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Histopathological and serological assessment of using rib lamb xenograft reinforced with and without hydroxyapatite nano gel for reconstruction tibial bone defect in dogs

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Abstract

This work aimed to assess repairing tibial bone defects using histopathological and serological examinations. Eighteen stray adult dogs of both sexes were used. The experiment was allocated into two equal groups, 9 of each. An experimental tibia defect of 2.5*0.7 cm was induced at the proxomedial aspect of the tibia. Deproteinized lamb's rib xenograft was used to reconstitute the defective area. The tissue and blood samples were collected for histopathological and serological investigations at different periods. On day seven post-surgery, the serological assessment indicated a significant increase in the level of Insulin-Like Growth Factor (IGF) of both the first and second groups, 0.2±0.03, 0.3±0.02 ng/ml, respectively; however, after 14 days, the levels were significantly reduced in both groups 0.08±0.03, 0.2±0.02 ng/ml, respectively. On day 7, the serum alkaline phosphatase level in the first group was lower, 31.6±3 u/l, than in the treated group, 54.2±1.86 u/l. However, at 14 days post-surgery, the serum alkaline phosphatase level in the first group slightly increased by 35.7±2.1 u/l; nevertheless, the treated group manifested a constant level of 54.1±5.24 u/l. At 60 days post-operation, the histopathological examination presented more organized tissue maturation in the second group. The histochemical results of all specimens of the hydroxyapatite group revealed an increase in calcified bone by showing a red reaction which indicates the formation of thick calcified compact bone at 60 days post defects. In conclusion, the hydroxyapatite Nano gel contributed to ossification across the bone defect and hastened the healing process; the serological investigations indicated an increase in the activity of bone tissue.

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Introduction

Bone grafting is a surgical technique that is used to replace bone loss by transferring an implant from a donor to the recipient. The graft could be from a patient's own body, an artificial (1). The bone graft must have some biological properties such as the absence of immunogenicity, fast combinability with the host, and more stable fixation capability. The agent used in bone grafting can be broadly categorized into several types including allografts xenografts, autografts, natural or synthetic materials, and combinations of these agents (2,3). Different materials like hydroxyapatite, coral, eggshell (4), xenograft bone material, and bone tissue engineering scaffolds, have been used as a filling material to reconstitute the bone defect (5,6). The utilized bone implants should be characterized as biocompatible, bioresorbable, osteoconductive, and osteoinductive. Besides, it should be easy to use, low cost,

and resistant to mechanical services on the surgical site. Moreover, it should activate and improve new bone development and combination by the host and it should be as firm and solid as an intact bone (e.g. load-bearing ability). Additionally, it can be kept for a long period, easily formed desired into the proper shape and form, should not possess an antigenic property, and is fully synthetic. However, the materials that are used today can only meet some of these properties (7). Demineralization of bone is a procedure commonly used in the bone grafting process; therefore, this method destroys the antigenic surface structures of bone. It is an indispensable process for the elimination of antigenicity in xenograft bones (8). Demineralization prevents rejection of implant as it has osteoinductive characteristics, but has no osteoconductive properties, which is produced by a careful selecting mineral from the bone without destroying the growth factors and other bone morphogenetic proteins (9). The demineralized bone matrix was used as a bone xenograft in the repair of non-critical bone defects in rabbit tibias (10) and xenogeneic bone putty is used with the carrier of hydrogel derived from the demineralized bone matrix (11). Hydroxyapatite (HAp) was widely used as a bone implant for more than four decades in orthopedics and this may be due to their similarity to a bone structural matrix including mineral composition it is such as biocompatibility, bioactivematerial, osteoconduction, slow-degradation, osteoinduction, and osteointegration (12). HAp is commercially available either from a natural or a synthetic source. Hydroxyapatite (HAp) is used with autologous bone marrow to repair segmental radial defects in rabbit radius (13). Also, hydroxyapatite nanoparticles are used as bone substitute biomaterial in a vertical bone augmentation model (14).

The objective of this study was to assess the histopathological, and serological examinations after using deproteinized xenograft with or without HAp Nano gel for repairing the tibial defect.

Materials and methods

The present work included Eighteen adult local breed dogs of both sexes (weighing 21±0.3 kg and aged 2.1±0.9 years). Animals were randomly allocated into two equals first and second (control and treated) group with 9 animals for each Deproteinized rib lamb xenograft was used to reconstitute the defective bone. A protocol of anesthesia included ketamine HCl (Rotexmedica, Germany) and xylazine HCl (Interchemie, Holland), 15mg and 5mg /kg BW, respectively as a mixture injected by intramuscular route (15).

An experimental tibia defect 2.5×0.7 cm was induced at the proxomedial aspect of the tibia. The skin of the proxomedial aspect of the tibia was incised 12 cm. Then, subcutaneous tissue and muscle separated bluntly to access the tibia. By electrical saw, a 2.5×0.7 cm bone defect was induced. Lamb's ribs are deproteinized and used as xenografts to replace the lost bone, xenograft prepared according to Pengfei *et al.* (16). Surgical interventions were done to repair the lost bone using a prepared deproteinized implant 2.4 × 0.6 cm in the first group, the implant was firmly fixed using cerclage steen less steel wire, while in the second group all steps were performed as similar to the first group except using hydroxy apatite Nano gel as a filling material (Figure 1).

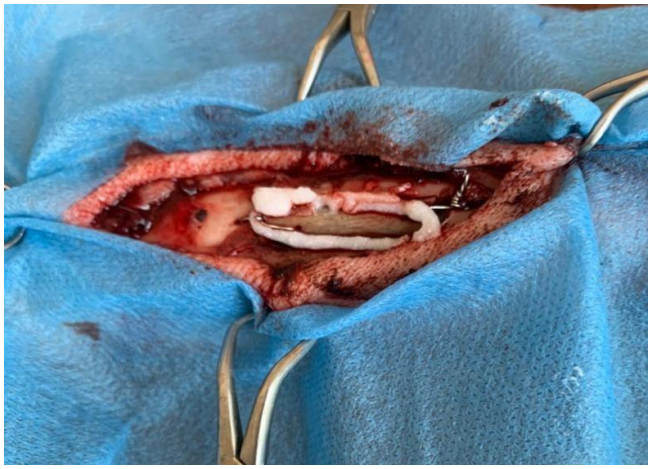

Figure 1: shows repairing the defect by using lamb rib bone xenograft and immobilized with 2-0 sterile cerclage stainless wire suture material, supported with 1ml hydroxyapatite Nano gel (black arrow) as filling materials.

Blood samples 5 ml were collected from the Jugular vein with an anticoagulant agent at various periods 0, 7-, 14-, 30-, and 60-days post-surgery for serological investigations including the level of insulin-like growth factor (IGF). The serum sample was analyzed by enzyme-linked immunosorbent assay technique (ELISA) using of specific dog insulin-like growth factor 1, IGF1 ELISA Kit (MyBioSource, USA). Alkaline phosphatase (ALT) (Biolabo reagents, France) was used (17). Histopathological investigations were done and new bone tissue formation were detected at 15-, 30- and 60-days post operation. Two-way ANOVA for means and standard errors of the obtained data was used to detect the effect of different parameters. The analysis was done using the IBM SPSS version 25.0 where significance was considered at $P \leq 0.05$ (18).

Ethical approve

The Research was approved by Ethics Committee of Faculty of the College of Veterinary Medicine, Mosul University No UM.VET.2021.060.

Results

The serological investigations indicated that the data for serum insulin-like growth factor levels within the first group and second groups at zero time exhibited significant differences. On day 7 post-surgery, the levels of IGF increased and their highest rates in both groups 0.2±0.03, 0.3±0.02 ng/ml, respectively, was noticed. Whereas on day 14, these levels gradually decreased in both groups and reached 0.08±0.03, and 0.2±0.02 ng/ml, respectively. After 60 days post-surgery, the reduction in rates continued in both groups 0.03±0.03, 05±0.03 ng/ml, respectively (Table 1).

Table1: Mean values (ng/ml) of insulin-like growth factor (IGF) during the various period of the study in both groups

days	Xenograft	Xenograft with hydroxyapatite
0	0.12^{Ac}±0.05	0.3^{Dd}±0.1
7	0.2^{Ad}±0.03	0.3^{Dc}±0.02
14	0.08^{Ab}±0.03	0.2^{Cb}±0.02
30	0.06^{Cb}±0.04	0.04^{Ba}±0.03
60	0.03^{Aa}±0.03	0.05^{Ba}±0.03

Different small letters and capital letters are significantly different at P≤0.05.

The results of the of serum alkaline phosphatase levels in both groups at zero time revealed significant differences. The level of serum alkaline phosphatase increased at 7 and 14-days post-operative in both groups, the highest rates were on day seven 31.6± 3, 54.2±1.86 IU/l, respectively, while those rates for day 14 were 35.7± 2.1,54.1±5.24 IU/l, respectively. Then, at 60 days postoperatively, the rates were gradually decreased and returned near the normal values 22±5.7, 37.1±2.4 IU/l, in first and second group respectively (Table 2).

Table 2: The Mean values of Serum Alkaline phosphatase (IU/L) during the period of the study in both groups

Time	Xenograft	Xenograft with hydroxyapatite
0	25^{Ab}±2	26.8^{Aa}±1.43
7	31.6^{Ac}±3	54.2^{Cc}±1.86
14	35.7^{Ad}±2.1	54.1^{Cc}±5.24
30	31.6^{Ac}±2.25	46.6^{Cb}±2.2
60	22^{Aa}±5.7	37.1^{Ca}±2.4

Different small letters and capital letters are significantly different at P≤0.05.

Histological results

The histological sections image of first group at 15 days post-surgery was filled with granulation tissue, abundant new blood vessels, macrophages, fibroblast, and lymphocytes cells with a small amount of new bone formation which was less dense and irregular in shape (Figure 2) and small amount of less dense newly bone lamellae (Figure 3). While at 15 days images of second group show a higher number of bone lamellae and less inter lamellar space for hydroxyapatite Nano gel group in between the bone lamellae surrounded by connective tissue (Figure 4). Moreover, at 30 days post-surgery, the xenograft group showed a normal healing process with a moderate amount of new bone lamellae scattered through the connective tissue (Figure 5). but it was still in the early stage of mineralization, while the hydroxyapatite nano gel group showed a normal healing process with bone lamellae such as woven bone formation, cellular lacunae, layers of osteoblast and osteoclast activity with osteocytes within lacunae also bone marrow was observed (Figure 6). In contrast, at 60 days post-surgery the xenograft group showed the formation of woven bone which was composed of abundant osteocytes, and a small number of mineral compounds with collagen fibers (Figure 7).

At 60-day post-surgery, the hydroxyapatite Nano gel treatment showed more organized tissue maturation with good healing detected. The defect area generally contained a compact bone formation with several Haversian canals surrounded by concentric lamellae of the matrix; in addition to the presence of Volkmann's canals, which contain the blood vessel (Figure 8), it means that there is complete ossification across the bone defect and the quality of the repair of the bone defect was distinguished from the xenograft group.

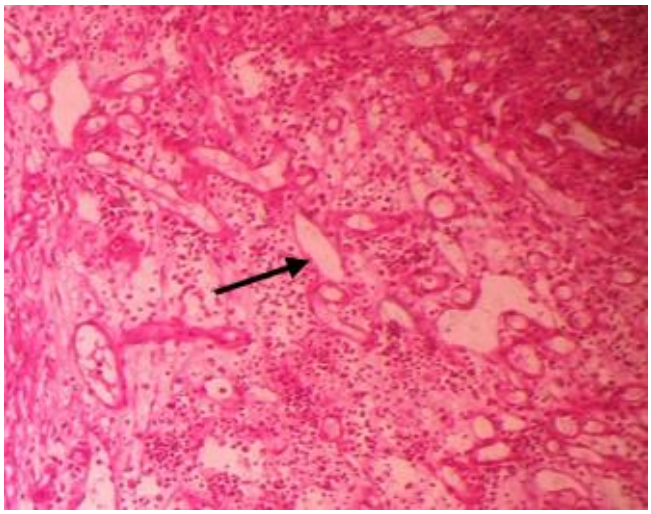

Figure 2: Micrograph section image of a tibia bone, H&E-stained section of bone tissue taken at 15 days post-surgery from xenograft group show granulation tissue formation with abundant of new blood vessels (Black arrow) (10X).

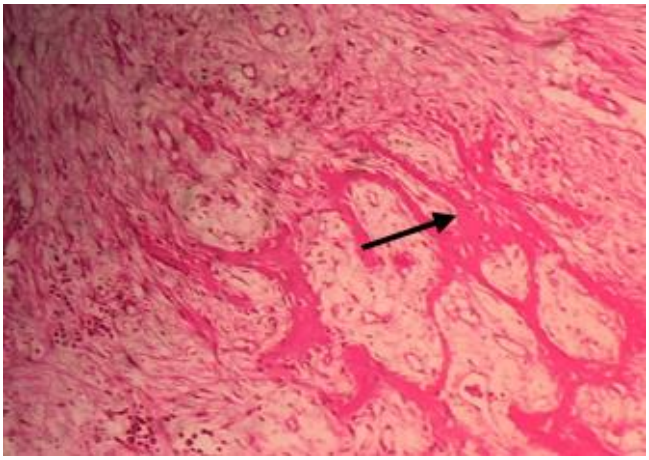

Figure 3: Micrograph section image of a tibia bone, H&E-stained section of bone tissue taken at 15 days post-surgery from xenograft group showing a small amount of less dense newly bone lamellae (Black arrow) (10X).

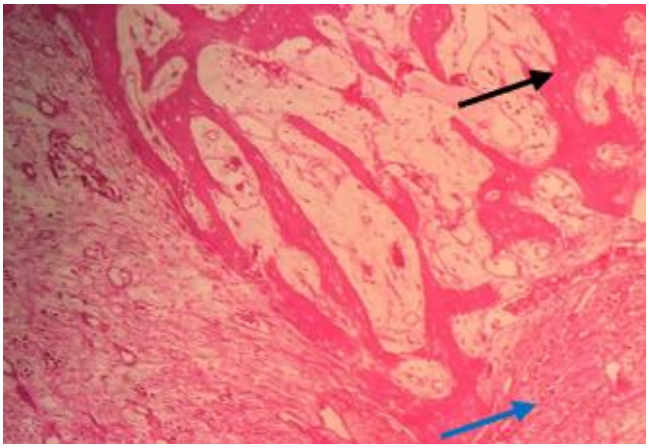

Figure 4: Micrograph section image of a tibia bone, H&E-stained section of bone tissue taken at 15 days post- surgery from hydroxyapatite Nano gel showing bone lamellae formation (Black arrow) surrounded by connective tissue (Blue arrow) (10X).

Histochemical results

Masson trichrome histochemical stain used for mature normal bone which showed two main reactions, a green reaction mainly localized in osteoid tissue and collagen fibers distribution, and the red reaction for lamellar bone formation, the new bone formation was shown in green color**.** The histochemical results of the xenograft group images show that the green reactivity was more evident than the red color in all periods 15, 30, 60 days. The histochemical results of all specimens of the hydroxyapatite group revealed an increase in calcified bone by showing a red reaction, which indicates the formation of thick calcified compact bone at 60 days post-surgery. The figures below show the expression of collagen stained in green color (green arrow) newly formed blood vessels at 15 days post-surgery taken from xenograft group (Figure 9), the expression of collagen stained in green color (green arrow) newly formed blood vessels at 15 days post operation taken from xenograft group (Figure 10). The newly bone lamellae stained in green color (green arrow) taken from hydroxyapatite Nano gel group 15 days post-surgery (Figure 11) The increased calcified bone with red reaction (red arrow) 30 days post-surgery taken from hydroxyapatite Nano gel group (Figure 12).

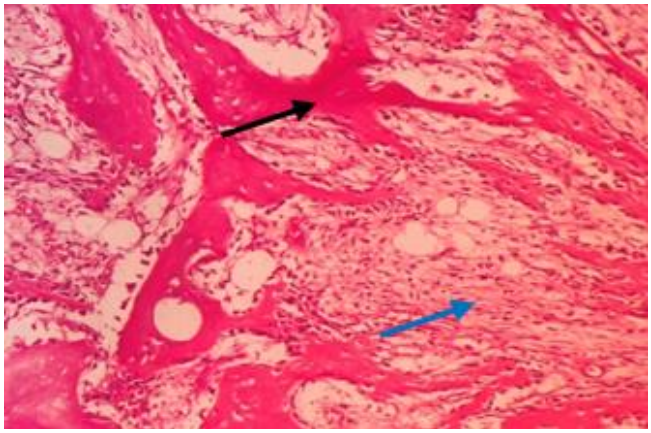

Figure 5: Micrograph section image of a tibia bone, H&E-stained section of bone tissue taken at 30 days post-surgery from xenograft group showing a moderate amount of new bone lamellae (Black arrow) scattered through the connective tissue (Blue arrow) (10X).

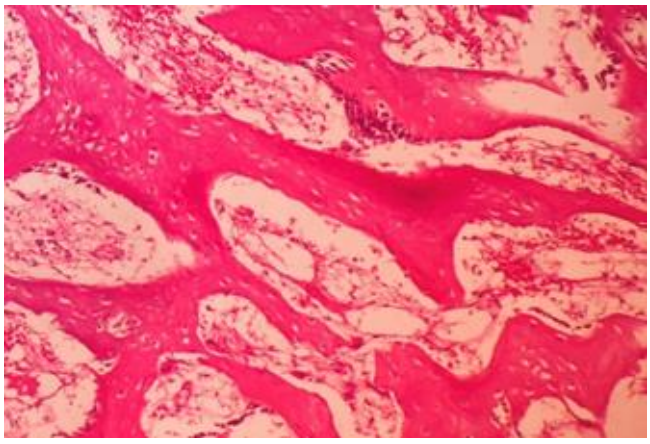

Figure 6: Micrograph section image of a tibia bone, H&E-stained section of bone tissue taken at 30 days post-surgery from hydroxyapatite Nano gel showing woven bone formation note the osteocytes with lacunae and layers of osteoblasts (10X).

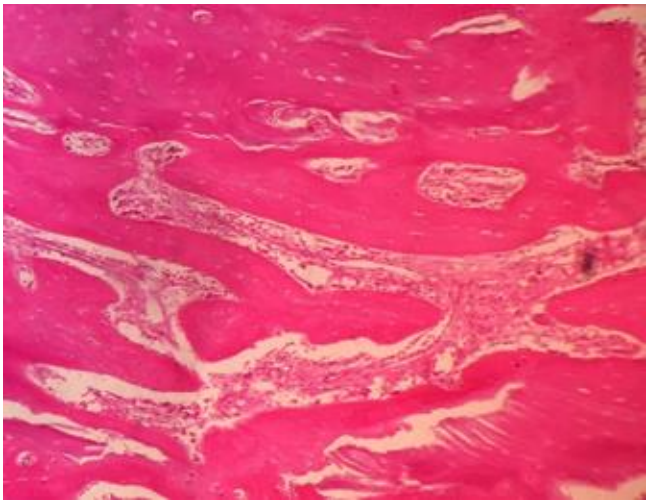

Figure 7: Micrograph section image of a tibia bone, H&E-stained section of bone tissue taken at 60 days post-surgery from xenograft group showing woven bone formation (10X).

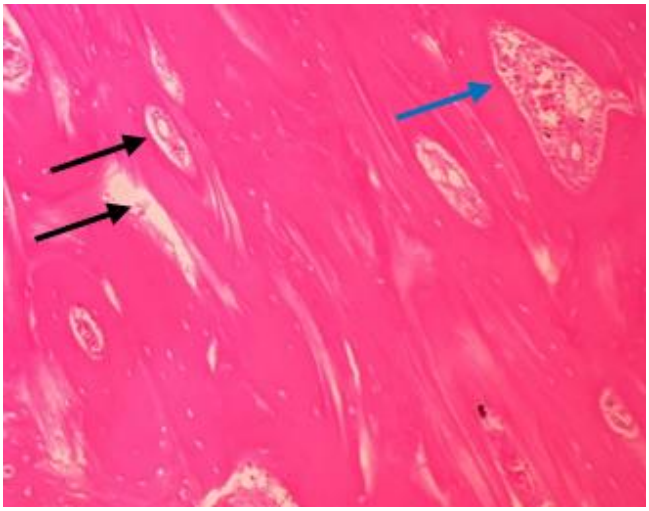

Figure 8: Micrograph section image of a tibia bone, H&E-stained section of bone tissue taken at 60 days post operation from hydroxyapatite Nano gel showing the formation of compact bone containing several Haversian canals (Black arrow) of Volkmann's canal (Blue arrow) (10X).

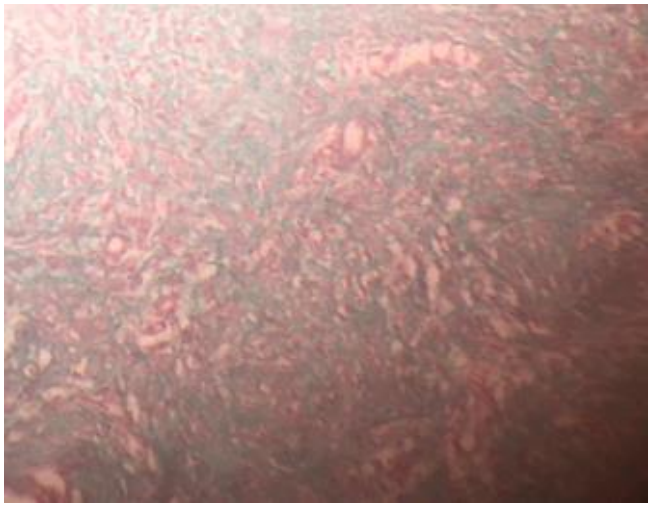

Figure 9: Micrograph image section of a tibia bone, Masson's trichrome stained section show the expression of collagen stained in green color (green arrow) newly formed blood vessels at 15 days post-surgery taken from xenograft group (10X).

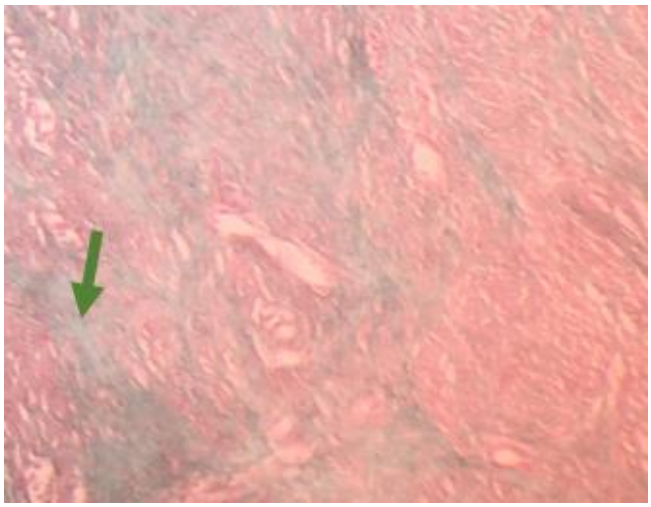

Figure 10: Micrograph image section of a tibia bone, Masson's trichrome stained section show the expression of collagen stained in green color (green arrow) newly formed blood vessels at 15 days post operation taken from xenograft group (10X).

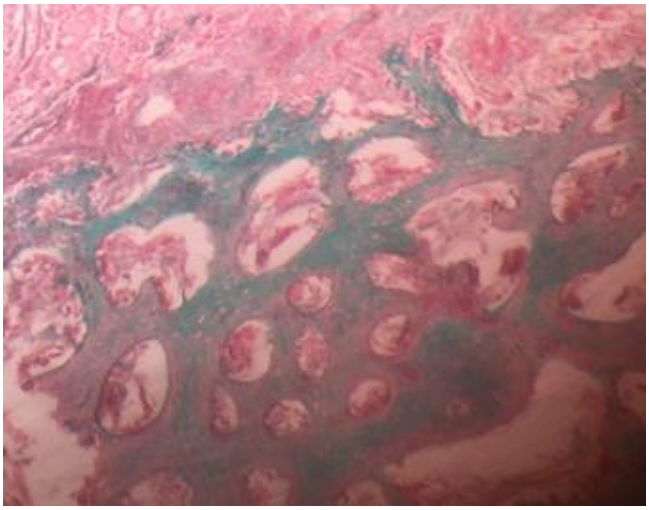

Figure 11: Micrograph image section of a tibia bone, Masson's trichrome stained section show the newly bone lamellae stained in green color (green arrow) taken from hydroxyapatite Nano gel group 15 days post-surgery (10X).

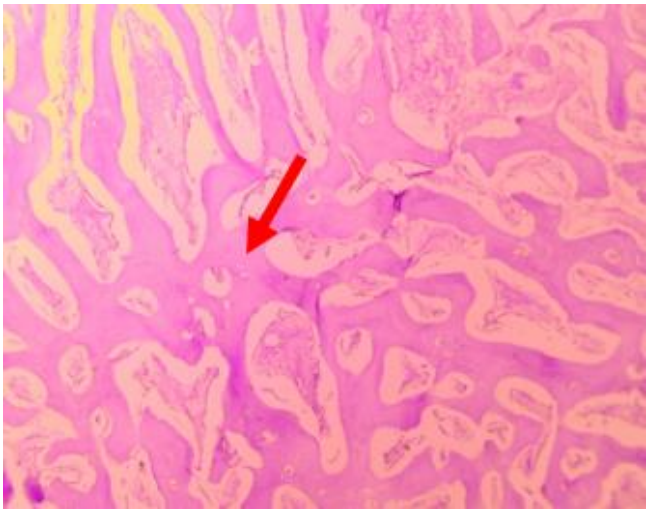

Figure 12: Micrograph image section of a tibia bone, Masson's trichrome stained section show increased calcified bone with red reaction (red arrow) 30 days post-surgery taken from hydroxyapatite Nano gel group (10X).

Discussion

The dog was used as a perfect animal's model for many operative surgical procedures as repairing Achilles tendon defect (19) and repairing of bone defect (20). In this study the implanted hydroxyapatite Nano gel and xenograft were well placed and well accepted in all animals group. The role of the Insulin-like growth factor is important in bone regeneration. It forms in the bone matrix, chondroblasts, and osteoblast and is categorized into two forms I and II (21). The level of IGF increased on day 7 post-surgery in both groups, this result indicated an increasing activity of osteoblast in which the osteoblast and bone matrix secretes large quantities of Insulin-like growth factors, which play a crucial role in bone matrix synthesis osteoblast proliferation, and bone resorption that gets involved in process of mineralization and bone defect formation especially with hydroxyapatite Nano gel group this results also pointed with Fowlkes *et al.* (21) and Tsiridis *et al.* (22). The level of serum alkaline phosphatase in both groups at 7- and 14-days post-surgery was significantly increased. These results are exhibited through the increased osteoblastic activity when osteoblast secretes large quantities of alkaline phosphatase that get involved in process of mineralization and bone defect formation as a result of adding the hydroxyapatite nano gel as a biomaterial, these findings agreed with previous works Thanoon *et al.* (23) and Zebon *et al.* (24). The tissue sections from the xenograft group 15 days post-surgery showed the presence of granulation tissue with inflammatory reaction without bone formation at the site of bone defect this occurs as a result of secretion of inflammatory cytokines such as vascular endothelial growth factor (VEGF) transforming growth factor beta (TGFB) fibroblast growth factor 2 (FGF2) and angiopoietin (25). The group of animals treated with xenograft and hydroxyapatite Nano gel showed earlier and greater newly formed bone lamellae at the site of bone defects at all periods than the xenograft group this might be due to the role of the hydroxyapatite Nano gel which causes cell migration, proliferation and differentiation at the defect site and enhances extracellular matrix organization, rapid new bone lamellae formation, and high rate of collagen maturation at 15 days post- surgery also positive effect of this biomaterial which has a potential effect of bone regeneration with very minimal toxicity or inflammatory response this results also reported with Mondal and Pal (26) and Ghiasi *et al.* (27). The small size of hydroxyl apatite acts as an excellent material for promoting bone regeneration (28). This study agreed with Zhou *et al.* (29) which showed that the nanostructure of hydroxyl apatite had higher osteogenic activity in vivo compared with other substances. Xenografts may lose part of their osteoinductive and osteoconductive abilities (30). The hydroxyapatite Nano-gel used in this study possessed the advantages of being chemically and structurally close to the natural structure of bone. In this work, at 60 days post-surgery hydroxyapatite Nano gel showed well-developed lamellar bone containing a Haversian canal and Volkmann's canals this result agreed with (31). Moreover, signs of healing developed earlier in the HAp group than in the xenograft group. At 30- and 60-days post-surgery, bone healing progressed, bone lamellae were organized also

remodeling was faster in the HAp, which means that HAp contributed in an acceleration of osteointegration (28). Post bone defect many enzymes are released by inflammatory cells, which accumulate at the site of the bone defect and lead to an increase in the reactive oxygen metabolites. Oxygen free radicals are negatively implicated in the repair of defective bone (32). It has been suggested that the increased free radicals are eliminated at 2-3 weeks after bone defect, therefore hydroxyapatite and bone grafts not only accelerated healing but also decrease the production of free radicals (33).

Conclusion

The microstructure of hydroxyapatite Nano gel provided biomimetic bone material that contributed in ossification across the bone defect and hasten healing process. The histopathological result of this study showed that the healing of bone defect can be repaired by using synthetic and xenograft implantation, however hydroxyapatite Nano gel had a better effect on osteointegration as compared with xenograft and could be used in bone tissue engineering. The serological investigations indicated increase in activity of bone tissue.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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التقييم النسجي المرضي والمصلي لاستخدام رقع مغايره للضلع مع أو وبدون صغائر الهايروكسي ابتيت لإعادة تشكيل أذى قصبة الساق في الكلاب

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الخلاصة

هدف هذا العمل إلى تقييم أذى عظم الساق المصلح باستخدام الفحوصات النسيجية المرضية والفحوصات المصلية. تم استخدام ثمانية عشر من الكلاب السائبة البالغة من كلا الجنسين. تم تقسيم التجربة إلى مجموعتين متساويتين ٩ لكل منهما. تم إحداث أذى تجريبي في قصبة عظم الساق ٢,٥ × ٠,٧ سم في الجانب الداني لعظم الساق. تم استخدام رقع مغايره منزوعة البروتين من ضلع الحمل لإعادة تشكيل منطقة الأذى المحدث. تم اخذ عينات من النسيج والدم لإجراء الفحوصات النسيجية المرضية والفسيولوجية في فترات مختلفة. في اليوم السابع، أشارت نتائج التقييم المصلي زيادة معنوية في مستوى عامل النمو الشبيه بالأنسولين في المجموعتين الأولى والمعالجة ٠,٠٣±٠,٢ ، ٠,٠٢±٠,٣ نانوغرام/مل، على التوالي؛ ومع ذلك ، بعد ١٤ يوما ، انخفضت المستويات بشكل ملحوظ في كلا المجموعتين ٠,٠٣±٠,٠٨ ، ٠,٠٢±٠,٢ نانوغرام/مل ، على التوالي. وفي اليوم السابع، كان مستوى الفوسفاتيز القلوي في المصل للمجموعة الأولى ٣±٣١,٦ وحدة/لتر اقل من المجموعة المعالجة ١,٨٦±٥٤,٢ وحدة/لتر وعلى كل حال في اليوم الرابع عشر بعد العملية ازداد قليلا مستوى الفوسفاتيز القلوي في مصل الدم ٢,١±٣٥,٧ وحدة/لتر ومع ذلك أظهرت مجموعة المعالجة مستوى ثابتا ٥,٢٤±٥٤,١ وحدة/لتر بعد ستون يوما من المعاملة. أظهر الفحص النسيجي المرضي نضجًا أكثر تنظيماً للأنسجة في المجموعة الثانية. أظهرت النتائج الكيميائية النسيجية لجميع عينات مجموعة الهيدروكسي اباتيت زيادة في العظام المتكلسة من خلال إظهار تفاعل أحمر يشير إلى تكوين عظم متكتل متكلس سميك بعد ٦٠ يومًا من إحداث الأذى وخلاصة ذلك، ساهم هلام النانو هيدروكسي اباتيت في أحداث التعظم خلال أذى العظم المحدث والإسراع في عملية الشفاء كما وأشارت الفحوصات المصلية إلى زيادة نشاط أنسجة العظام.

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