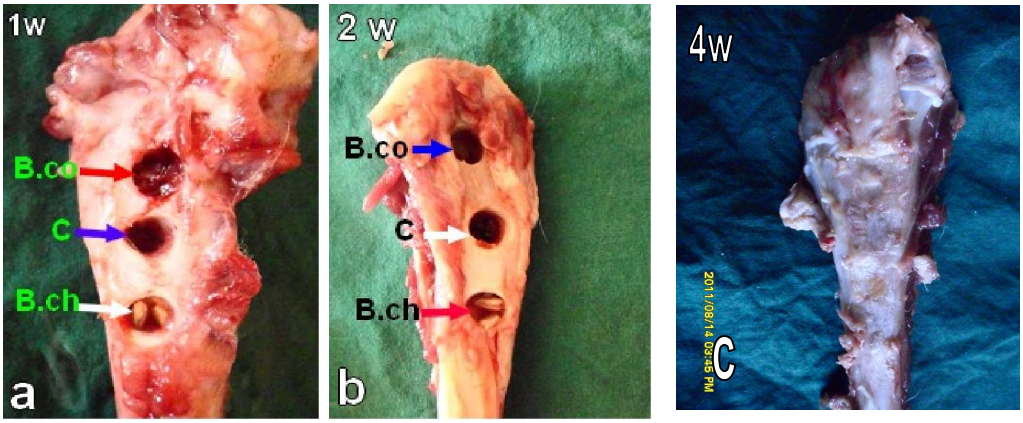


Plate (1):

Fig. a: The harvested tibia at one week post operation showing, remnants of B.co (red arrow) and B.ch (white arrow)

Fig. b: The harvested tibia at two weeks post operation showing, complete resorption of the grafted materials in the first hole. But it could be easily detected in the third one.

Fig. c: The harvested tibia at four weeks post operation showing periosteal reaction which completely masking the implantation site.

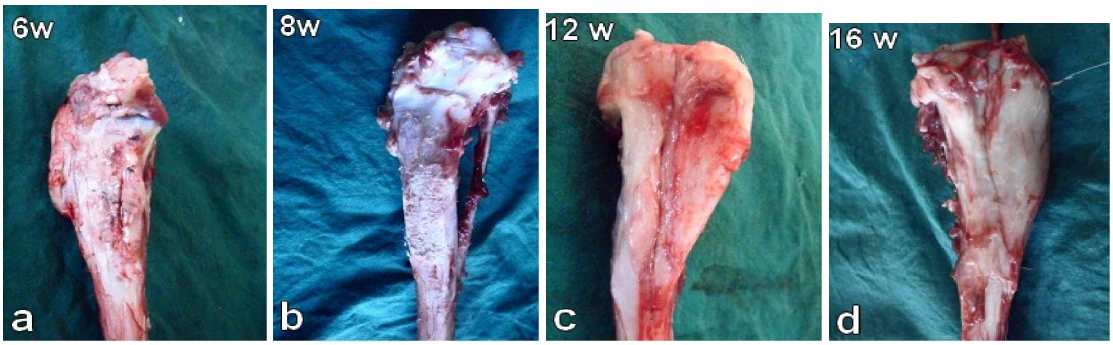


Plate (2):

Fig. a; The harvested tibia at 6 weeks post operation showing thick callus covering the implantation site.

Fig. b: The harvested tibia at 8 weeks post operation showing the beginning of callus remodeling.

Fig. c: The harvested tibia at 12 weeks post operation showing, complete remodeling of the periosteal callus

Fig. d: the tibia at 16 weeks post operation showing, complete remodeling of the periosteal callus and the tibia returned to its normal appearance.

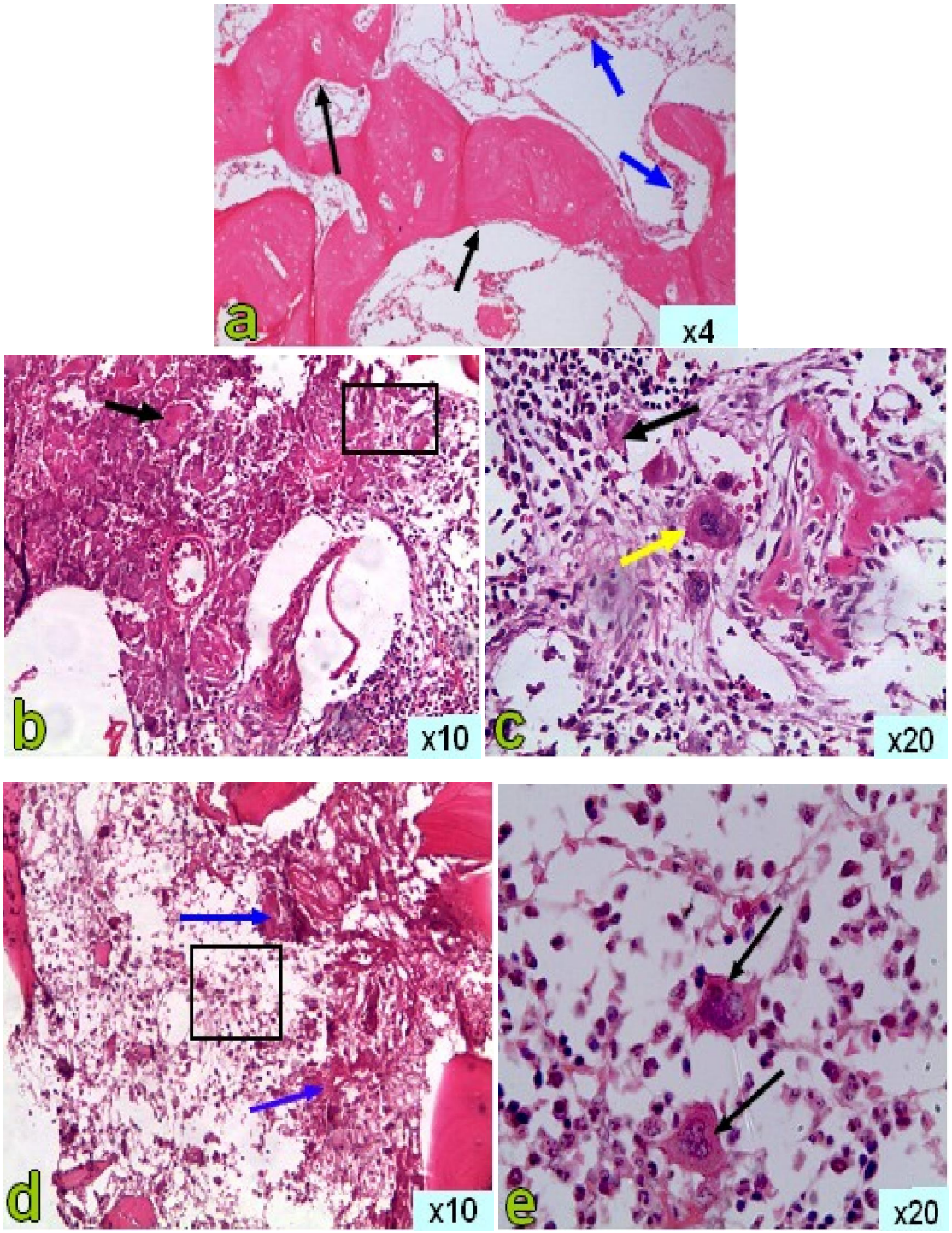


Plate (3):

Fig. a: Control defect at one week post operation showing, red blood cells (blue arrows) in the cavity and fibroblast (black arrow) lined the cavity, H&E ×4.

Fig. b& c: B.ch defects at one week postoperation showing; B.ch particles in the defect cavities (black arrow). The osteoclasts (yellow arrow), H&E. ×10 and x20.

Fig. d& e: B.co defect at one week postoperation showing; B.co particles in the defect cavity (blue arrows), and some osteoclasts (black arrows), H&E. ×10 and x20.

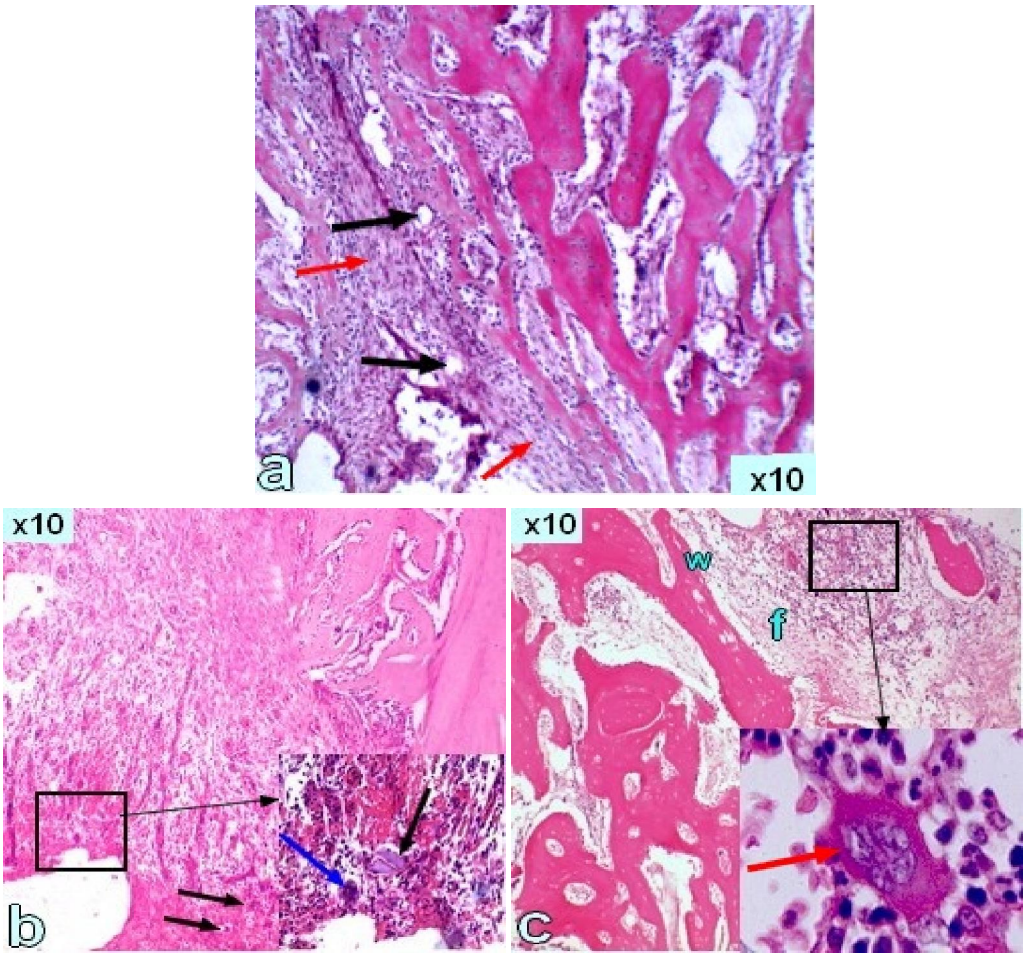
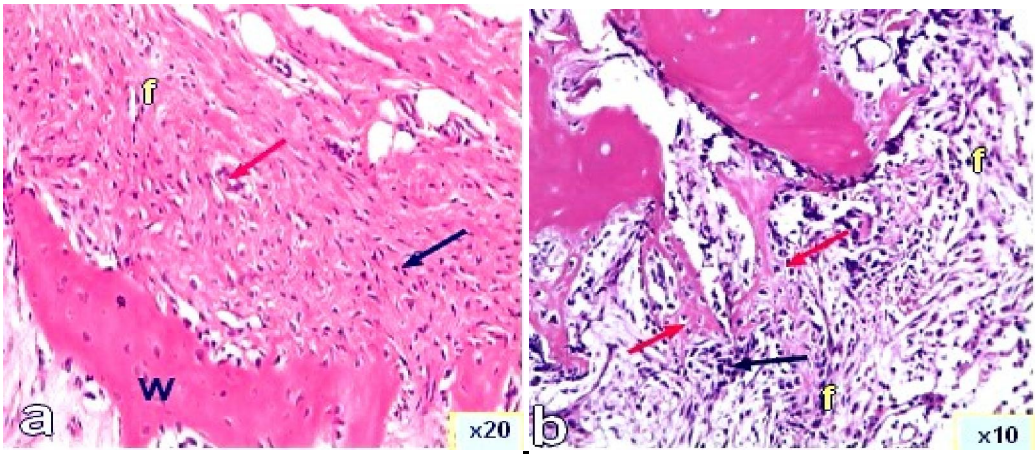


Plate (4):

Fig. a: Control defect at two weeks post operation showing, dense layer of fibrocellular tissue (arrows) filling the bony spaces and containing dilated blood vessels (black arrow) and large amount of fibroblasts, H&E ×10.

Fig. b: B.ch at two weeks post operation showing; increased amount of the fibrocellular tissues (f) inclosing bone chips in between (black arrow). Osteoclasts (red arrow) H&E ×10 &×20

Fig. c: B.co defect at two weeks post operation showing; fibrocellular tissue (f) enclosing thick woven bone trabeculae (w) and osteoclasts (red arrow), H&E ×10 &×20.



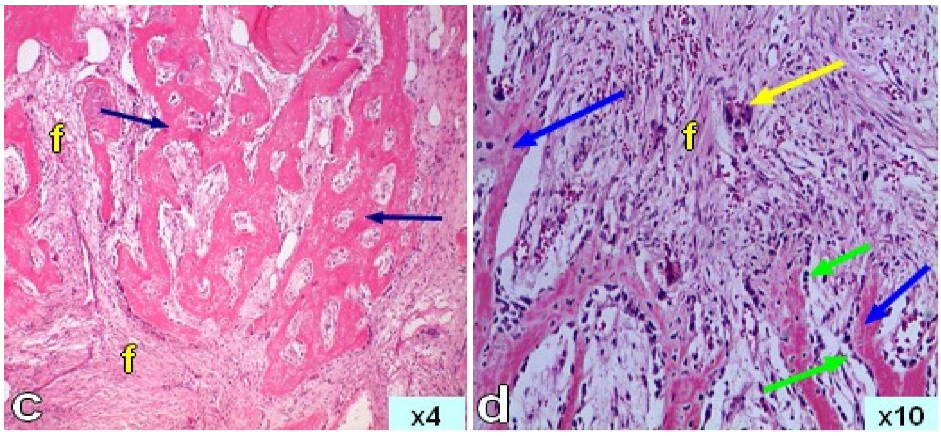


Plate (5):
Fig. a: Control defect at three weeks postoperation showing, fibrocellular tissues (f) containing fibroblasts (black arrow), dilated blood vessels (red arrow) and thin trabeculae of woven bone (w), H & E ×20.
Fig. b: B.ch defect at three weeks post operation showing, fibrocellular tissues (f) surrounding the remnants of B.ch particles (B.ch), and containing thin woven bone trabeculae (red arrows) at the surface of B.ch particles also osteoclastic activities (black arrows), H & E ×10.
Fig. c& d: B.co defect three weeks post operation showing: fibrovascular tissues (f) containing woven bone trabeculae (blue arrows), osteoclasts (yellow arrow) and osteoclastic activities and the osteoblasts arranged in an epithelioid manner (green arrows) around the woven bone H & E ×4 & ×10.

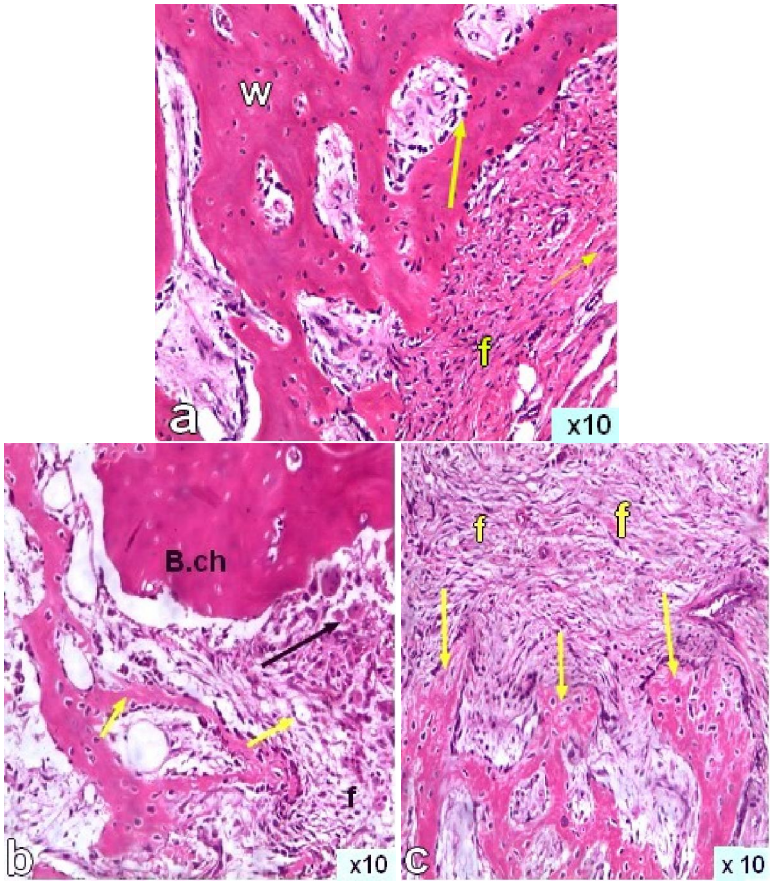


Plate (6):
Fig a: Control defect at four weeks postoperation showing; fibrocellular tissue (f) containing dilated blood vessels (arrows), and woven bone trabeculae (w), H&E ×10.
Fig. b: B.ch defect at four weeks postoperation showing; newly formed woven bone trabeculae (yellow arrows) at the surface of B.ch particles (B.ch), fibrocellular tissue (f) and osteoclast cells (black arrow). H&E. ×10.
Fig. c: B.co defect at four weeks postoperation showing; thick woven bone trabeculae (yellow arrows) enclosing fibrocellular tissue (f) in between, H&E. ×10.

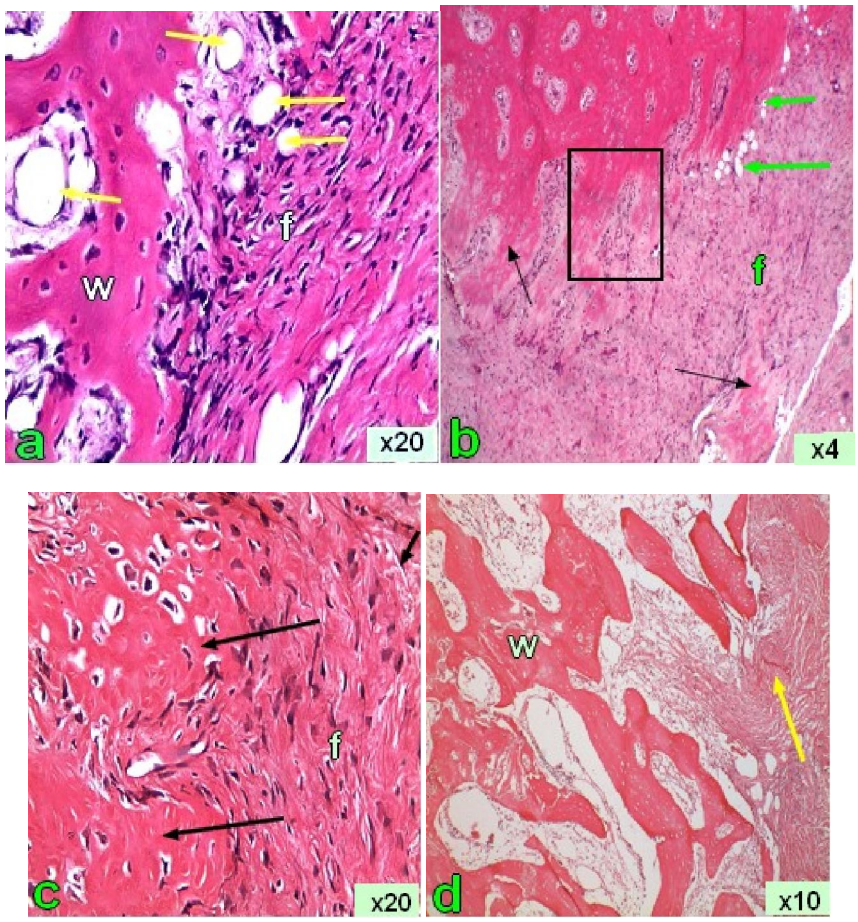
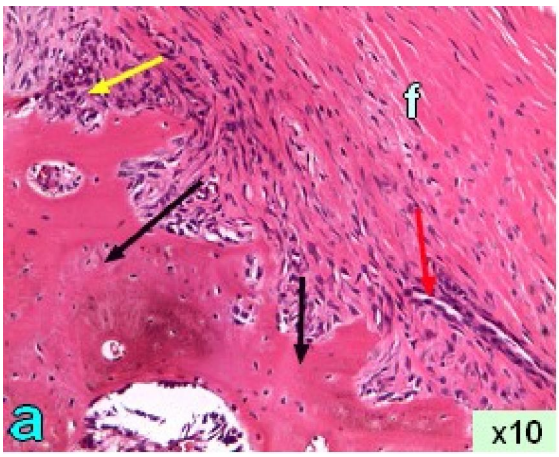


Plate (7):

Fig a: control defect at six weeks postoperation showing; fibrocellular tissue (f) containing woven bone trabeculae (w), also resorption spaces (arrows), at the surface of the formed woven bone H&E X 20.

Fig. b & c: B.ch at six weeks postoperation showing; thick woven bone trabeculae (black arrow), enclosing fibrocellular tissues (f) in between and resorption spaces (green arrow) at the surface of the formed woven bone, H&E. X 4 & X 20.

Fig. d: B.co at six weeks postoperation showing; newly formed bone matrix (yellow arrows) and the woven bone trabeculae (w), H&E. X 10.



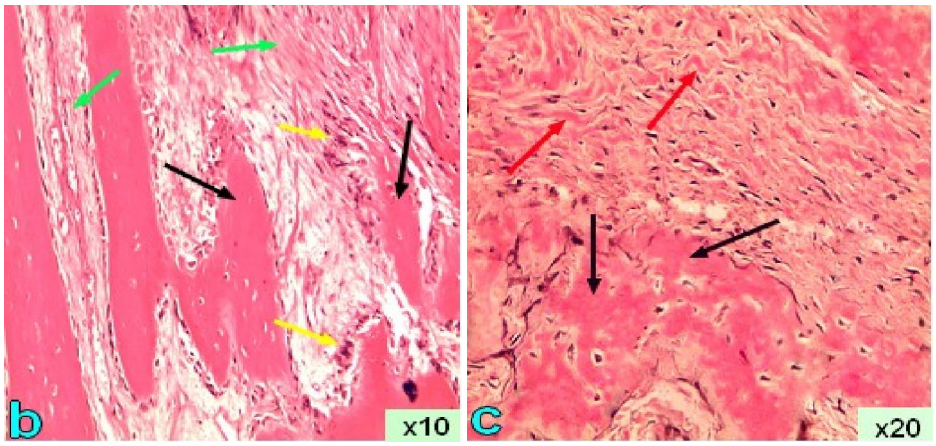


Plate (8):

Fig. a: Control defect at 8 weeks postoperation showing; fibrocellular tissue (f) enclosing dilated blood vessels (red arrow) and woven bone trabeculae (black arrow). Osteoclastic activities at the surface of the formed woven bone (yellow arrow) H&E..X10.

Fig. b: B.ch defect at 8 weeks postoperation showing; newly formed collagen bundle (green arrows) at the surface of the formed woven bone trabeculae (black arrows). Osteoclastic activities at the surface of the formed woven bone (yellow arrows) H&E.. X10.

Fig. c: B.co defect at 8 weeks postoperation showing; newly formed lamellar bone (red arrows) beside the formed woven bone (black arrows), H&E X20

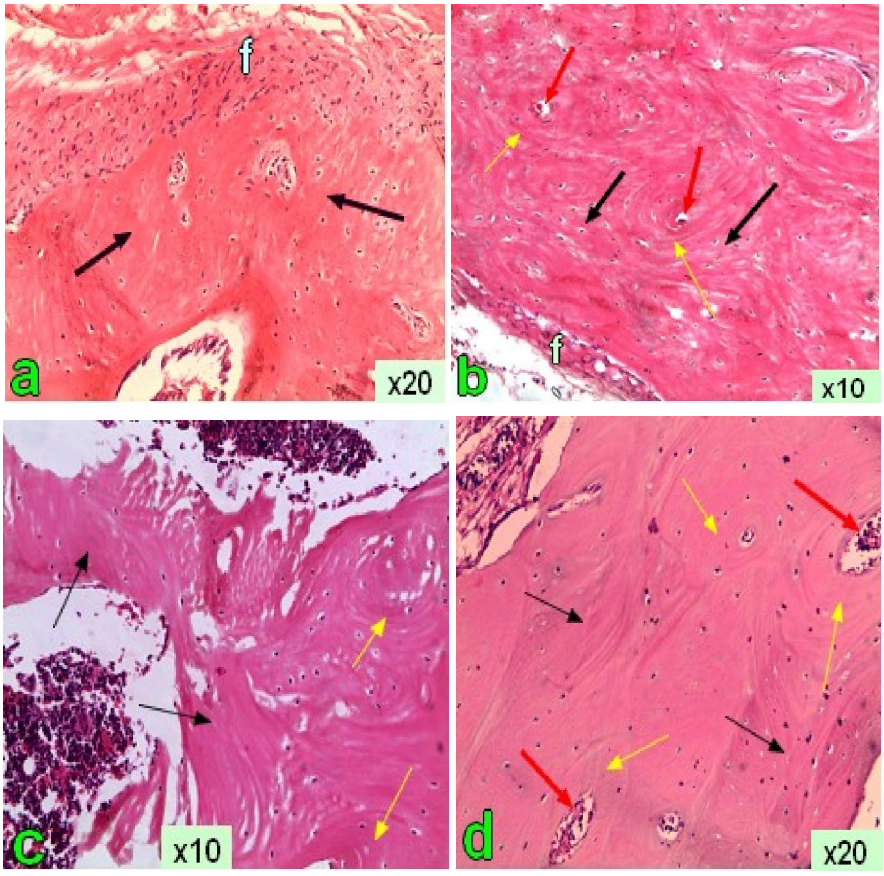


Plate (9):

Fig. a: control defect at 12 weeks postoperation showing, fibrocellular tissue (f) enclosing woven bone trabeculae (black arrow) H&E. X20.

Fig. b&c: The B.ch defect at 12 week postoperation showing, lamellar bone forming Haversian systems (yellow arrows) and Haversian canals (red arrows) and enclosing woven bone trabeculae (black arrows) in between. It was surrounded by loose layer of fibrocellular tissues (f), H&E. X10 & X10.

Fig. d: The B.co defect at 12 week postoperation showing, lamellar bone forming Haversian systems (yellow arrows) and Haversian canals (red arrows) & enclosing remnants of woven bone trabeculae (black arrows), H&E. X20.

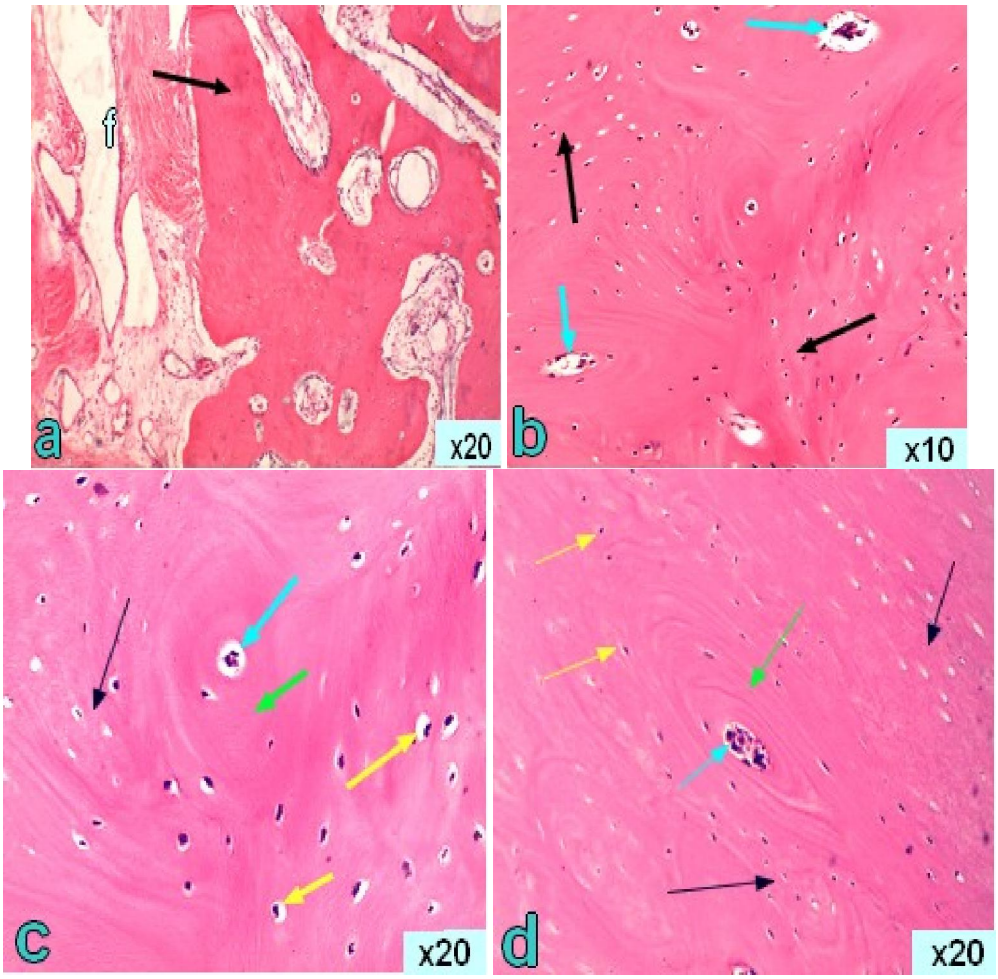


Plate (10):

Fig. a: The control defect at 16 weeks post operation showing: fibrovascular tissue (f) enclosing woven bone trabeculae (arrows), H&E. X20.

Figs. b&c: The B.ch defect at 16 weeks post operation showing: lamellar bone forming Haversian system (green arrows) enclosing Haversian canal (blue arrow), and osteocytes (yellow arrows), remnants of woven bone (black arrow) in between the formed Haversiansystems, H&E. X10 & X20.

Fig.d: The B.co defect at 16 weeks post operation showing: lamellar bone forming Haversian system (green arrows), enclosing Haversian canal (blue arrow), osteocytes (yellow arrows), interstitial lamellar bone (black arrows) in between the formed Haversian systems, H&E. X20.

4-Discussion

The animals used in this experiment were adult mongrel dogs of both sexes because the biological repair process of mature dogs is similar to those of human **(Burchardt, 1987).**

In the present study, a critical size hole defects (10 mm Ø) were artificially induced in canine tibia. **Arnaud *et al.*, (1999)** reported that a 9mm trephine defect in rat skull considered as a critical size defect. **Griffon *et al.*, (2001)** added that this technique allow preliminary evaluation of the biological properties of several agents with in the same animal and minimize the effect of biologic differences between animals. Moreover, this technique dose not require internal fixation, and optimize the use of the animals **(Pratt *et al.*, 2002).**

Selection of the duration of this study (16 weeks follow-up period) as the findings recorded by **Johnson *et al.*, (1996),** where the complete regeneration of created defect occurred**.** In the contrary **Naglaa Abdel Wahed (2003),** found that 24 weeks were necessary for complete incorporation of the grafted materials in the host bed.

The histological results revealed that the beginning of new bone formation originate at the host-graft interface and directed toward the center of the implantation site. As that reported by **Petite *et al.*, (2000)**.

The histological findings proved that bovine bone composite and autoclaved bovine bone chips xenograft have osteoinductive and osteoconductive prosperities. **Dehaghani & Baizaei (2001)** and **Bohner *et al.*, (2005)** stated that cortical autograft, allograft and xenograft were used primarily to promote osteogenesis,

The histological results of, all (B.co) defects showed progressive reduction at the end of each first and second weeks and reach the highest degree at the third week. These findings attributed complete resorption of bone composite by the end of the second week. while **Saraswathy *et al.*, (2004);** found partial resorption of the composite includes deglude bone, chitosan and gelatin occurred by the end of the third week while complete resorption occurred by the end of the sixth weeks.

The present study showed greater amount of the newly formed fibrocellular tissues at the control defects than that formed at the implantation site filled with B.co. This fact attributed to the inhibitory effect of chitosan upon fibroblasts proliferation while, stimulating osteoblastic activities **(Lee *et al.,* 2002).** We also, found that the control defects contained fibrocellular tissues and small amount of woven bone which, not adequate to fill the entire defects (**Bolander & Balian, 1986 ; Singh, 1998; Gil-Albarova *et al.,* 2004; Nandi *et al.,* 2009).** The control group always showed better bone neoformation. We attribute this to the slower evolution of bone repair when bone grafts are utilized in a bone cavity or defect, an observation previously made by others, **(Torricelli *et al.,* 2002; Granjeiro *et al.,* 2005 and Calasans-Maia *et al.,* 2007).**

Bone repair depends on an adequate vascular supply with osteoblasts working in the regions adjacent to the blood vessels, where the formation of highly organized bone tissue requires a mechanically stable and solid surface upon which the newly formed bone can be laid **(Sverzut *et al.,* 2008)**. Our histological picture revealed presence of thick trabeculae of woven bone by the end of the fourth week, while at sixth week the increased osteoclastic activities at the surface of the woven bone which accompanied with its resorption. At 8^{th} week, lamellar bone formation, the same results were mentioned by **Naglaa Abdel Wahed (2003)** and **Saraswathy *et al.,* (2004).**

The present study showed that the defect cavities were filed with thick lamellar bone enclosing haversian systems. In contrary **Naglaa Abdel Wahed (2003),** in canine tibia reported that the optimum time for complete disappearance of the defects was 24 weeks. We concluded that an early disappearance of the defect cavities filled with B.co may be attributed to osteoinductive prosperities chitosan and gelatin **(Lee *et al.,* 2002)**.

Sarupinder *et al.*, (1989) mentioned that ultraviolet light is efficient method for sterilization of bone graft materials. While, the process of preparation (chopping) and autoclave sterilization enhance incorporation of the B.ch into the host bone. Because of the process of chopping increase the surface area which, in turn improve the osteoclastic activities besides, the process of autoclaving destroying the antigenicity and minimize the rejection rate. So the present study recommended the use of ultraviolet light of sterilization for B.co and autoclave sterilization for B.ch. The morphohistological observations of the present study confirmed the post-operative clinical evaluation of the animals, without any complication.

5-Conclusion:

The present study demonstrated that the bovine bone composite and chips xenograft have osteoinductive and osteoconductive effect.

References

1. **Arnaud, E., De Pollak, C., Meunier, A., Sedel, L., Damien, C., Petite, H.(1999):** Osteogene-sis with coral is increased by BMP and BMC in a rat cranioplasty**.** Biomaterials 20 :1909-1918.
2. **Bancroft, J.D., Steven, A. and Turner, D. (1996):** Text book of Theory and Practice of Histological Technique. 4th ed., Lea& Febiger, Pheladelphia, USA.
3. **Bohner, M., Gbureck, U., Barralet, J.E. (2005):** Technological issues for the development of more efficient calcium phosphate bone cements: a critical assessment. Biomaterials ;26:6423–9.
4. **Bolander, M.E., Balian, G., (1986):** The use of demineralized bone matrix in the repair of segmental defects. Journal of Bone and Joint Surgery 68A, 1264–1274.
5. **Burchardt, H. (1987):** Biology of bone transplantation. Orthop. Clin North Am, 18: 187-196.
6. **Calasans-Maia MD, Fernandes GVO, Rossi AM, Dias EP, Almeida GDS, Mitri FF *et al.*, (2007)**: Effect of hydroxyapatite and zinc-containing hydroxyapatite on osseous repair of critical size defect in rat calvaria. Bioceramico; 20 273-6.
7. **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, vol.17 no.6 São Paulo
8. **Dehaghani, S.N. & Baizaei, F.E. (2001):**Using xenografts bovine bone pin for fracture repair in dogs 27^{th} world Small. Vet. Assoc. Congress proceeding. Vancouver, British Columbia, Canada.

9. **Finkemeier, C.G. (2002):**Bone-grafting and bone-graft substitutes. J Bone Joint Surg Am; 84A: 454-64.
10. **Gil-Albarova, J., Garrido-Lahiguera, R., Salinas, A.J., Roma´n, J., Bueno- Lozano, A.L., Gil-Albarova, R., Vallet-Regı´, M., (2004):**The in vivo performance of a sol–gel glass and a glass–ceramic in the treatment of limited bone defects. Biomaterials 25, 4639–4645.
11. **Granjeiro JM, Oliveira RC, Bustos-Valenzuela JC, Sogayar MC, Taga R. (2005):** Bone morphogenetic proteins: from structure to clinical use. Braz J Med Biol Res.;38:1463-73.
12. **Griffon, D.J., Dunlop, D.G., Howie, C.R., (2001):** An ovine model to evaluate the biological properties of impact morsellised bone graft substitutes. J. Biomed Mater Res, 56(3): 444-51
13. **Johnson, K.D., Frierson, K.E., Keller,T.S., Cook, C., Scheinberg, R., Zerwekh, J., Meyers L. & Sciadini, M.F. (1996):**Porous ceramics as bone graft substitutes in long bone defects: A Biomechanical,Histological and Radiographic Analysis. J. Orthop. Res., 14:351-369.
14. **Lane, J.M. & Sandhu, H.S. (1987):** Current approaches to experimental bone grafting. Orthop. Clin. North Am. 18:213-225.
15. **Lee,J,;Nam, S., Im, S., Park, Y., Lee,Y., Seol,Y., Lee, S. (2002):** Enhanced bone formation by controlled growth factor delivery from chitosan-based biomaterials Journal of Controlled Release 78:187–197
16. **Naglaa Abdel Wahed, (2003):**Experimental studies on allo-implants in canine model. PhD. Thesis, Faculty of Oral and Dental Medicine, Cairo University, Egypt.
17. **Nandi, S.K., Kundu, B., Datta, S., Basu, D. (2009):** The repair of segmental bone defects with porous bioglass: An experimental study in goat.J.Research in Veterinary Science; 86:162-173
18. **Nandi, S.K., Roy, S., Mukherjee, P., Kundu, B., De, D.K. & Basu, D.,(2010):** Orthopaedic applications of bone graft & graft substitutes: a review. Indian J Med Res July,132, pp 15-30
19. **Petite, H., Viateau, V., Bensaid, W., Meunier, A., de Pollak, C. (2000):** Bourguignon M, et al., Tissue-engineered bone regeneration. NatBiotechnol; 18:959–63.
20. **Pratt, J.N., Griffon, D.J., Dunlop, D.J., Smith, N., and Howie, C.R. (2002):** Impaction grafting with morsellised allograft and tricalcium phosphate- hydroxyapatite: incorporation within ovine metaphyseal bone defect. J. Biomaterials: 23, 3309, 3317.
21. **Saraswathy, G., Pal, S., Rose C. and Sastry, T.P. (2001):** a novel bio-inorganic bone implant containing deglued bone, chitosan and gelatin. Bull. Mater. Sci., Vol. 24 (4): 415-420.
22. **Saraswathy, G., Sastry, T.P., Pal, S., Makeena, S., Suresh, R.V. (2004):** A new Bio-inorganic composite as bone grafting material: in vivo study. Trends Biomater. Artif. Organs. 17(2):37-42.
23. **Sarupinder, D.D.S., Norman, G., Schaaf, D.D.S. (1989):** Dynamic sterilization of titanium implants with ultraviolet, j. Oral Maxillofac. Implants. 4:139-146.
24. **Shibata, Y., Fujita, S., Takahashi, H., Yamaguchi, A. & Koji, T. (2000)**: Assessment of decalcifying protocols for detection of specific RNA by non-radioactive in situ hybridization in calcified tissues. Histochem Cell Biol: 113:153–159
25. **Singh, S., (1998):** Reconstruction of segmental ulnar defect with tricalcium phosphate, calcium hydroxyapatite and calcium hydroxyapatite-bone matrix combination in rabbit. MV Sc Thesis. Indian Veterinary Research Institute, India.
26. **Sinibaldi, K.R. (1989):** Evaluation of full cortical allografts in 25 dogs.Journal of the American Veterinary Medical Association, 194 (11):1570-1577.
27. **Schena, C.J.; Graham, D.L.; Hoefle, W.D. (1985):** Segmental freeze-dried and fresh cortical allografts in the canine femur. II.A sequential histologic comparison over one year time interval. J. Am. Anim. Hosp. Assoc, 21: 193-204.
28. **Sverzut CE, Lucas MA, Sverzut AT, Trivellato AE, Beloti MM, Rosa AL, *et al.*, (2008)**: Bone repair in mandibular body osteotomy after Using 2.0 miniplate system-histological and histometric analysis in dogs. Int J Exp Pathol.;89:91-7.
29. **Torricelli P, Fini M, Rocca M, Giavaresi G, Giardino R. (1999)**: Xenogenic demineralized bone matrix: osteoinduction and influence of associated skeletal defects in heterotopic bone formation in rats. Int Orthop.;23:178-81.
30. **Torricelli P, Fini M, Giavaresi G, Rimondini L, Giardino R. (2002)**:- Characterization of bone defect repair in young and aged rat femur induced by xenogenic demineralize d bone matrix. J Periodontol;73:1003-9.