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***Pathology***

# Comparative Evaluation of Bone Regenerating Capacity Using Nanocrystalline Hydroxyapatite and Coral Composite: A Canine Model Study

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## ABSTRACT

Natural and synthetic graft materials have been studied *in vivo* and *in vitro* in osseous tissue repair. This study was carried out to investigate and compare the ability of the nanohydroxyapatite (Nano-HA) and coral composite (C-co composite) containing coral, gelatin and chitosan to induce new bone growth when implanted in a critical size defect in canine tibia. Fifteen adult mongrel dogs were used in this study. Groups 1, 2, 3, 4 and 5 were investigated after 2, 4, 8, 16 and 24 weeks, respectively. Three holes (10 mm diameter) were made at the upper third of the tibia. The first hole was implanted with C-co composite, the second one was left empty; while, the third hole was filled with Nano-HA. Healing of the implanted holes was evaluated using sequential radiography and histopathological evaluation at the end of each observation period. The holes implanted with C-co composite and Nano-HA were filled with mature compact bone; on the other hand, the control holes were filled with dense connective tissue. Both C-co composite and Nano-HAP behaved in a similar manner concerning their pattern of resorption. In conclusion, addition of chitosan and gelatin to coral improves its osteoinductive properties. Furthermore, both C-co composite and Nano-HA had a similar pattern for formation of new bone when used to fill critical size bone defect in canine tibia. C-co composite is cheap, available, easily prepared and can replace Nano-HA in bone grafting.

**Keywords**: Chitosan, Composite, Coral reef, gelatin, Nanohydroxyapatite.

## INTRODUCTION

Bone is biochemically and structurally complex (Brannigan and Griffin, 2016). It has a unique ability to interpret mechanical stress such as exercise by increasing bone mineral content and reducing the risk of fracture (Dutta and Dutta, 2013). Under normal circumstances, it has a good ability to repair, regenerate and remodel itself without scar formation (Vajtai, 2013). When a large bone defects are created as result of severe trauma or resection of tumor, bone loses its ability to regenerate itself. Which in turn require more sophisticated procedures such as bone grafting techniques (Stanovici *et al.*, 2016).

Bone healing is divided into direct and indirect healing (Claes *et al.*, 2012). Direct bone healing, the fracture is connected by osteons or Haversian system with absence of inflammatory response. Indirect bone healing occurs through three phases (inflammatory phase, repair phase, and the remodeling phase (Marsell and Einhorn, 2011). The inflammatory phase happens immediately after bone fracture as the trauma leads to rupture of blood vessels inside and around the fracture site forming a hematoma. The hematoma acts as a scaffold for the recruited inflammatory cells and inflammatory cytokines to initiate the

inflammatory response (Walters *et al.,* 2018). The repair phase is characterized by Callus formation and then collagen matrix is actively laid down through the area. The formation of callus has two types: the intramembranous ossification and the endochondral ossification (Kenkre and Bassett, 2018). During the remodeling phase bone restore its original shape and structure. Activity of osteoblast and osteoclast helps lamellar bone formation (Marsell and Einhorn, 2011; Kenkre and Bassett, 2018).

Bone graft substitutes help management of localized bone loss, enhance some of the desired mechanical qualities of bone, and provide unlimited availability. Moreover, bone graft substitutes possess osteointegrative and osteoconductive properties (Nandi *et al.,* 2008; Rahaman *et al.,* 2011; Emara *et al.,* 2013). On the other hand, most of the known bone graft substitutes lakes osteoinductive properties which made them unable to heal large bone defects when used alone (Ajeesh, 2010).

Coral as marine invertebrates has been used as bone substitute since more than 20 years ago (White, 1997; Clarke *et al.,* 2011). More than 2000 coral species exist but only 14 types have been studied to be a bone graft substitute (Bouchon *et al.,* 1995). Coral possesses good osteoconductive properties with limited osteoinductive/ osteogenic capabilities (Zhang *et al.,* 2007).

Hydroxyapatite (HA) either natural derived or synthetic is currently used for bone repair and regeneration in the form of granules, blocks and scaffolds (Yelten and Yilmaz, 2017). HA has excellent biocompatibility, bone bonding ability and its structural and composition is similar to the mineral phase of human bones (Santos *et al.,* 2007). Although natural HA is more closely matched to human bone composition, synthetic one is often used to overcome and reduce hydroxyapatite graft associated risks such as lack of durability, slow resorption rate, poor quality x-ray image and risk of migration from the recipient site (Rajendran *et al.,* 2014).

This study aimed to investigate and compare the ability of coral composite containing gelatin, chitosan and coral powder (C.co composite) as a cheap available source to replace Nanohydroxyapatite (Nano-HA) in inducing new bone regeneration when applied for repair of artificially induced critical size bone gap defects in dog tibia.

## MATERIALS AND METHODS

***Preparation of C.co Composite:***

Natural coral reef fingers (genus *Porites Astreoides*) were powdered alone, packed, and sterilized by autoclaving at 121 °C for 20 min. Chitosan (150 mg) purchased from (Chitolab. company, Egypt) and 3 gm of gelatin powder purchased from (Adwic company, Egypt) were dissolved individually in 6 ml hydrochloric acid, then mixed together in a water bath at 55°c. Finally, the coral powder was added to chitosan/gelatin mixture and formulated into small cylinder paste measuring 1 cm length and diameter. The pastes were adjusted to the size of the holes (10mm) and left at room temperature for 2-3 hours (hrs) to dry, then incubated at 55 °c for 12 hrs and finally incubated at 100 °c for another 4 hrs. After complete dryness, pastes were stored in a sterile double wrapped plastic roll and sterilized directly before implantation by ultraviolet radiation (UV) for 10 min.

***Nano-HA***

Nano-HA used in this study was purchased from Nanotech Company, Egypt. Prior to its use, it was placed in a double plastic wrap and sterilized by UV for 10 min.

***Dogs and surgical procedures***

Fifteen adult male mongrel dogs weighing 20 to 25 kg were used in this study. Dogs were acclimatized for 2 weeks before surgery in individual cages (130-cm width x 150-cm height x 150-cm depth) and had free access to food and water. The dogs were free from any musculoskeletal problems.

Dogs were pre-medicated by intravenous (i.v) injection of a mixture of atropine sulfate 0.05 mg/kg (Atropine sulfate®: 1mg/ml Adwia company, Egypt) and diazepam 1 mg/kg (Neuril®: 0.5% sol. Memphis Company, Egypt). Anesthesia was induced immediately through i.v injection of a mixture of Ketamine hydrochloride 10 mg/kg (Ketalar®: 5% sol. Amoun company, Egypt) and Xylazine 1 mg/kg (Xylaject®: 2% sol. Adwia company, Egypt). The anesthetic depth was maintained with i.v administration of 2.5 % thiopental sodium (Thiopental®: Epico company, Egypt). Hair shaving, septic preparation, and 10-cm skin incision were made at the proximal third of the inner surface of the

tibia of hind leg. The incision was made through skin and periosteum to show the bone. Three 10 mm holes with 1 cm distance between them were drilled under continuous saline irrigation to avoid thermal necrosis (Figure 1A). The holes were filled using sterile gauze to stop hemorrhage from the medullary cavity. The first hole was implanted with C.co composite, the second one was left empty as control, while the third hole was filled with Nano-HA (Figure 1B). The subcutaneous tissue was sutured using polyglactin 910 (Vicryl®) in simple continuous pattern and the skin was sutured using silk in a horizontal mattress pattern. Each dog received a prophylactic course of cefotaxime sodium (Cefotax®: Epico Company, Egypt) at dose of 4.5 mg/kg body weight by i.v route before the operation and repeated every 8 hrs for five successive days post-operation. Skin stitches were removed 10 days later. Dogs were randomly divided into 5 groups and investigated after 2, 4, 8, 16 and 24 weeks, respectively. The study protocol was approved by the Animal Care and Use Committee, University of Sadat City.

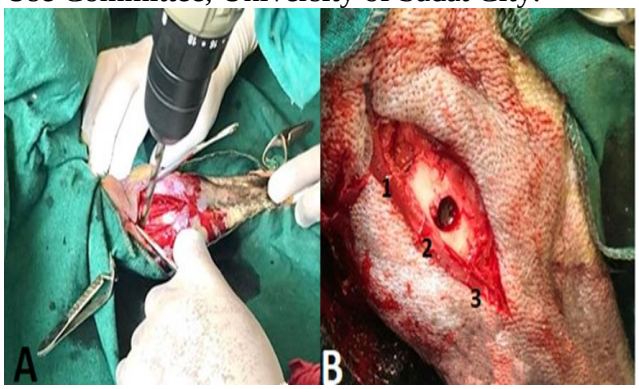


Fig. 1: A) Showing skin incision and drilling of three 10 mm holes 1 cm apart at the proximal third of the tibia under contentious saline. B). showing implantation of the formed holes with graft materials, the first hole was implanted with C.co composite, the second one was left empty as control, while the third hole was filled with Nano-HA powder .

***Post-Operative Follow-Up Evaluation***

***Clinical evaluation***

Clinical signs were observed daily for all dogs including body temperature, heart rate, respiratory rate, and appetite. The tibia was examined for swelling, inflammation, wound drainage, and regional lymph nodes swelling.

***Radiographic evaluation***

Medio-lateral radiographs were taken to the operated tibia using X-ray apparatus (Semens300) at zero day and then at 2, 4, 8, 16, 24-weeks post operation. In each radiograph the implantation sites were evaluated for their radiographic density and the degree of implant integration to surrounding host bone.

***Histopathology examination***

Three dogs were euthanized at each time point (2, 4, 8, 16, and 24 weeks) post operation. The tibial holes were examined grossly for partial or complete filling. Bone blocks including the implanted holes were collected, immediately fixed in 10% buffered formalin for 72 hrs. The samples were decalcified using 10% EDTA (El Nasr pharmaceutical chemical company, Egypt) for a month at 37°c. Decalcified samples were washed, processed and embedded in soft paraffin. A 3- 5µm sections were mounted on glass slides, deparaffinized, and stained with hematoxylin and eosin stain (H&E) for histopathological examination (Bancroft and Gamble, 2002).

## RESULTS

***Clinical assessment:***

There is no evidence of infection or seroma reaction in the operated animals as assessed by absence of swelling, hotness of the operated limbs and regional lymph node swelling.

***Radiographic results***

On examination of the radiographs taken for the operated tibia directly after the operation and throughout the observation period, it was noticed that; immediately post-operation (day zero), the control holes appeared radiolucent while the holes implanted with C-co composite appeared radiopaque. The holes implanted with Nano-HA appeared homogenously radiodense with surrounding host bone and radiolucent than C-co implanted holes (Figure 2A). By the end of the 2$^{nd}$ week post-operation, a slight radiolucent zone could be detected at the margin of C-co implanted holes separating between the implanted material and the host bone. The entire radiodensity of the hole is reduced indicating resorption of the implanted materials. Nano-HA implanted holes showed mild resorption of the implanted materials which appeared as a radiolucent zone at one side. The control holes showed no detectable changes comparing to day zero (Fig. 2B). By the end of the 4$^{th}$ weak post-operation; the implanted C-co composite tends to aggregate at one side. The holes implanted with Nano-HA showed presence of a radiolucent zone separating between the implanted materials and

the host bone indicating resorption of the implanted materials. The control holes appeared irregular with presence of a faint radiopaque zone at its peripheral margins (Figure 2C). At 8th week post-operation, the holes implanted with C-co showed complete resorption of the implanted materials. Its center appeared radiolucent with a marked radiopaque peripheral zone with marked reduction in its diameter comparing other holes. A slightly radiopaque zone was detected at the margins of the control holes and the holes implanted with Nan-HA. The implanted Nano-HA could be easily detected at the center of the hole (Figure 2D). At 16th weeks post-operation, the holes implanted with C-co showed marked reduction in its diameter. The control holes and holes implanted with Nan-HA showed no detectable changes comparing to previous period (Fig. 2E). At 24th week post-operation the holes implanted with C-co completely disappeared and the implanted area appeared homogenously radiodense comparing to the surrounding host bone. Although they showed marked reduction in its diameters, the control holes and the holes implanted with Nano-HA, could be easily detected radiographically with increased radiodensity at its center (Fig. 2F).

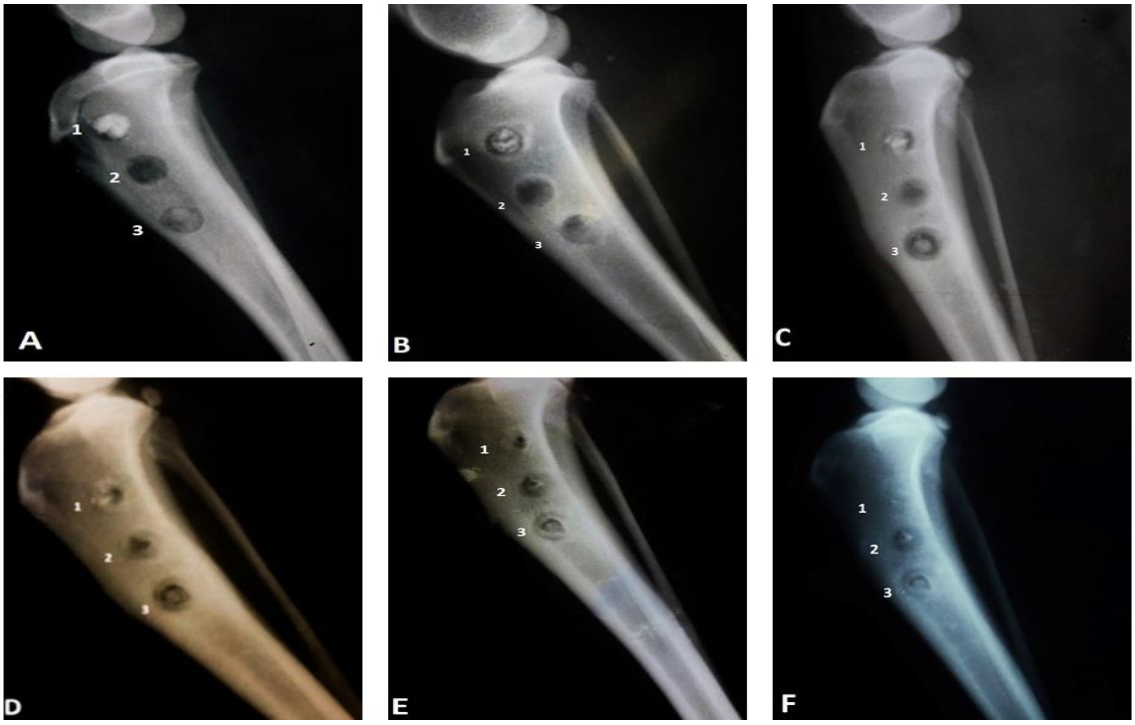


**Figure 2**: Showing sequential radiography of the operated tibia: (1) hole implanted with C-co (2) control hole (empty), and (3) hole implanted with Nano-HA. A) Day zero, the control hole appeared radiolucent while the hole implanted with C-co appeared more radiopaque comparing to hole implanted with Nano-HA. B) Two weeks' post-operation, the implanted materials at C-co and Nano-HA implanted holes begins to be resorbed which marked by presence of radiolucent zone separating the implanted materials from the host bone C) At 4 weeks post-operation, the implanted C.co material tends to aggregate at one side of the hole with marked reduction of the hole size. The implanted Nano-HA aggregated at the center of the hole with presence of radiolucent zone separating the implanted materials from the host bone. D) At 8 weeks post-operation, the C-co implanted holes showed marked reduction in its size comparing to other holes. E) At 16-week post-operation, the C-co implanted holes showed marked reduction in its size comparing with other holes with complete resorption of the implanted materials. F) At 24 weeks post operation, the holes implanted with C.co completely disappeared and the area appeared homogenously radiodense comparing to the surrounding host bone. The control holes and the holes implanted with Nano-HA showed marked reduction in its diameters with increase radiodensity at its center.

### *Histopathology examination*

Microscopical examination of bone holes from control and implanted groups with C.co composite and Nano-HA at each time point post-operation showed the following: At 2 weeks post-operation, bone holes from control group showed loose granulation tissue, inflammatory cells infiltration, hemorrhage, and congested blood vessels (Fig. 3a). Bone holes from C.co composite group showed well-arranged dense granulation tissue and congested blood vessels.

(Fig. 3b) Bone holes from Nano-HA group showed dense granulation tissue and bony cartilaginous matrix filling the entire bone holes (Fig. 3c). At 4 weeks post-operation, bone holes from control group showed dense granulation tissue, inflammatory cells, congested blood vessels and remnants of necrosed bone (Fig. 3d). Bone holes from C.co composite group showed dense arranged connective tissue and osteoblastic activity (Fig. 3e) Bone holes from Nano-HA group showed osteoblastic and osteoclastic activity, more cartilaginous matrix mixed with few dense connective tissue (Fig. 3f). At 8 weeks post-operation, bone holes from control group showed osteoclastic activity and denser connective tissue (Fig. 4a). Bone holes from C.co composite group showed well organized connective tissue, osteoblastic activity and new woven bone formation (Fig. 4b). Bone holes from Nano-HA group showed soft callus formation (Fig. 4c). At 16 weeks' post-operation, bone holes from control group had dense connective tissue (Fig. 4d). Bone holes from C.co composite group had osteoblastic activity and woven bone formation (Fig. 4e). Bone holes from Nano-HA group showed complete filling with soft callus formation and start of haversian system formation (Fig. 4f). At 24 weeks' post-operation, bone holes from control group were filled with connective tissue and formation of woven bone at the margins (Fig. 5a). Bone holes from C.co composite group were completely filled with mature compact bone with Haversian system (Fig. 5b). Bone holes from Nano-HA group had mature bone with haversian system formation (Fig. 5c).

## DISCUSSION

It is a generally accepted that a good bone substitutes should mimic cancellous bone characters, having high osteoinductive, angiogenic potentials and good biological safety. Moreover, the scaffolds should be biocompatible, biodegradable, easily eliminated with no immune response (Haugen *et al.,* 2018).

The Nano-HA and coral scaffold biomaterials are widely used as bone substitutes. Nano-HA has mineral structure and surface properties very similar to that of bone. Moreover, its smaller grain size increases its surface area causing rapid osseo integration (Sullivan *et al.,* 2014). Coral is composed primarily of calcium carbonate (more than 97% of its weight) (Pountos *et al.,* 2016). Coral has good osteoconductive properties with limited osteoinductive/ osteogenic capabilities (Zhang *et al.,* 2007). Coral has been mixed with chitosan and gelatin to fill artificially induced critical gap bone defect in canine tibia (Emara *et al.,* 2013).

In this study, C.co composite and Nano-HA were selected to reconstruct critical size gap defect in canine tibia and to compare between them radiologically and histopathologically especially C.co composite is cheap, available and could be a good alternative to Nano-HA in bone grafting.

The use of animal models for testing new formulations in bone repair remains the golden standard in the pre-clinical phase. Animal models provide a great opportunity to follow up the effect of newly developed bone formulations and to determine the extent of new bone formation radiographically and histologically (Peric *et al.,* 2015; Abdel-Salam et al, 2020). In the present study, 10 mm gap defect was made in the canine tibia which coincided with Arnaud et al. 1999 that a 9 mm trephine defect is considered as a critical size defect that cannot heal spontaneously when left empty. In addition, the technique performed in this study allows preliminary evaluation of the biological properties of several agents within the same animal and minimize the effect of biologic differences between animals (Griffon *et al.,* 2001).

Concerning our radiological results, the Nano-HA implanted holes appeared homogenously dense with the surrounding host bone, while; the holes implanted with C-co composite appeared radiopaque directly after the operation until the end of the 4$^{th}$ week post implantation. This is due to the higher calcium content of coral which exceed 97% of its weight (Vuola, 2001). Both C-co composite and Nano-HAP behaved in a similar manner concerning their pattern of resorption. Both started to be resorbed from the periphery and preceded toward the center taking along period. Nano-HA was resorbed in a slow rate comparing to C-co composite. The later was completely resorbed by the end of the 4$^{th}$ week while the Nano-HA was detected radiographically till the end of the 16$^{th}$ week.

These results disagree with (Meskinfam *et al.*, 2018), who stated that Nano-HA marked by rapid absorbability comparing to its conventional counterpart. The reason for rapid absorption of C.co composite may be attributed to the addition of chitosan and gelatin to coral improved the overall composite osteoinductive properties, cell adhesion, differentiation, and rapid bone healing by stimulating new bone growth (Saraswathy *et al.*, 2001,2004; Emara *et al.*, 2013).

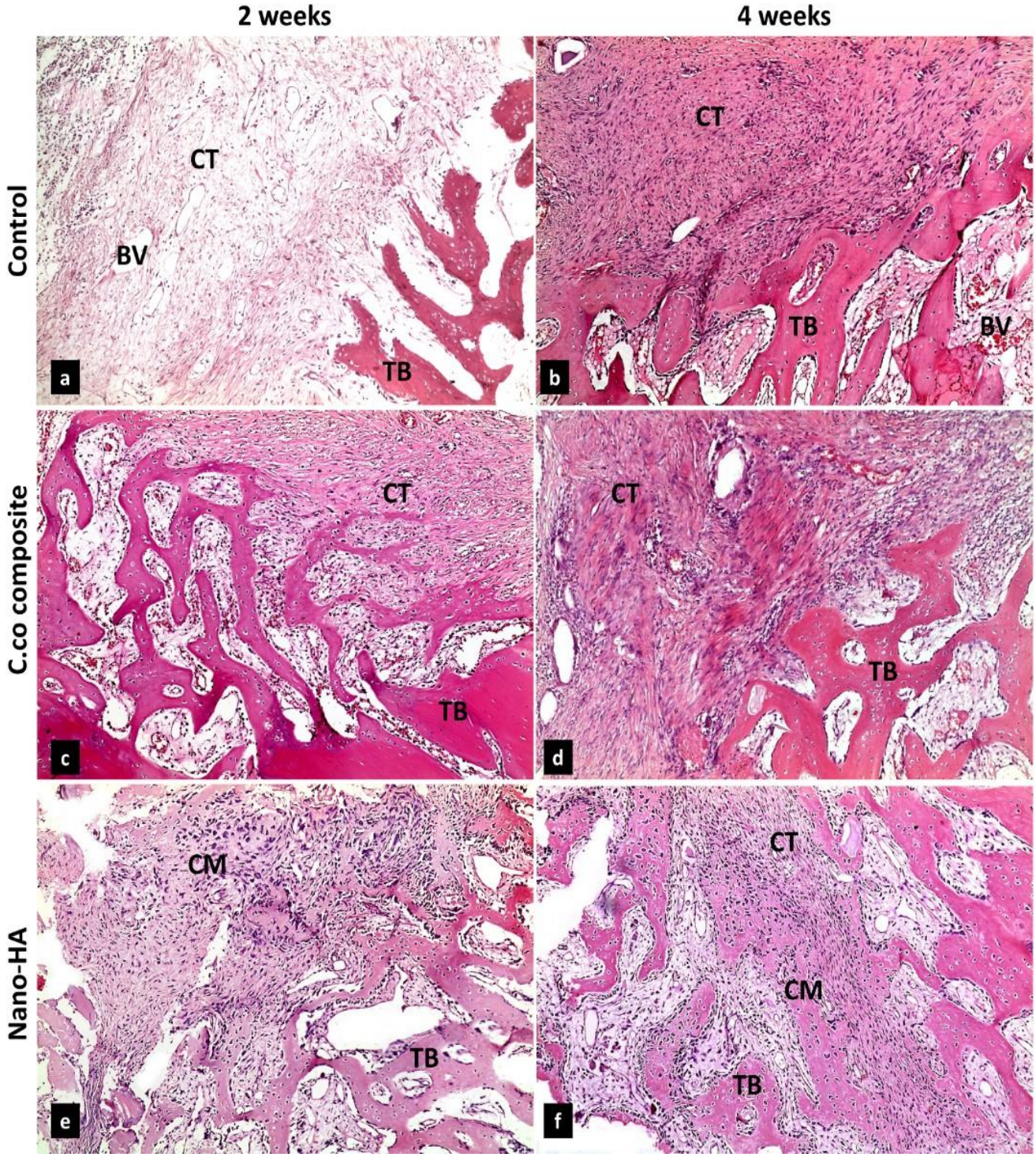


**Fig. 3:** Dog, tibial bone (TB). a, b and c represent control, C.co composite, and Nano-Ha group at 2 weeks post operation, respectively**.** a; bones holes had loose connective tissue (CT), inflammatory cells infiltration hemorrhage, and congested blood vessels (BV). b; bone holes showed well-arranged dense CT and congested blood vessels. c; bone holes dense CT and bony cartilaginous matrix filling the entire bone holes (CM). H&E stain. X10. d, e, and f represent control, C.co composite, and Nano-Ha group at 4 weeks post operation, respectively**.** a; bone holes showed dense CT, inflammatory cells, congested blood vessels and remnants of necrosed bone. e; bone holes showed dense arranged CT and increased osteoblast activity. f; bone holes showed osteoblastic and osteoclastic activity, more cartilaginous matrix mixed with few dense CT. H&E stain. X10**.**

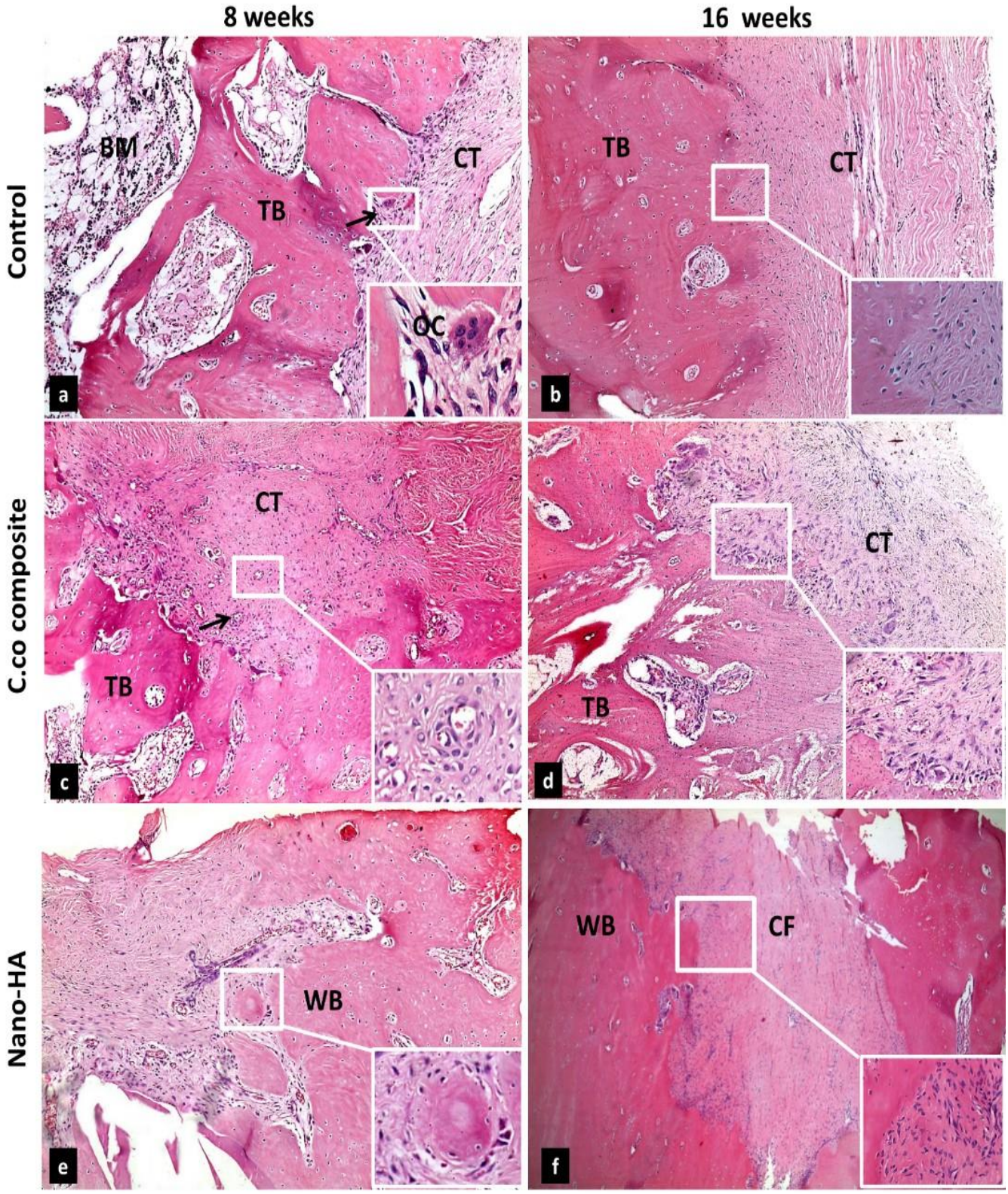


**Fig. 4:** Dog, tibial bone (TB). a, b and c represent control, C.co composite, and Nano-Ha group at 8 weeks post operation, respectively. a; bone holes showed osteoclastic activity (inset) and denser CT. b; bone holes showed well organized CT (inset), osteoblastic activity and new woven bone formation. c; bone holes showed new formation of woven bone (WB) (inset). H&E stain. X10. d, e, and f represent control, C.co composite, and Nano-Ha group at 16 weeks post-operation, respectively. d; bone holes had dense CT (inset). e; bone holes showed osteoblastic activity and woven bone formation (inset). f; bone holes showed complete filling (CF) with WB formation and new haversian system (Inset). H&E stain. X10 (control, C.co composite), x4 (Nano-HA).

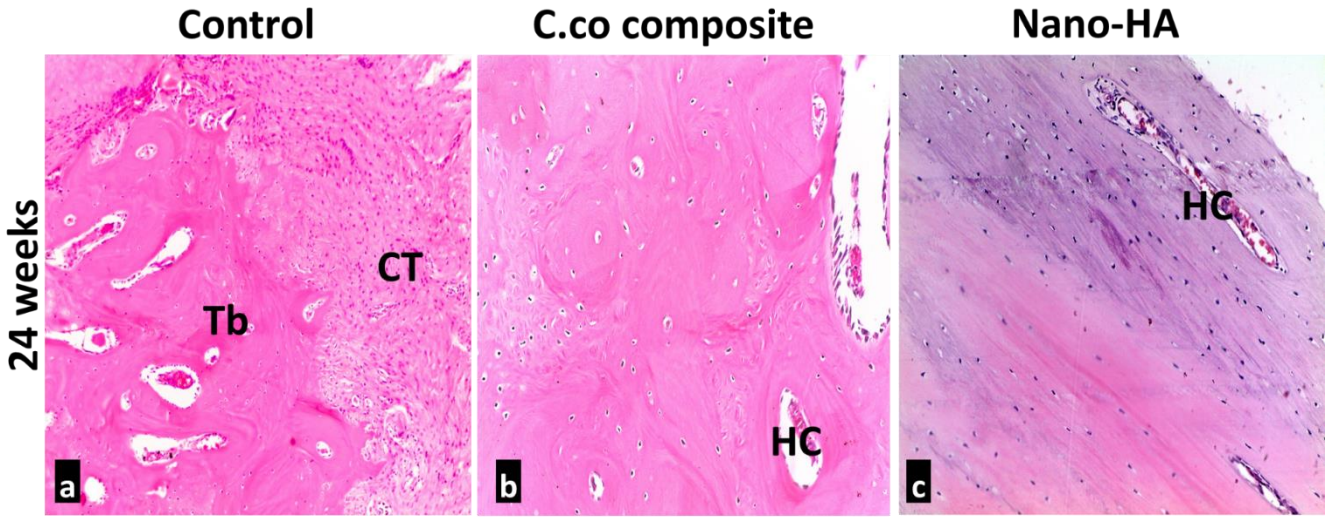


**Fig. 5:** Dog, tibial bone (TB). a, b and c represent control, C.co composite, and Nano-Ha group at 24 weeks post operation, respectively. a bone holes were filled with CT and formation of woven bone at the margins. b; bone holes were completely filled with mature compact bone with Haversian system (HC). c; bone holes had mature bone with haversian system formation. H&E stain. X20.

Histological results confirmed the former radiological findings. It showed marked increase of osteogenesis with new bone formation at the holes implanted with C-co composite and Nano-HA comparing to the control ones which was filled with connective tissue and small amount of woven bone which is not enough to fill the whole defects (Nandi *et al.*, 2008). Osteogenesis and new bone formation of C-co composite and Nan-HA holes started by the end of the 2nd week and became mature by the end of the 24th week post-operation. This may be attributed to the biocompatibility, biodegradability and bioactivity of chitosan and Nano-HA granules respectively (Martino *et al.*, 2005). Microscopic examination successed to explain why Nano-HA could be detected radiographically till the end of the 16th weeks post observation? It can be stated that the healing pattern of the Nano-HA occurred by partial replacing of its granules by the newly formed bone through a creeping substitution process (Wang and Yeung, 2017).

Concerning the amount of the newly formed fibrocellular tissue, the control holes showed marked connective tissue comparing to C-co composite and Nano-HA implanted holes which appeared more prominent by the end of the observation period. This is due to the suppressing effect of chitosan upon fibroblasts proliferation and enhancing osteoblastic activities (Lee *et al.*, 2002). Also, the promoting effect of Nano-Ha granules on osteoblast adhesion, proliferation, osseointegration, and the deposition of calcium-containing minerals on the implant surface (Von Doernberg *et al.*, 2006).

## CONCLUSION

In conclusion, addition of chitosan and gelatin to coral improves its osteoinductive properties. Furthermore, both C-co composite and Nano-HA had a similar pattern for formation of new bone when used to fill critical size bone defect in canine tibia. C-co composite is cheap, available, easily prepared and can replace Nao-HA in bone grafting.

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**Conflicts of interest:**

The authors declare that they have no conflict of interest.

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