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Evaluation of the effect of calcium phosphate nanoparticles in accelerating healing process of an induced femoral bone defect in dogs

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ABSTRACT

To investigate the suitability of calcium phosphate nanoparticles (CaP-NPs) as bone graft substitute in an experimental critical-size femoral bone defect (0.5 cm), six apparent clinically healthy mongrel male dogs of, average age 2 years, average weight 10-20 kg.b.w.t were randomly allocated into two equal experimental groups (3dog each).Control group without any implants, and treated group received CaP-NPs implant. Firstly, transverse femoral diaphyseal osteoctomy was made by Gegli saw. Then diaphyseal critical-size femoral bone defect (0.5 cm) was fixed by 3.5mm dynamic compression plate with 6 holes. Finally, the defect was filled with CaP-NPs. The observation period extend to 90 days post operation. During which the operated animals were evaluated clinically, radiologically and histopathologically. And also biochemical evaluation and computed tomography examination were also conducted to confirm the result. . Clinically, CaP-NPs group was superior to control in full weight bearing, soft tissue healing and resolution of inflammation at 10-15 days post-operation. Radiologically, the defect was completely filled with new bone formation after 10 weeks in CaP-NPs group. The control group showed a relatively slow healing process, and the union was complete after 13 weeks. Biochemical analysis showed a significant elevation of ALP activity, Ca, and Ph in CaP-NPs group. Computed tomography and histopathological examination at 13 weeks revealed better bone healing through marked bridging callus formation in CaP-NPs group than control one. The obtained result revealed CaP-NPs is a promising bone grafting material for treatment of bone defects due to its biocompatibility, osteoconductive and osteoinductive properties.

1. INTRODUCTION

The repair of large bone defects resulting from trauma, disease, oncologic resection, or mal confirmation is a great challenge in veterinary practice. Therefore, there is an increasing demand for the development of effective therapies or bone grafts for bone regeneration and for regaining high quality of life.

Bone grafts aim to enhance bone healing by acting as a scaffold for clot development, maturation, and remolding that supports bone formation in defects. It also acts as a structural framework and metrics for osteoblast attachment and proliferation. (Brydone 2010). The ideal characteristics of bone graft material are non-antigenic, nontoxic, easily adaptable, strong, resilient, resistant to infection, sufficiently available, able to induce osteogenesis, and minimal surgical procedures.

Several investigations were applied for bone grafts including the application of autografts, xenografts, and alloplasts (Alves et al., 2012). The usage of autologous bone is limited, as it can result in many complications during harvesting (Keating and McQueen, 2001). Immune reactions and disease transmission are additional hazards associated with allogeneic bone transplantation. For these reasons, there is an urgent need for the creation of an efficient synthetic bone substitute (Sun et al., 2011).

In the last decades, nanoparticles have been widely used in the field of bone grafting. (Levingstone et al., 2019). The size of nanoparticles used for therapeutic purposes typically ranges from 10 to 100 nm as smaller particles are easily eliminated by the kidneys (Walmsley et al., 2015). From the therapeutic point of view, nanoparticles offer a number of benefits. They have a high surface-to-volume ratio, which enables a larger driving force for diffusion and higher particle solubility and tolerability by the body. The high surface-to-volume ratio allows specific proteins to adherence differently, which makes them especially well-suited for the delivery of therapeutic agents (Dorozhkin, 2010).

Calcium phosphate nanoparticles have been investigated for biomedical applications. Among the biomaterials, Calcium phosphate (CaP) and crystalline phase of calcium phosphate (hydroxyapatite), have received particular attention, due to their resemblance to the mineral phase of natural bones that confirms their biocompatibility (Cross et al., 2016). Moreover, hydroxyapatite nanoparticles|(HA) most closely mimic the type of calcium phosphate present in naturally healthy bones. Alternatively, CaP is the most common type of bone graft material due to its higher biocompatibility, greater osteoinductivity, and increased osteoconductivity (Chen et al., 2017). The present study aimed to evaluate the effect of calcium phosphate nanoparticles (CaP-NPs) on the healing of fractures in dog-

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model according to the following parameters: clinical, radiological, biochemical, computed tomography, and histopathological evaluations.

2. MATERIAL AND METHODS

2.1. Ethical approval statements

All experiment procedures were approved by the ethical committee of the Faculty of Veterinary Medicine, Benha University, for animal care and experimentation under approval number (BVFVTM 22-02-23).

2.2. Preparation of calcium phosphate nanoparticles (CaP-NPs)

Calcium phosphate nanoparticles were prepared by Sol gel by a solution casting technique using 30 gm of Na3PO4 dissolved in 250 ml deionized water at room temperature for 5 minutes with continual stirring until Na_3PO_4 was fully solved. Then, freshly prepared 30 gm of $CaCl_2$ solution in 250 ml deionized water with continuous stirring for 5 minutes at ambient temperature was added to Na_3PO_4 solution and dropped wisely. The reaction was kept for 4 hours weekly to form a white precipitate then make filtration and 4 times washing processes then dry in the oven at 60 °C. The mixture was ground well, and Cap is formed after calcination in the oven at 500 °C (Qiu et al., 2022)

2.3. Experimental design

Six apparent clinically healthy mongrel male dogs, average age 2 years, average weight 10-20 kg.b.w.t were randomly allocated into two equal experimental groups (3dog each). The Control group was left empty without any implant. The other group was treated by CaP-NPs. After surgical intervention, clinical, plain radiographic, and biochemical (alkaline phosphatase activity, and inorganic calcium and phosphorus levels) investigations were done at 0, 15, 30, 45, 60, and 90 days. Computed tomography (CT) and histopathological examination were carried out at 90 days post-operation. The experiment was done at the Department of Surgery, Radiology, and Anesthesiology, Faculty of Veterinary Medicine; Benha University

2.4. Surgical operation

Animals were generally anesthetized by Xylazine (Xyla-Ject® 20 mg/ml Adwea Pharmaceutical, Egypt) at a dose of 11 mg/kg B. Wt. and Ketamine (Ketamine 500 mg/10 ml., EIPICO, Egypt) at a dose of 10 mg/kg B. Wt. (Hall et al., 2001) and maintained by a continuous infusion with Propofol (Diprivan®, Pfizer Inc. USA) at a dose of 0.15 mg/kg/min (Njoku, 2015). The right hind limb was prepared for aseptic surgery. The surgical intervention was performed on the right femur which was exposed through the craniolateral approach. The periosteum was stripped by periosteal elevator, then a critical size defect (0.5 cm) was induced in the middle third of femoral diaphysis. Then fixed by 3.5 mm dynamic compression plate with 6 holes (Hayashi et al., 2018).

In the control group, the defect was left empty without any biomaterial implantation. In the treated group, CaP-NPs were used to fill the space between the two osteomatized bone fragments. (Fig.1). the incisions were closed as routine surgical procedures. All dogs were daily given cefotaxime (Cefotax®, EPICO Pharmaceutical, Egypt) at a dose of 30 mg/kg B. Wt. IM and Meloxicam (Mobitil®, MUP Pharmaceutical, Egypt) at a dose of 0.2 mg/kg B. Wt. for 5 days post-operation. Food and water were maintained ad libitum.

2.5. Clinical evaluation

All dogs were routinely monitored post-operatively for the following: wound healing, swelling at the site of operation, atrophy of the muscle, and weight bearing capacity. Scoring the operated limb during movement was done according to Maiti et al. (2007) as follows:

Table (1) showed scoring the operated limb during movement

Score	Response
1	The limb not touching the ground
2	Toe of test limb touching the ground occasionally
3	Toe of test limb touching the ground frequently
4	Paw touching the ground with partial weight bearing
5	Paw touching the ground with full weight bearing

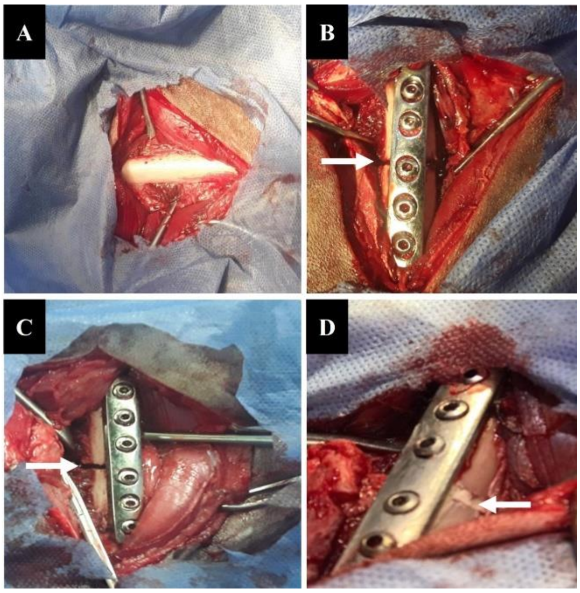

Figure 1 Surgical exposure of femur bone between vastus lateralis and biceps femoris muscle (A), critical size bone defect (CSD) in femur diaphysis (B), control group left without any biomaterial implantation (C), CAP-NPs group was treated by calcium phosphate nanoparticles (D).

2.6. Radiological evaluation

Sequential radiography was done before and immediately after the operation and at 15-, 30, 45-, 60- and 90-days post-operation (PO) using Simply HP X-ray machine (Simply HP 32, ITALY). Two radiographic projections were taken (lateromedial and craniocaudal views). All radiographs were evaluated for callus formation and the presence of osseous unions.

2.7. Serum biochemical analysis

Serum alkaline phosphatase activity, and inorganic calcium and phosphorus content were analyzed using Microlab ARX 3+ 1 machine as previously mentioned at 0-, 30-, 60- and 90-days post-operation at Clinical Laboratory of Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Benha University.

2.8. Computed tomography examination.

Computed tomography was conducted after euthanasia (using magnesium sulfate 12.5% through intravenous injection according to Sallam et al. (2020) at the 90^{th}-day post-operation by CT scanner (TOSHIBA 600 HQ, third-generation equip TCT, JAPAN) at Private Radiology Center, Benha, Egypt. The images were started at the level of the proximal extremity of the femur and continued 1 cm distally in a row below the stifle joint till the level of the distal extremity of the femur.

2.9. Histopathological examination

At the end of observation period (90 days), all operated dogs were euthanized, and implantation site were harvested, freed from any adhering soft tissue, fixed in 10% buffered formaldehyde solution, and decalcified using a formic-hydrochloric acid solution. Following proper dehydration and clearing, the tissue pieces were processed and embedded in paraffin wax. Sequential pieces 5 mm thick were cut perpendicular to the bone axis and stained with Meyer's hematoxylin and eosin (H&E) according to Sterchi (2019) to evaluate the bone healing pattern.

2.10. Statistical analysis:

Biochemical analysis data were presented as mean (± SEM) and analyzed using an independent student *t*-test to compare the treated and control groups. Besides, One-Way ANOVA was used to compare mean changes before and after surgery for the same group. All statistical analyses were conducted using SPSS (Ver. 21). The P value was set at 0.05 to denote the significant differences.

3. RESULTS

3.1. Clinical evaluation

In the Control animals, the surgical wound healed by the first intention within 10-15 days except one animal healed by the second intention due to surgical stitch breakdown. Atrophy of the muscle didn't appear in any animal. Mild to moderate swellings were noticed at 5-12 days post-operation in two dogs. Weight-bearing was good in all animals at Day-4 post-operation. However, after five days, weight bearing was low in two dogs that suffered from pain and moderate swelling, and it improved rapidly after 15 days (Table 1).

In CaP-NPs group, the surgical wound healed by the first intention within 7-11 days in all animals. Atrophy of the muscle wasn't evident in any animal. Mild to moderate swellings were noticed during the first 3 days post-operative in one dog. Weight- bearing was good in all animals from the 2nd day post-operation.

Table (2) show Differences in operated limb scoring during movement in control and treated groups.

Behavioral item	Control group	CaP-NPs group
Test limb not touching the ground	Present	Absent
Toe of test limb touching the ground occasionally	Present	Absent
Touching the ground frequently	Present	Present
Paw touching the ground with partial weight bearing	Present till 15 days	Present till 10 days
Touching the ground with full weight bearing	Present after 15 days	Present after 10 days

3.2. Radiological evaluation

Radiographically all animals lacked any bone or joint illness before intervention. After surgery there was a good reduction, fixation, and alignment of osteotomized bone fragments in animals of both groups (Fig. 2). On the 15th day, there was little periosteal reaction and gap between osteotomized bone fragments in both groups. On the 30th day, control dogs showed little periosteal reaction and gap between osteotomized bone fragments, while CaP-NPs group showed a clear periosteal reaction and narrowing in the gap between osteotomized bone fragments. On the 45th day, the control group showed further periosteal reaction and little narrowing in the gap, while in CaP-NPs group there was a good periosteal reaction and more narrowing in the gap between osteotomized bone fragments.

On the 60th day, control dogs showed relatively more periosteal reaction and narrowing in the gap between osteotomized bone fragments, Meanwhile, CaP-NPs group showed an obvious periosteal reaction and incomplete obliteration of the gap. On the 75th day, control animals showed an obvious periosteal reaction and incomplete obliteration of the gap, Nevertheless, CaP-NPs group showed complete obliteration of the osteotomies line. On the 90th day, the control group showed a complete obliteration of the fracture site (Fig. 2).

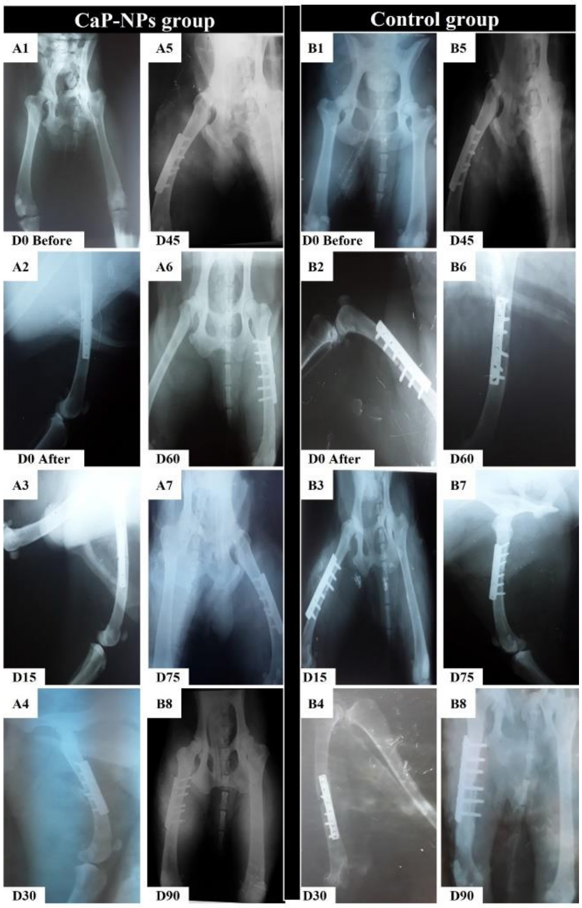

Figure 2 Sequential radiograph of the right femur of both the CaP-NPs group (A1-A8) and the control group (B1-B8) at 0-, 15-, 30-, 45-, 60-, 75- and 90-days post operation, respectively.

3.3. Serum biochemical analysis

In the control group, there was no significant difference in ALP activity from 0 to 60 days post-operation, but it decreased significantly thereafter. The activity of ALP was substantially higher on Day 30 and 60 than at Day 0 and 90 post-surgery in CaP-NPs group. The inorganic Ca showed a significant increase in control and treated groups at Day-30 and -60 before returning to normal levels at Day-90. In the control group Ph decreased significantly from Day-30 to Day-60 and returned to the ordinary level on Day 90th. CaP-NPs treated dogs showed a progressive drop in Ph from Day-0 to Day-90 post-operation. (Fig..3)

3.4. Computed tomography examination.

Computed tomography showed a good reduction and fixation process, significant bridging callus formation, increase in bone density, normal alignment of osteotomized bone fragments, and clear periosteal reaction in CaP-NPs group than the control group.

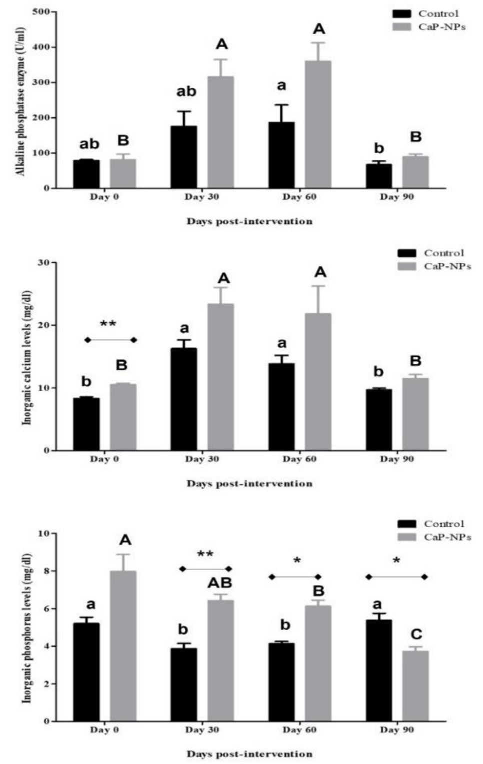

Figure 3 Variation in the serum (a) alkaline phosphatase activity, (b) inorganic calcium and (c) inorganic phosphorous in control and CAP-NPs groups.

3.5. Histopathological examination:

After 13 weeks of implantation, the Haversian system in the treated group was fully restored at the level of the defect with clear Haversian canals which contain some amount of bone marrow and normal periosteum with complete absence of fibrocartilage tissue. The defect was entirely filled with bone tissue, and a sizable amount of mineralized tissue was seen. Bone tissue apposition was indicated by the presence of osteoid tissue and mature osteoblast along the Haversian system. Control animals showed a marked formation of fibrocartilage tissue with the presence of woven bone, immature osteoblast, and less amount of fibrous tissue formation.(Fig.4)

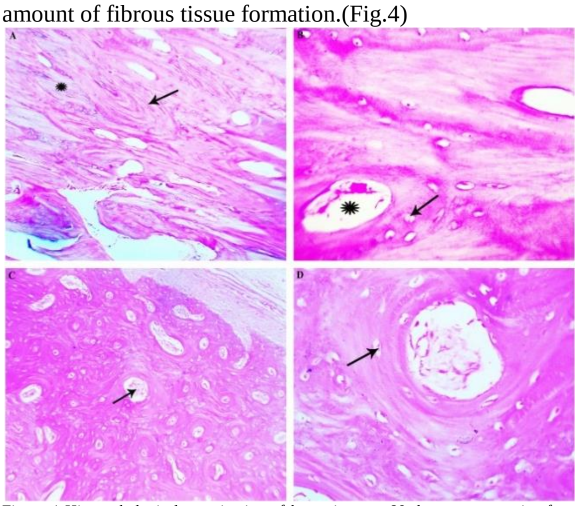

Figure 4 Histopathological examination of bone tissue at 90 days post-operative from control (a-b), CaP-NPs- (c-d) groups stained with hematoxylin and eosin. Notice (a) formation of fibrocartilage tissue with presence of woven bone (asterisk, ×100), (b) formation of harversian system with presence of immature osteoblast (asterisk, ×200). (c) complete healing of the osteoid tissue and normal periosteum and formation of harversian system with clear harversian canal. (d) normal haversian canal that surrounded by mature osteoblast.

4. DISCUSSION

In the last decades, nanoparticles have been widely used in the field of bone grafting (Tang et al., 2014; Levingstone et al., 2019; Sallam et al., 2020). Nanoparticles are characterized by improved physiochemical and biological electrical properties, and increased cell adhesion and proliferation (Albinali et al., 2019). CaP-NPs have a similarity with the mineral content of bone that confirm its biocompatibility, osteoinductivity, and osteoconductivity (Cross et al., 2016; Chen et al., 2017). The osteoconductive features of bone grafts e.g., CaP-NPs through acting as supporting structures for the host cells which migrate throughout, attached, and differentiate into osteogenic cells. The results of the present study showed that CaP-NPs can be used as a bone graft in orthopedic surgery as it provides a large amount of Ca and Ph ions and can act as a structural framework for bone regeneration. These findings come in agreement with Tang et al. (2014). A variety of biological, medical, and therapeutic characteristics of CaP-NPs were reported e.g., antimicrobial (Bastari et al., 2014), anti-inflammatory, and growth factors to promote osteogenesis (D'Mello et al., 2017). The results of the present study revealed that CaP-NPs animals showed surgical wound healing by the first intention within 7-11 days without muscle atrophy in any animals. Mild to moderate swellings were noticed by Day-2 post-operation in one dog and the bone healing was faster than in the control group. This finding came in agreement with Liang et al. (2021), who referred to the medical and therapeutic capabilities of CaP-NPs as bone morphogenetic factors to promote osteogenesis.

Regarding clinical evaluation, there was no tenderness or abnormal activity around the region of the bone defect and weight bearing was good in the treated group within 10 days post-operation which indicates a good reduction and fixation process. These results were approved by Maiti et al. (2007) and Tang et al. (2014), who used CaP-NPs in the canine tibiofibular model for accelerating the healing process of femur Osteotomies in dogs.

. The radiological evaluation here showed new bone formation and bridging callus at 10- and 13 weeks post-operation in the treated and control groups, respectively. These findings coincided with Balaguer et al. (2018), who used calcium phosphate nanocomposite in accelerating the healing process of fracture of the canine radius and ulna. The healing pattern in our study matched with Sallam et al. (2020), who used crystalline phase calcium phosphate nanocomposite in canine radius and ulna model in which callus formation was completely established at 13 weeks post-operation.

Alkaline phosphatase activity in the control group didn't change significantly between days 0 and 60 following surgery, but it started to decline at day 90. In the CaP-NPs group, ALP activity considerably increased at days 30 to 60 days correlated with the formation of osteoblast-like cells and indicated a superior healing process in the treated group. This finding was supported by Manjubala et al. (2005), who demonstrated that ALP is crucial for the formation and development of new bone as it is firmly attached to the cell membrane. Calcium levels increased significantly in control and CaP-NPs groups from 30 to 60 days following surgery. Likewise, Malard et al. (2005) stated that Ca content can be considered as a direct tool for measurement of the extent of mineralization during the fracture and healing processes. They added that the high circulating Ca level resulting from the withdrawal of calcium for callus formation would enhance the healing process. Our finding agreed with Malard et al. (2005), who accused the elevation of serum Ca level to its release from CaP-NPs. Phosphorus levels sharply dropped in the control groups from Day 30 and 60 post-surgery, while it decreased gradually in the CaP-NPs group during the experimental period (from 0 to 90 days). The high quantities of Ca and Ph ions play a role in promoting the osteogenic differentiation of pluripotent cells into osteoblasts .This inverse relationship between Ca and Ph levels and the osteoinductive effect of calcium due to bioresorbability was indicated in former studies (Kalita et al., 2007; Habraken et al., 2016).

CT examination in this experiment indicated superior bone healing in the CaP-NPs than the control as a result of new bone and callus formation, and periosteal reaction at 90th days post-operation These findings agreed with Balaguer et al. (2018), who found accelerated healing of canine radius and ulna under the effect of CaP-NPs.

In histopathological examination, the high potential of CaP-NPs for osteogenesis compared with the control group was evidenced by the complete formation of the haversian system and mature osteoblast as it was indicated in previous studies (Balaguer et al., 2018; Sallam et al 2020).

5. CONCLUSION

Based upon clinical, radiological, biochemical, CT, and histopathological presented data, CaP-NPs application is a promising synthetic biomaterial for bone grafting through enhancing faster calcification and callus formation of the fractured femur after 75 days post-operatively minimal adverse effects, due to its biocompatibility, osteoconductive and osteoinductive properties.

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