Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

The Effects of nano hydroxyapatite and nano hydroxyapatite doped by magnesium on fracture healing in dogs

Samira Mohamed Sallam[1], L. M. Ahmed[1], Aziza Amin[2], A. M. Al-akraa3, A.H. El-kasapy[3]

[1] *Department of Physics, Science College, Benha University, Benha, Egypt.*

[2] *Department of Pathology, Faculty of Veterinary Medicine, Benha University, Egypt.*

[3]*Department of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Benha University, Egypt.*

ARTICLE INFO

Keywords

Dogs

Fractured bone

Healing

Histopathology

Nano Hydroxyapatite

Received 28/04/2020

Accepted 23/06/2020

Available On-Line 08/09/2020

ABSTRACT

The aim of this study was to investigate and compare the effect of the Nano Hydroxyapatite (NHA) and Nano Hydroxyapatite doped by magnesium (NHA-Mg) on healing of fracture in dogs. Twenty-seven adult dogs of both sexes aging between 2-3 years old were randomly assigned into three groups. Group one (control group), group two (NHA group), and group three (NHA-Mg). An experimental model of diaphyseal complete radial fracture was surgically created by gegli saw. The fractured radius was fixed by extra-medullary 6-hole dynamic compression plate (6-hole DCP), with addition of the previously mentioned materials to fill the radial fracture defect in the corresponding groups. Data of clinical observation, plain radiography, computed tomography (CT) with histopathological examination and scoring were recorded on 45, 75, 90 days after induction of fracture. Dogs of all groups achieved the full bearing weight and stance 5 days postoperatively. The radiographs admit little knowledge about the healing scoring rather than the CT which declares that N HA-Mg was improve the filling of fracture defect. The histopathological examination and scoring revealed that NHA-Mg was superior than Nano HA alone in fracture healing. Data of the present work showed that Nano hydroxyapatite doped by (0.007mol) magnesium concentration allows complete calcification and healing of the fractured radius during the experimental time frame.

1. INTRODUCTION

The healing of fracture showed a great challenge either in veterinary or human orthopedic practice because spontaneous regeneration is limited to relatively small defects (Xiebo et al, 2018). According to the World Health Organization approximately 5-10% of fracture resulted in delayed union or nonunion (Childs, 2003). Several investigations were demonstrating the positive effect of many materials to enhance the healing of fractured bone (Zahra et al, 2012). Hydroxyapatite, a crystalline form of calcium phosphate which resembles one the component of bone substance, has shown acceptable promise as a graft material. It was initial mechanical and structural rigidity and demonstrate osteoconductive as well as angiogenic properties *in vivo* (Appleford et al., 2009).

The presence of Mg in HA is very important, apart its structural role in the HA crystals, it is a contributor factor to bone health increasing the bone density, mineral metabolism, formation and crystallization processes (Castiglioni et al, 2013; Nabiyouni et al, 2015).

Mg-e HA improves cell adhesion, proliferation, and metabolic activation compared with HA (Tamimi et al, 2011 and Park et al, 2013).

The experiment was designed to compare the healing of N HA-Mg with that of N HA alone on thin radius fracture in dog model.

2. MATERIAL AND METHODS

2.1. Ethical approval statements

The study protocol appropriated by the ethical committee of the faculty of veterinary medicine, Benha University, for animal care and experimentation.

2.2. Materials

In present works magnesium concentration was prepared by doped 2 g of magnesium in hydroxyapatite using wet precipitated method. Hydroxyl apatite prepared by wet precipitation method and doped with different concentrations of magnesium ions. The samples were then characterized by XRD, FTIR, SEM and EDX, as described in previous work (Hanafi et al, 2016).

2.3. Experimental Animals and treatment

Twenty-seven clinically healthy dogs of both sexes, weighting 15-20 kg, aged from 2-3 years old were arranged in 3 groups randomly each of 9 dogs. Each group was subdivided into three subgroups, each with 3 dogs.

The first group was the control group, the second group was treated by using Nano hydroxyapatite and the third one was treated by addition of Nano hydroxyapatite doped by magnesium. Data of clinical observation, plain

* Corresponding author: Aziza Amin, Department of Pathology, Faculty of Veterinary Medicine, Benha University, Egypt.

radiography, computed tomography (CT) and histopathological examination and scoring were recorded on 45, 75 and 90 days after induction of fracture.

2.4. Operation

The forearms of all dogs were subjected to surgical intervention according to Fossum (2013). The animals were anesthetized by Xylazine (Xyla-Ject 20 mg/ml Adwea Pharmaceutical, Egypt), Ketamine (Ketamine 500mg/10ml, EIPICO, Egypt) mixture in a rate of 2-1 and maintained by a continuous rate infusion of Propofol (Diprivan, Pfizer Inc.USA) in a dose rate of 0.15 mg/kg/min (Waelbers et al, 2009; Jia et al, 2015). The radius was exposed through craniolateral approach by incision of skin, subcutaneous tissue and separation between extensor carpi radialis and extensor digitorum communis muscle (Done et al, 1996 and Johnson and Dunning2005). Diaphyseal fracture was induced by gegli saw. All fractures were fixed by 6-hole DCP. In the second group HA used to fill the space between the two fracture fragments. On the other hand, the Nano magnesium enriched hydroxyapatite was used in the third group.

2.5. Clinical evaluation:

All dogs were observed all over the experimental period (90 day). Estimation of healing was done at 45, 75and 90 days by using X-rays and histopathological examination. X-ray was done in Department of Surgery, Radiology and Anesthesiology, Faculty of Veterinary Medicine, Benha University using simply HP X-ray machine.

2.6. Postoperative care

All dogs were subjected to local and systemic management for 10 days after surgical intervention. Three dogs from each group at each sampling time were euthanized using magnesium sulphate 12.5% through intravenous injection (AVMA, 2020). The radius of all animals at each time point were harvested and subjected to CT and histopathological examination.

2.7. CT Examination

The limbs were undergoing the shaft of the radius bone in dogs more than that consecutive CT scan using CT scanner (TOSHIBA 600 HQ, third-generation equip TCT, Japan) at Ahmed Faried Radiology Center, Benha, Egypt. The acquisition settings were 120 kv, 130 Ma and 1.5 seconds, thickness of 3 mm, pitch of 0.625, field of view of 45 cm and matrix size of 512×512 pixels. The images were started at the level of the proximal extremity of the radius and continuing 1cm distally in a row below the elbow till the level of the distal extremity of the radius. The investigation of CT images was compared according to Hounsfield grey scale (Jainil et al, 2018).

2.8. Histopathological examination

Tissue sections from the bone of all managed groups at each sampling time were firstly fixed in 10% buffered neutral formalin for histopathological examination, then decalcified in EDETA. Following proper dehydration, clearing then the samples were embedded in paraffin wax. Sections about 5μm thickness were prepared and stained with Meyer's hematoxylin and eosin (H&E) for histopathological examinations according to Suvarna et al. (2018). Histopathological scale of 0 to 7 points (Table 1) was established for the quality of healing at the site of bone fracture on the basis of the predominant type and amount of tissue (Emery et al. 1994).

2.9. Statistical analysis

Data were analyzed with two-way analysis of variance (ANOVA; experimental groups: duration) using (SAS software, 2004). Significant results were statistically evaluated with Tukey's post-hoc test and p-value ≤ 0.001 was considered

Table 1 Histopathological scoring system for bone healing

Score	Tissue Present
7	Bone only
6	More bone than fibrocartilage
5	More fibrocartilage than bone
4	Fibrocartilage only
3	More fibrocartilage than fibrous tissue
2	More fibrous tissue than fibrocartilage
1	Fibrous tissue only
0	Empty cleft

3. RESULTS

The dogs of all group exhibited mild to moderate degree of lameness where their operated limbs just touch the ground and the animal prefer the recumbent position. After three days, the dogs complete carry weight equally distributed in four legs. The wound of the legs showed the normal pattern of healing except two animals showed wound dehiscence and healing by mixed intension.

The radiographic follow up revealed no different changes of radius fracture healing in all groups. The statistical analysis showed a significant effect on density of bone (p ≤ 0.001) with the NHA-Mg that recorded the highest mean for bone density (Fig. 1).

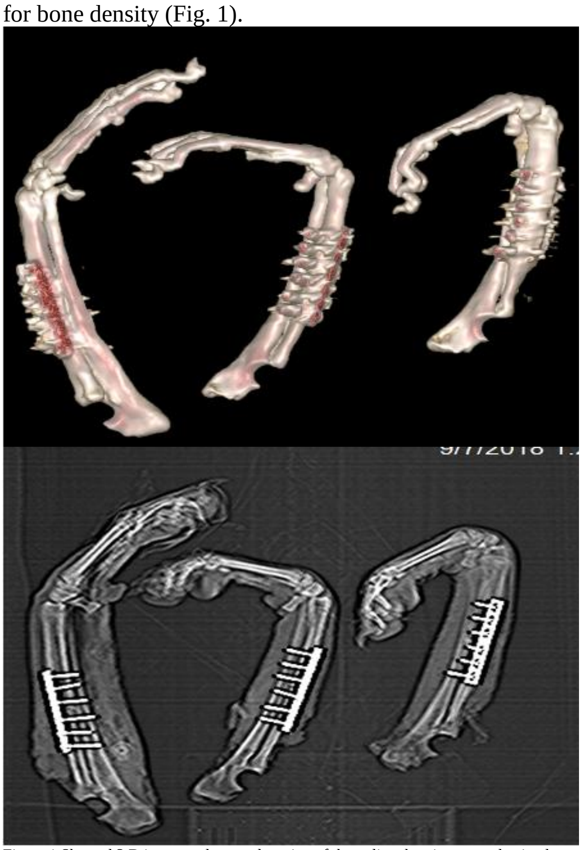

Figure 1 Showed 3 D image and coronal section of the radius showing control animal at 90 day (left), HA at 90 day (middle) and NHA- Mg at 90 day (right)

The duration showed a significant effect on density of bone (p ≤ 0.001) with the duration 90 days recorded the highest mean for bone density (Table 2).

Table 2 Generalized least square means ± standard errors for the effect of groups and duration on density of bone

	Density of bone	
Group	LSM	SE
Control	156.67[c]	2.30
NHA	269.33[b]	2.30
NHA-Mg	316.00[a]	2.30
Duration	LSM	SE
45 day	196.00[c]	2.30
75 day	252.11[b]	2.30
90 day	293.89[a]	2.30

Means Within the same column bearing different superscripts are significantly different at p≤ 0.001

Histopathological examination:

45 days' post-surgical operation

The histopathological examination of control group revealed fibrous tissue formation (score = 1) in the fracture gap of all dogs in this group (Fig. 2a). Multifocal, the proliferated fibrous tissue was infiltrated by variable numbers of inflammatory cells mainly macrophages (Fig. 2b). However, the healing scores of the animals treated with Nano hydroxyapatite or Nano Hydroxyapatite-Mg-groups were improved and are higher than those of the animals of the control group, as the treated dogs with NHA and NHA-Mg- group showed more advanced healing criteria than those of the control group. The gab in-between the two ends of fractured bone of two dogs treated with NHA was filled with excessive fibrous tissue formation with fewer amount of fibrocartilage tissue (score = 2) (Fig. 2c&d), however the third animal showed fibrous tissue formation only (score = 1) in the fracture gab. Meanwhile, all animals in NHA-Mg-group showed marked formation of fibrocartilage tissue with occasional fewer amount of wavy bone (score = 5) in the fracture gab (Fig. 2e&f).

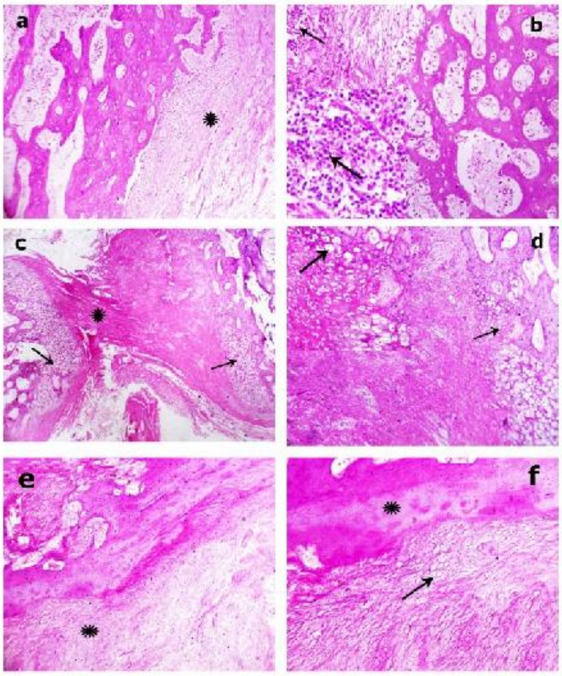

Figure 2 H&E stained sections of bone tissue taken at 45 days post-operative from control group (a-b), Nano Hydroxyapatite-group (c-d) and Nano Hydroxyapatite-Mg-group (e-f) showing, (a)fibrous tissue formation (asterisk,x100), (b) infiltration of inflammatory cells mainly macrophages in the proliferated fibrous tissue (arrow, x200, insert x400), (c) excessive fibrous tissue formation (asterisk) with fewer amount of fibrocartilage tissue (arrow) filling the gap between two fractured ends (x200), (d) fewer amount of fibrocartilage tissue(arrow,x200, insert, x400), (e) marked formation of fibrocartilage tissue (asterisk) with fewer amount of wavy bone (x100), (f) formation of fewer amount of wavy bone (asterisk) with fibrocartilage tissue formation (arrow, x200).

75 days post-surgical operation

Bone healing in control group revealed marked formation of fibrocartilage tissue with occasional fewer amount of fibrous tissue formation (score = 3) in between the two ends of fractured bone of all investigated animals of this group (Fig. 3a&b). Animals in NHA-group showed mild improvement in the healing process in comparison to the control group with average score of all treated animals among this group was 3.7 in addition one treated dog showed excessive fibrocartilage tissue with fewer amount of fibrous tissue (score = 3) were observed in the fracture gab (Fig. 3c&d). While the lesion in the remaining two dogs, the microscopic examination showed accumulation of fibrocartilage only in the gap between two fractured ends (score 4). Meanwhile, all animals in NHA-Mg-group showed marked healing by formation of wavy bone with few fibrocartilage tissue (score = 6) (Fig. 3e&f).

90 days post-surgical operation

The animals in control group revealed marked formation of fibrocartilage tissue (score = 4) in-between the two ends of fractured bone (Fig. 4a&b). All treated animals in NHA-group showed average score 4.7 where one treated dog showed formation of fibrocartilage tissue (score = 4) in the fracture gab (Fig. 4c), while the microscopic findings in the rest two from three treated dogs were accumulation of more fibrocartilage than bone in the gap between two fractured ends (score = 5). Healing quality in NHA-Mg-group had the highest score in comparison to the other investigated groups as marked formation of bone (score = 7) was filling the fractured gab and fused with the old bone (Fig. 4d).

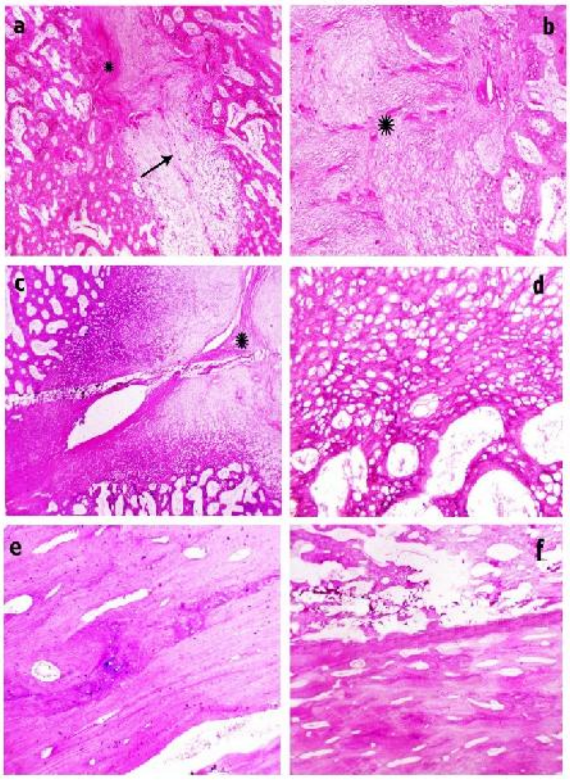

Figure 3 H&E stained sections of bone tissue taken at 75th day post-operative from control group (a-b), Nano Hydroxyapatite-group (c-d) and from Nano Hydroxyapatite-Mg-group (e-f) showing, (a) formation of fibrocartilage tissue (arrow) with fewer amount of fibrous tissue formation (asterisk) in-between the two ends of fractured bone (x200), (b) excessive formation of fibrocartilage tissue in-between the two ends of fractured bone (asterisk, x200), (c) marked fibrocartilage tissue with fewer amount of fibrous tissue (asterisk) in the fracture gab (x100), (d) marked fibrocartilage tissue formation (x400), (e) marked healing by formation of wavy bone tissue (x200), (f) clear formation of wavy bone tissue (x200).

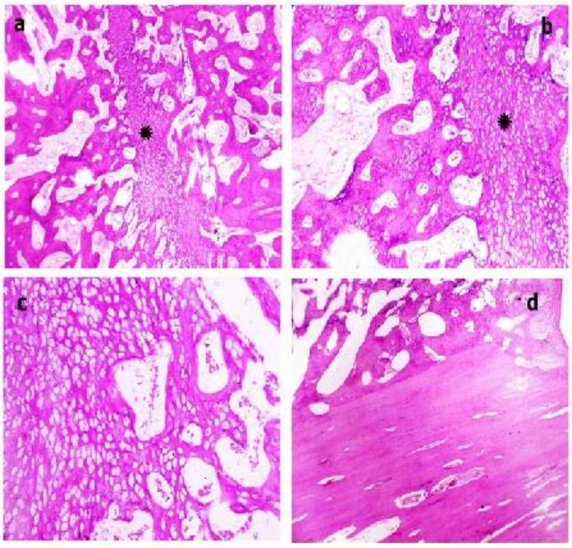

Figure 4 H&E stained sections of bone tissue taken at 90 days post-operative from control group (a-b), Nano Hydroxyapatite-group (c) and Nano Hydroxyapatite-Mg-group (d) showing, (a) formation of fibrocartilage tissue in-between the two ends of fractured bone (asterisk, x100), (b) note to fibrocartilage tissue formation (asterisk, x200), (c) formation of fibrocartilage tissue in the fracture gab (x400), (d) marked formation of new bone filling the fractured gab and fused with the old bone (x200).

4. DISCUSSION

In the current research, the results of the radiological and histopathological examinations showed that bone healing were enhanced when Mg was doped with hydroxyapatite. The obtained results indicated that Mg in doped with hydroxyapatite stimulates a favorable reaction in the injured area of the long bones. The pathologic evaluation revealed that the healing of bone fracture in NHA-Mg group was healed before that of the other two groups. The lesions of the control animals even at the end of 75 days post-operative were still in the fibroplasia stage, while the healing of fractured bone in hydroxyapatite-Mg group was in osteogenesis stage even at 45 days post-operative. These findings could be attributed to the implantation of Mg in bone that activate calcitonin gene-related polypeptides via Substance P neurons (McArthur et al., 2013). As the activation of Nociceptors, resulting in Substance P release (Ohgushi, 1989 and McArthur et al., 2013).

CT provides cross-sectional image of the area of interest in three dimensional reconstructions and can detect small fragment that may be obscured by surrounding bone in radiograph which facilitate early treatment of deformities. The CT images were not represented directly as they are in conventional X-ray images but was made of grey scale.

The computed tomography images up to 75 days of the control group revealed radiolucent density of the bone cleft, and somewhat increased at 90 days. On the other hand, the degree of density of control group which previously recorded at 90 days appeared at 75 days of the HA group and 45 days on NHA-Mg. These results co-inside with that reported by Xia et al. (2016).

The animals of NHA-Mg group showed priority of density pattern (75 days) where it resembles the pattern of NHA group at 90 days. The density configurations of NHA-Mg group at 90 days resemble the density of normal bone, similar results were concluded by Wang et al 2016 who reviewed that the nanomaterial's mimicking the Nano-features of bone and offering unique smart function are promising for better bone fracture repair.

Regarding to the obtained results of histopathological examination, the healing scores of the animals treated with NHA or NHA-Mg were improved and it was higher than those of the animals of the control group, as the treated dogs with NHA and NHA -Mg groups showed more advanced healing criteria than those of the control group. Hydroxyapatite, a crystalline phase of calcium phosphate found naturally in bone minerals, has shown great promise as an implant material as it exhibits initial mechanical rigidity and structure, and demonstrates osteo-conductive as well as angiogenic properties *in vivo* (Kilian et al. 2008; Appleford et al. 2009; Yoshikawa et al. 2000).

In osteoperiosteal gaps bridged with hydroxyapatite only, the porosities were invaded with fibrous tissue or fibrocartilage tissues were more than bone tissues. Occasionally, bone formation was observed in direct contact with hydroxyapatite, confirming its osteo-conductive ability, but it was insufficient to allow union. These findings were similar to those reported using hydroxyapatite. When the gap reaches a critical size the osteo-conductive properties of the material are insufficient to fill the gap with formation of new bone (Ohgushi et al. 1989).

5. CONCLUSION

CT and histopathological findings demonstrated that the NHA-Mg material showed superior and faster bone formation after 90 days post-operatively and the density of the bone defect is similar to the normal bone.

6. REFERENCES

1. Appleford, M.R., Oh S., Oh N., Ong J.L., 2009. *In vivo* study on hydroxyapatite scaffolds with trabecular architecture for bone repair. J. Biomed Mater Res A, 89 (4):1019–1027.
2. AVMA, 2020: AVMA guidelines for the euthanasia of animals. 2013 Edition (American Veterinary Medical Association, Schaumburg, Illinois, USA, 2013).
3. Childs, S., 2003. Stimulators of bone healing. Biologic and biomechanical. Orthop Nurs, 22 (6): 421–428.
4. Castiglioni, S., Cazzaniga, A., Albisetti, W., Maier, J.A., 2013. Magnesium and osteoporosis: current state of knowledge and future research directions. Nutrients, 5: 3022–3033.
5. Done, S. H., Goody, P.C., Evans, E. S., Stickland, N. C., 1996. Color atlas of Veterinary Anatomy, the dog and cat. Volume 3, Elsevier Limited, pp 183.
6. Emery, S.E., Brazinski, M.S., Koka, A., Bensusan, J.S., Stevenson, S. 1994. The biological and biomechanical effects of irradiationon anterior spinal bone grafts in a canine model. J Bone Jt Surg, 76 (4): 540.
7. Fossum, T.W., 2013. Small animal surgery. Fourth edition Mosby Elsevier, Pp 1142-1233.
8. Hanafi, M.E., Sallam, S.M., Mohamed, F.A., 2016. Characterization of Hydroxyapatite doped with different concentrations of magnesium ions. International Journal of Research Studies in Science, Engineering and Technology, 3 (8): 1-7.
9. Jia, N., Zhao, C., Wang, L., Li, Y. Cui, J. Cao, S., Li, R., Wang, C., Wu, Y., Wen, A. 2015. The effects of a propofol/alfentanil admixture on total intravenous anaesthesia in dogs undergoing splenectomy. Veterinarni Medicina, 60 (4): 194-201.
10. Jainil, P. S, Steveve, D.M., Randolph, L.M. and Martin, P.T., 2018. characterization of CT Hounsfield Units for 3D Acquisition Trajectories on a dedicated Breast CT system. J Xray Sci Technol.;26(4):535-551

11. Johnson, A. and Dunning, D., 2005. Atlas Of Orthopedic Surgical Procedures Of The Dog And Cat, Elsevier Saunders.
12. Kilian, O., Wenisch, S., Karnati, S., Baumgart-Vogt, E, Hild A, Fuhrmann R (2008). Observations on the microvasculature of bone defects filled with biodegradable nanoparticulate hydroxyapatite. Biomaterials, 29 (24-25): 3429–3437.
13. McArthur, N., Fomuki, W.T., Tanner, M.C., Huber, F.X., 2013. The nanocrystalline hydroxyapatite paste Ostim® may present a new possibility in the treatment of persistent nonunion. OA Case Reports, 2 (12): 118.
14. Nabiyouni, M., Ren, Y., Bhaduri, S.B., 2015. Magnesium substitution in the structure of orthopedic nanoparticles: a comparison between amorphous magnesium phosphates, calcium magnesium phosphates, and hydroxyapatites. Mater Sci Eng C Mater Biol Appl, 52: 11–17.
15. Ohgushi, H., Goldberg, V.M., Caplan, A.I., 1989. Repair of bone defects with marrow cells and porousceramic: experiments in rats. Acta OrthopScand, 60: 334-339.
16. Park, K.D., Lee, B.A., Piao, X.H., et al., 2013. Effect of magnesium and calcium phosphate coatings on osteoblastic responses to the titanium surface. J Adv Prothodont, 5: 402–408.
17. Suvarna, K.S., Layton, C. and Bancroft, J.D., 2018. Bancroft's Theory and Practice of Histological Techniques E-Book. Elsevier Health Sciences.
18. Tamimi, F., Nihouannen, D.L., Bassett, D.C., et al., 2011. Biocompatibility of magnesium phosphate minerals and their stability under physiological conditions. Acta Biomater, 7: 2678-2685.
19. Waelbers, T., Vermoere, P., Polis, I., 2009. Total intravenous anesthesia in dogs. Vlaams Diergeneeskundig Tijdschrift, 78: 160-169.
20. Xia, Y.,Zhou, P., Wang, F., Qiu, C., Wang, P., Zhang, Y., Zhao, L., Xu, S., 2016. Degradability, biocompatibility, and osteogenesis of biocomposite scaffolds containing nano magnesium phosphate and wheat protein both in vitro and *in vivo* for bone regeneration. International Journal of Nanomedicine¸11: 3435–3449.
21. Xiebo Y., Yueqin, S., Weihan, Y. and Yaohua, H., 2018. Enhancement of tendon-bone healing following rotator cuff repair using hydroxyapatite with TGFβ. Molecular Medicine Reports, 17: 4981-4988.
22. Yoshikawa, T., Ohgushi, H., Nakajima, H., Yamada, E., Ichijima, K., Tamai, S. 2000. *In vivo* osteogenic durability of cultured bone in porous ceramics: a novel method for autogenous bone graft substitution. Transplantation, 69 (1): 128–134.
23. Zahra, S.S., Oryan, A., Bigham, A.S., 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: 96-10