



The Impact of Natural Calcium Carbonate and Ubiquinone on Bone Mineral Density in Rabbits

Ahmed A. Hussein¹ and Ghada A. Taqa²

¹Ministry Of Health, Nineveh Health Directorate, Mosul, Iraq.

²Department of Dental Basic Sciences, College of Dentistry, University of Mosul , Mosul , Iraq.

*Corresponding Author, Ghada A. Taqa, E-Mail: ghadataqa@uomosul.edu.iq

ABSTRACT

This study investigates the effect of Ubiquinone and/or calcium carbonate (CaCO₃) on bone mineral density (BMD) in rabbits at the healing site in mandibular bone postoperatively. Sixty healthy white male New Zealand rabbits (almost the same age [5-6 months], weight [2.0±0.25 kg] and circumstance) were used in this study. The heavy-duty dental engine drilled a mandibular groove of 2mm diameter and 6mm length in all animals. Post-surgery, the rabbits were randomly divided into six groups (10 each) according to the treatment. According to the evaluation period, each group was further subdivided into two subgroups (5 rabbits each); 15 and 30 days. The groups included; control group (the groove was not filled by anything) and treated groups which included five groups as follows: group I (in which the groove was filled with Ubiquinone), group II (groove was filled with CaCO₃), group III (groove was filled with CaCO₃: Ubiquinone as a ratio [1:1]), group IV (groove was filled with CaCO₃: Ubiquinone as a ratio [1:2]) and group V (groove was filled with CaCO₃: Ubiquinone as a ratio [2:1]). The BMD was measured using Densitometry after 15 and 30 days. Statistical analysis showed significant differences at p≤0.05 in bone mineral density (BMD) between the control group (92±0.15) and all other treated groups except group III (125±3.54) and group IV (126±5.13) showed no significant differences at follow-up times at 15 days. Also, in the present study, we found there is a significant difference in bone mineral density among the control group (109±1.25), group I (144±3.45), group II (153±6.24) and group V (182±12.56) groups respectively. ANOVA test showed no significant difference in group III (160±8.94) and group IV (165±9.11) at follow-up times at 30 days. Adding the Ubiquinone and/or CaCO₃ showed a significant increase in BMD manifested by bone healing like space-filling, newly formed bone trabeculae and a high number of lacunas compared with the control group. It can be concluded that, the Addition of Ubiquinone and/or CaCO₃ to the mandibular fracture has a favourable effect on BMD and enhance bone healing in rabbits.

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INTRODUCTION

The bone is a hard organ that is part of the vertebrate skeleton. Bones assist and protect the body's various organs, manufacture red and white blood cells, store minerals, provide structure and support to the body, and allow mobility. Bones come in a variety of shapes and sizes, with a complicated internal and external structure. They are lightweight but powerful and tough to use, and they perform a variety of functions (Krousouloudi, 2019).

The mineralized bone-tissue matrix comprises an organic component called ossein, mainly collagen, with an inorganic bone-mineral component comprising many salts (Bigham *et al.*, 2020). Bone tissue is classified into two types: cortical bone and cancellous bone (Hunt *et al.*, 2021). A bone defect is a lack of bone tissue that can occur as a result of an injury, sickness, inflammation, congenital or traumatic process in the same portion of the body. It can also happen as a result of continuing illnesses or surgical operations in some situations (Walsh *et al.*, 2021).

Ubiquinone or coenzyme Q10 is a substance similar to vitamins that is naturally oily-soluble in most eukaryotic cells. It's a 1,4-benzoquinone molecular subunit with a quinone chemical and 10 isoprenyls on the tail (**Dawood et al., 2020**). Ubiquinone is an important component of the electron transport (ETC) aerobic energy production cycle through the ADP to ATP transition (**Knapp-Wilson et al., 2021**). Because of the largest energy need, Ubiquinone is present in organs like the heart, liver and kidney at the highest concentration (**Lu et al., 2020**). It is found mostly in mitochondria because of its position in the electron transport chain and its interaction with reactive oxygen species (ROS) (**Wang et al., 2021**). Ubiquinone should be considered in 2 forms: oxidized (Ubiquinone) and reduced (ubiquinol) (**Fullerton et al., 2020**).

Calcium carbonate is a chemical substance having the formula CaCO_3 . It is a natural substance found in rocks such as calcite and aragonite minerals (most notably calcite, a form of sedimentary rock composed mostly of calcite). It is the major component of eggs, snail shells, seashells, and pearls (**Barth and Jean, 2020**). It is used in medicine as calcium or antacid supplement, but too much can be hazardous and induce bad digestion (**Lattey et al., 2021**).

As the basic module, an eggshell as a natural calcium carbonate supply is extremely important in bone metabolism and growth, particularly bone substitution. Studies have demonstrated that using eggshells as a bone replacement material is a promising and cost-effective option (**Abdollahiyan et al., 2021**). A variety of procedures and materials were used to correct osseous abnormalities in order to find a suitable alternative to autologous bone. Autologous bone augmentation is the preferred method and, consequently, gold standard (**El-Gindy et al., 2021**).

Bone mineral density (BMD), is the amount of bone mineral in bone tissue. Bone density is measured as the mass of minerals per volume of bone in clinical medicine as an indirect predictor of osteoporosis and fracture risk. It is measured using a technique known as Densitometry. The procedure is painless and non-invasive, with minimal radiation exposure (**Cole, 2008**). There is an association between poor bone density and a higher probability of fracture. The aim of the study was to evaluate the effects of CaCO_3 and Ubiquinone on bone formation in rabbits by measuring BMD changes when the drug is given in therapeutic doses for different follow-up periods.

MATERIALS AND METHODS

In this study, sixty healthy white male New Zealand rabbits weighing 2.0 ± 0.25 kg, aged (5-6) months were used. The animals were housed under laboratory conditions at a temperature (23 ± 2) °C with

natural light/ dark cycle and fed with a standard diet, vegetables, crushed wheat, corn, and water during the experimental period. The habitat of the animal during the experiment was in a special cage in the animal house of the College of Dentistry, University of Mosul. This research was performed under the guidelines of the institutional animal research ethics committee of REC reference no. UoM.Dent/A.L.38/21 at 20/4/2021.

Preparation of Calcium carbonate from the eggshell:

The chicken eggshell used in this study was bought from local markets. Egg casings were removed and then the cover was lifted from the peel lining and then washed very well to ensure that the cover lining was removed and dried (**Al-Abdali et al., 2015**). 100 gram of eggshells were crushed by coffee grinder (**Isa et al., 2020**) and purification by special mash (100 μm); To produce fine crystals, the crushed eggshells were added to 100mL of distilled water and filtered by (No.6) Whatman filter paper. These were dried and sterilized by autoclave for one hour at 125 °C and finally, calcium carbonate crystals white powder was produced. The Calcium carbonate crystals were combined with Ubiquinone in different proportional as following CaCO_3 : ubiquinone ratio: (0:1, 1:0, 1:1, 1:2, 2:1) W/W respectively.

Experimental protocol:

Randomly selected animals were assigned to six experimental groups (10 rabbits each). General anesthesia was achieved using an intramuscular injection of Xylazine HCl (Interchemie werken "De Adelaar" BV, Holland) (5mg/kg) and Ketamine hydrochloride (Romvac Company, SA) (40 mg /kg) (**Ahirwar et al., 2021**). Complete anesthesia had been obtained within 5 minutes and lasted for about 50 minutes.

The hair was shaved from the operation site in the right mandibular region, and the skin was cleaned by povidone-iodine. Rabbits were placed in the left lateral position and a 5 cm longitudinal skin incision was made on the antero-lateral aspect of the mandibular area. The mandibular muscles were displaced by forceps to expose the bone. A groove of 2mm diameter and 6mm length groove was drilled by bone drill and irrigation with saline solution to reduce the heat. Each group was further subdivided according to the medication period into two subgroups (15, 30 days) (5 rabbits each)) as follow:

Control group: No treatment was received in the groove.

Treated groups were subdivided into:

Group I: the groove was filled with Ubiquinone.

Group II: the groove was filled with CaCO_3 .

Group III: the groove was filled with CaCO_3 : Ubiquinone as a ratio (1:1).

Group IV: the groove was filled with CaCO₃: Ubiquinone as a ratio (1:2).

Group V: the groove was filled with CaCO₃: Ubiquinone as a ratio (2:1).

The muscles were sutured with absorbable suture material and the skin wound was closed with a black silk suture(china). The wounds were disinfected with 2% iodine solution and for five days following surgery, a veterinary antibiotic comprising (0.5g) Oxytetracycline was administered IM once daily.

Radiographical analysis of bone densitometry:

The digital Densitometric Kodak software 9000C (kodak9000three-dimensional-3D) Imaging system was used to calculate bone density in the region of interest (Mandibular Region at the defect location in the right mandible of rabbit). The equipment measured the distance between two points as a linear distance, and the readings provided the average value of bone density at every particular point (Taneja *et al.*, 2015).

So, we obtained the information on the rate of bone formation and determined the variation in radiodensity between radiographs. Digital panoramic radiographs were made for all groups of rabbits at the time (15, 30 days) employing Kodiak 9000C 3D Extraoral Imaging System, V shape child jaw size, at 60 kv, 2.0 mA, 13sec After complete anesthesia was obtained, the position of the rabbit's head was standardized. The rabbit was put on a small box and its ears were fixed on the system. A laser beam was adjusted in a manner parallel to the mandibular plain of the mandible bone of the rabbit (Fig. 1).



Fig.1: Densitometric analysis with orthopantomography (OPG) for mandible bone of rabbits.

Statistical analysis:

All data were processed and analyzed by the use of the statistical package SPSS ver. 25 (SPSS Inc., Chicago, ILL). ANOVA test was used to compare the differences in mean bone density among six study groups and different follow-up periods (Ali and

Bhaskar, 2016). A p-value ≤0.05 was considered statistically significant.

RESULTS

BMD results

T-test showed a significant difference in BMD between the control group and all treated groups after 15 and 30 days of study. (Fig. 2).

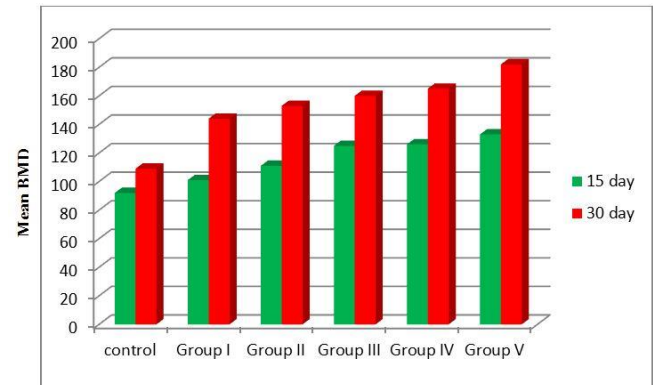


Fig. 2: Changes in BMD among control and treated groups at different follow-up periods.

After 15 days, statistical analysis showed significant differences at p≤0.05 in BMD between the control group (92±0.15) and all treated groups except group III (125±3.54) and group IV (126±5.13), which showed no significant differences (p>0.05). (Table 1). (Figures;3A [control group] ,4A [group I] ,5A [group II] ,6A [group III] ,7A [group IV] and 8A [group V]).

After 30 days, statistical analysis showed significant differences at p≤0.05 in BMD among the control group (109±1.25), group I (144±3.45), group II (153±6.24) and group V (182±12.56). ANOVA test showed no significant difference in group III (160±8.94) and group IV (165±9.11) at follow-up times at 30 days (Table 1).(Figures;3B[control group],4B[group I],5B[group II],6B[group III],7B[group IV],8B[group V]).

Table 1: Comparison of BMD (g/cm²) among all study groups at 15 and 30 days.

Groups	BMD at day 15	BMD at day 30
Control	92±0.15 ^A	109±1.25 ^A
Group I	101±1.05 ^B	144±3.45 ^B
Group II	111±2.23 ^C	153±6.24 ^C
Group III	125±3.54 ^D	160±8.94 ^D
Group IV	126±5.13 ^D	165±9.11 ^D
Group V	133±6.29 ^E	182±12.56 ^E

Different letters mean significant difference, at P ≤ 0.05.

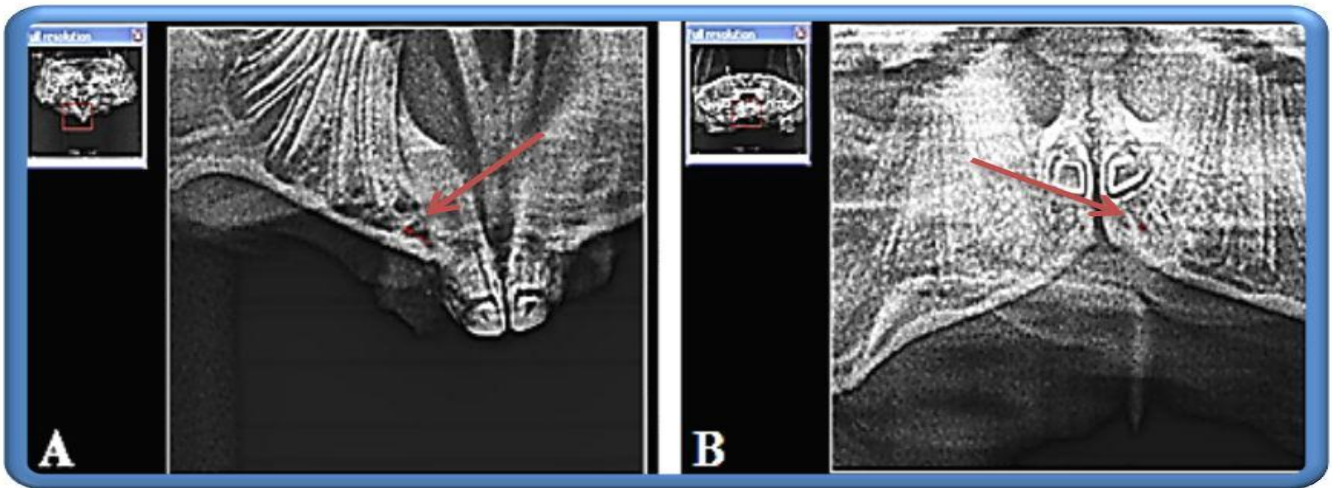


Fig. 3: **A.** Densitometric analysis for control rabbit group at day 15; **B.** Densitometric analysis for control rabbit group at day 30, (→) site of operation.

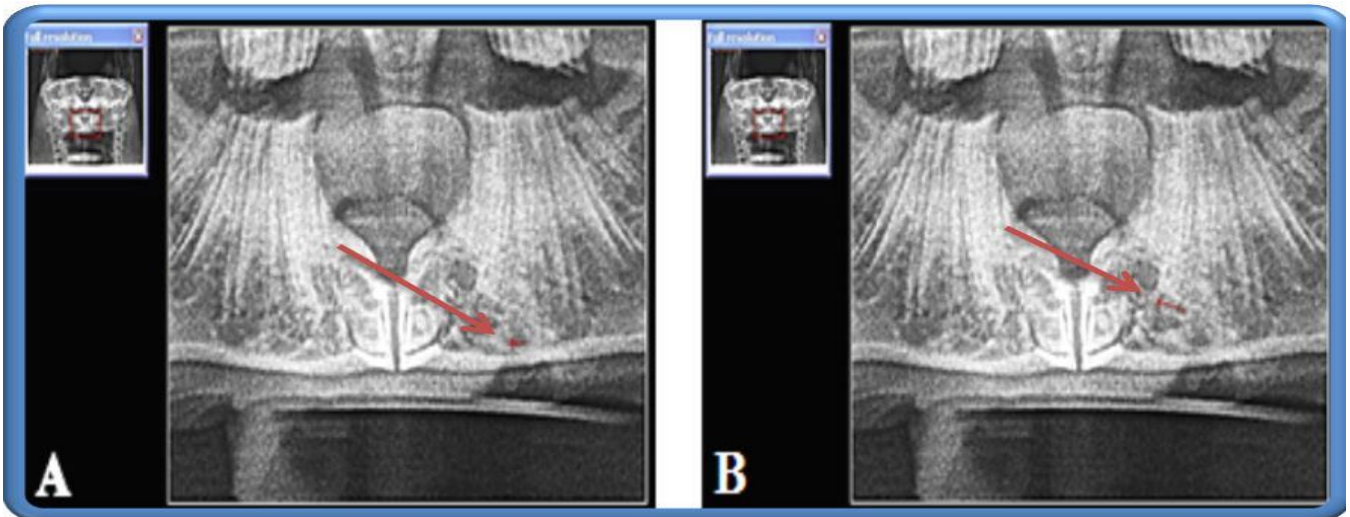


Fig. 4: **A.** Densitometric analysis for rabbit group I on day 15; **B.** Densitometric analysis for rabbit group I on day 30, (→) site of operation.

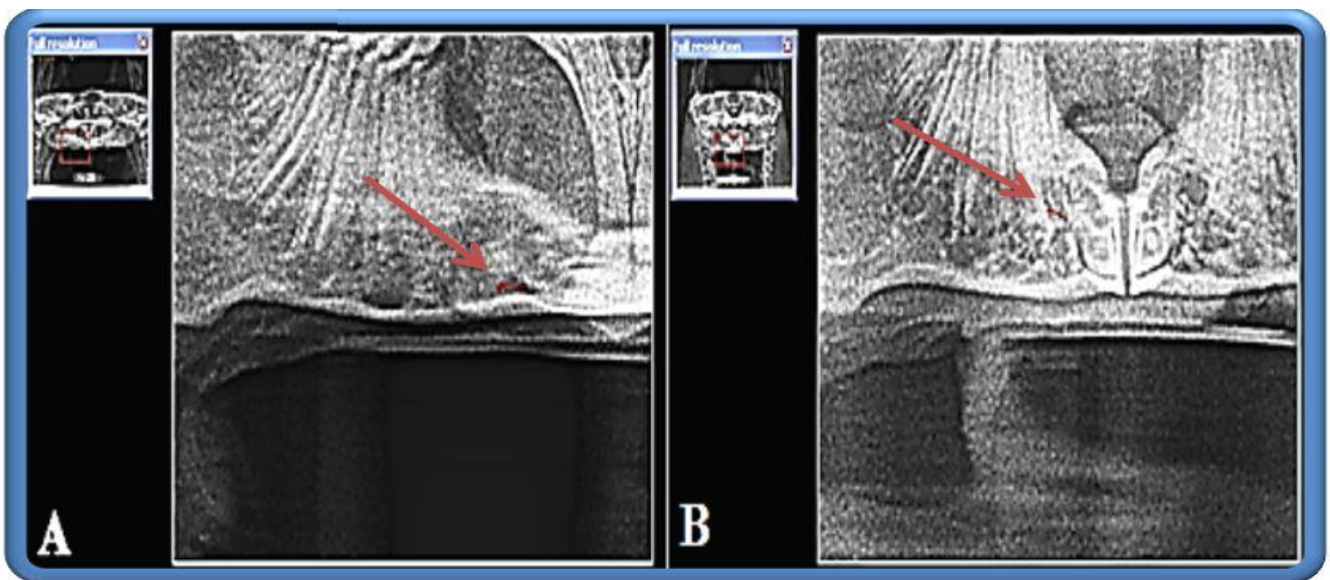


Fig. 5: **A.** Densitometric analysis for rabbit group II at day 15; **B.** Densitometric analysis for rabbit group II at day 30, (→) site of operation.

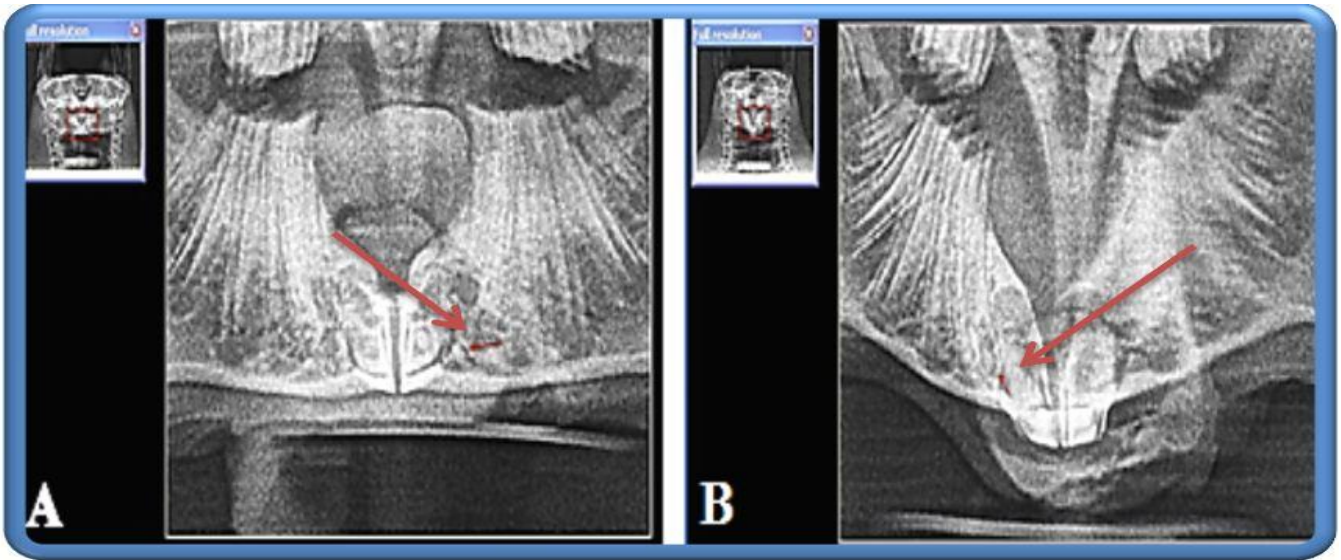


Fig. 6: **A.** Densitometric analysis for rabbit group III at day 15; **B.** Densitometric analysis for rabbit group III at day 30, (→) site of operation.

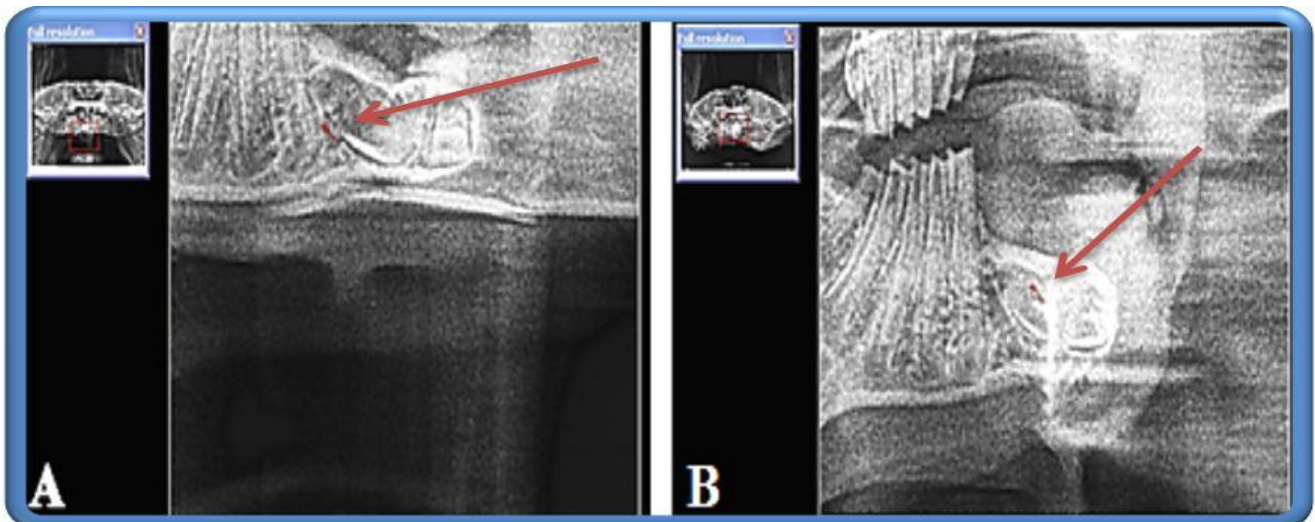


Fig. 7: **A.** Densitometric analysis for rabbit group IV at day 15; **B.** Densitometric analysis for rabbit group IV at day 30, (→) site of operation.

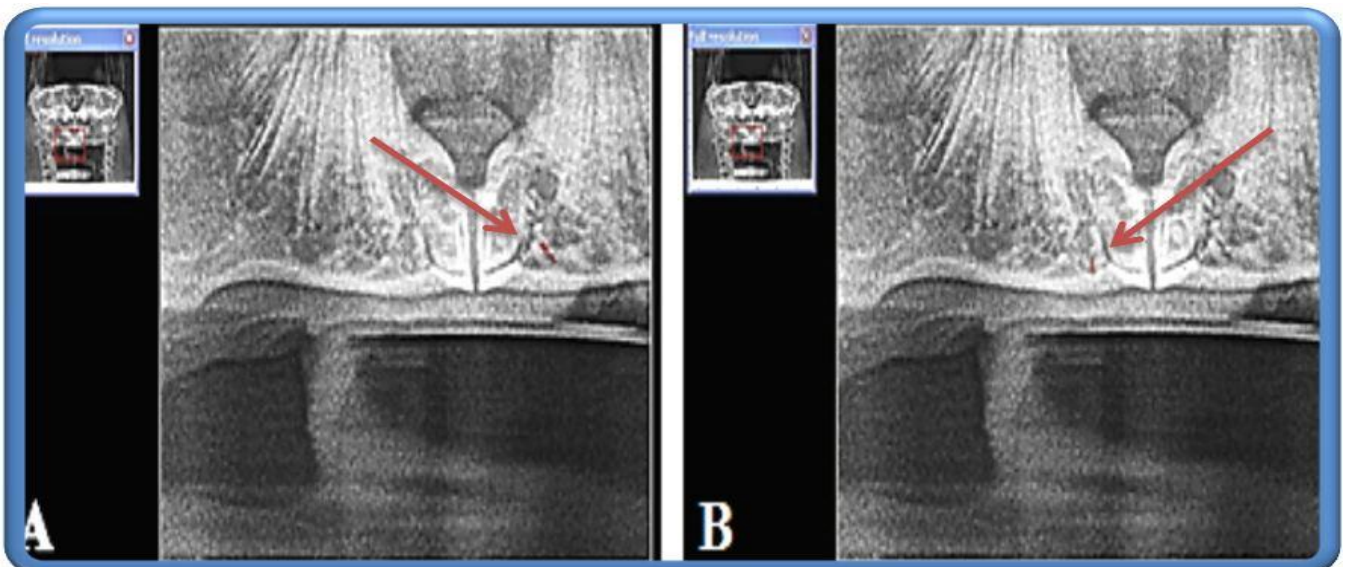


Fig. 8: **A.** Densitometric analysis for rabbit group V at day 15; **B.** Densitometric analysis for rabbit group V at day 30, (→) site of operation.

DISCUSSION

When the bone loss regeneration process is slowed or even stopped, the bone repair is frequently required to stimulate bone regeneration through surgical insertion of materials (**Fernandez et al., 2021**). In recent decades, autograft materials have largely been used for bone repair due to their superior bone conduction, inducibility, and osteogenesis (**Ou et al., 2020**). In terms of bone healing, an autologous bone graft remains the therapy of choice in practical practice (**Su et al., 2021**). However, complications involved with the harvesting process and the limited volume of donor's bone limit the clinical applicability of this approach (**Jeon et al., 2021**). As a result, various bone substitutes, such as alternate graft materials, are critical for efficient bone restoration (**Wang et al., 2021**).

The radiographical findings in this study showed significant differences in BMD between the control group and all treated groups except group III and group IV which showed no significant differences at day15. At the same time, on day 30, there are significant differences in BMD among the control group, group I, group II and group V. ANOVA test showed no significant difference in group III and group IV. Group I showed minimum BMD while group V showed maximum BMD at all periods. This means that the ratio of combination at group V increased the bone formation compared to control, also showed that the ratio of combination at group V was more effective in bone formation than CaCO₃ alone as in group I.

In this investigation, fresh bone development was seen at the peripheral 30days following surgery. This could be due to the osteoconductive qualities of eggshell CaCO₃, which allow osteoprogenitor cells to adhere and move on the scaffolds, differentiate, and eventually produce new bone. These findings, which agree with the findings of other investigations, demonstrated that the proposed hydroxyapatite scaffold is suitable for bone tissue regeneration (**Alhussary et al., 2020; Wang et al., 2021**). These findings concurred with the findings of other research, which said that hydroxyapatite (HA), which has exceptional mechanical qualities and outstanding osteoconductivity, should have a high potential for bone repair and regeneration (**Zhu et al., 2021**).

The healing process in defects filled with CaCO₃ is heavily reliant on the material's osteoconductive characteristics. Studies have reported similar findings (**Codrea et al., 2021**). Again these findings agree with findings reported by **Petrovova et al., (2019)** who mentioned that calcium carbonate scaffolds might represent a novel pattern in bone tissue engineering by encouraging osteogenesis as a means of mending bone defects and remodeling.

Ubiquinone is an antioxidant with free radical scavenging, membrane stabilizing, and cytoprotective properties (**Dawood et al., 2020; Suárez-Rivero et al., 2021**). In agreement with **Li et al., (2019)**, Ubiquinone can stimulate osteogenic differentiation and increase the proliferation of bone marrow stromal cells (BMSCs) in a dose-dependent manner. While the antioxidant Ubiquinone plays a preventative and protective effect in osteoporosis by boosting osteoblastic bone production, decreasing osteoclastic bone resorption and oxidative stress, and avoiding cell senescence (**Wu et al., 2021**).

A study in rats found that short-term Ubiquinone administration reduced bone and mineral content loss caused by spinal cord damage, indicating that Ubiquinone had a favourable effect on BMD (**Varela-López et al., 2017**). The current study found that Ubiquinone's in vivo antioxidant activity may aid in the prevention of bone loss by lowering ROS levels in osteoclasts and osteoclast progenitor cells. This work supports **Valentino et al., (2021)** findings's who stated that Ubiquinone boosted matrix mineralization and Alkaline phosphatase (ALP) activity in murine osteoblast-like cells. The results from our study showed an increase in bone formation when using Ubiquinone alone or when combined with CaCO₃. The ratio of bone formation will increase as the ratio of Ubiquinone increases. This may be due to the effect of Ubiquinone as an antioxidant.

CONCLUSION

The results of the current in vivo study demonstrate the high potential of the combination of eggshell calcium carbonate and Ubiquinone as basic components for bone regeneration and enhanced bone formation in mandibular defects in rabbits.

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Declaration of Conflicting Interests

The authors revealed that there was no potential conflicts of interest.

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