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EFFECTIVENESS OF COMBINATION OF VITAMIN D WITH CHITOSAN HYDROXYAPATITE VERSUS CHITOSAN HYDROXYAPATITE ON BONE REGENERATION IN CRITICAL SIZE DEFECT (AN EXPERIMENTAL STUDY IN RABBITS)

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ABSTRACT

BACKGROUND: It is challenging for bone to heal in a critical-size lesion. Tissue engineering produces better results but necessitates using costly additives such as stem cells and bone morphogenic proteins. Vitamin D3 plays an important role in bone turnover and mineralization using osteoblast and osteoclast activation. Chitosan is extensively employed in bone tissue regeneration. Using chitosan in conjunction with other bioactive materials, such as hydroxyapatite, has demonstrated efficacy in promoting bone repair. Hydroxyapatite is a synthetic, ceramic substance that has demonstrated efficacy in producing new bone.

AIM OF THE STUDY: Aim to assess and compare histologically and histomorphometrically the effect of vitamin D3 chitosan hydroxyapatite composite hydrogel versus chitosan hydroxyapatite hydrogel alone on healing of bone in critical size defect in rabbits

MATERIALS AND METHODS: 36 Male rabbits were used in the study. A critical-size osseous defect was induced in the tibia of each animal. The sample was divided into 3 groups. The first group was left empty as a negative control, the second group was filled with chitosan hydroxyapatite composite hydrogel, and the third group was filled with vitamin D3 chitosan hydroxyapatite hydrogel. Scarification was done after 4 and 6 weeks. The results obtained from this study include histological results and histomorphometric analysis

RESULTS: This study finds that vitamin D3 accelerates bone formation and promotes bone maturation. According to our findings, applying vitamin D 3 (calcitriol) locally speeded up the production of new bone as shown by histological and histomorphometric analysis. The mean value of bone surface area in group III (chitosan hydroxyapatite vitamin D hydrogel) was higher than group II (chitosan hydroxyapatite composite hydrogel.) and control group (empty bone defect) at 4 weeks and 6 weeks.

CONCLUSION: Applying vitamin D3 (calcitriol) locally speeded up the production of new bone as shown by histological and histomorphometric analysis.

KEYWORDS: Chitosan, hydroxyapatite, Vitamin D.

RUNNING TITLE: Vitamin D3 with chitosan hydroxyapatite versus chitosan hydroxyapatite on bone regeneration

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INTRODUCTION

Critical size defect (CSD) is recognized as an abnormality that needs additional surgical intervention, such as bone grafting and does not heal on its own after surgical stabilization. The primary goal of bone augmentation surgery should be achieved in the absence of hard tissue volume by employing graft materials to the patient's needs (1-3).

Natural polysaccharide copolymer chitosan is frequently employed in bone tissue regeneration and medication administration. Combining chitosan with other bioactive materials has been shown to enhance its ability to promote bone healing and to effectively build new bone in the alveolar socket after tooth extraction (4).

Chitosan has a wide range of potential uses. It might be found in various forms, including films, fibers, gels, sponges, and beads. Chitosan can be used in hydrogel form. The capacity of hydrogels to carry proteins, growth factors, cells, and other elements required for tissue engineering makes them appealing materials. Injectable hydrogel systems have shown effective in encapsulating a range of biological components, including growth factors, medicines, and cells, in minimally invasive surgical procedures. Furthermore, after injection, they can create gels that can fill any target region of any shape (5,6).

In oral and maxillofacial surgery, hydroxyapatite (HA), a synthetic and biodegradable ceramic substance, is frequently employed in a variety of applications. The benefits of incorporating HA into chitosan hydrogels suggest their possible use in clinical settings for bone regeneration (7,8).

One type of calcium phosphate ceramic is hydroxyapatite (Ca10(PO4)6(OH)2). Tricalcium phosphates and bioglass ceramics are other family members and are commonly utilized as materials to replace bone. Because of its structural and functional resemblance to the mineral composition found in teeth and bones, HA is preferred over other calcium phosphates (9,10).

Vitamin D is a steroid hormone that can be effectively oxidized in the liver and kidneys. It can be synthesized by the skin when there is enough sun exposure (290 –315 nm).VitaminD3 increases the activity of osteoclasts and osteoblasts in bone A lack of daytime sun exposure combined with contemporary work environments like COVID -19 and lifestyle choices makes people deficient in vitamin D3 (11- 13).

Deficits in vitamin D3 can result in conditions like osteomalacia and rickets that impact bone metabolism and mineralization. One critical decision that needs to be taken before the choice of grafts is determining whether the bone defect is one of critical size or not. Literature suggests that when the length of the defect is greater than 8 mm and there is more than 50% loss of the circumference of the bone, the defect is considered a critical size defect (2,10).

The null hypothesis of this study was that there is no significant difference between the healing of critical-sized bone defects treated with chitosan hydroxyapatite composite hydrogel alone versus vitamin D3chitosan hydroxyapatite hydrogel.

Aim of the study is to assess and compare histologically and histomorphometrically the effect of vitamin D3 chitosan hydroxyapatite composite hydrogel versus chitosan hydroxyapatite hydrogel alone on the healing of bone in critical size defect in rabbits.

MATERIAL AND METHOD

The study has been approved by the Research Ethics Committee, Faculty of Dentistry, Alexandria University (IORG0008839). The approval number by the ethical committee is (0474-7 / 2022)

Thirty -six adult male rabbits 6 months old weighted 3 kg were used in this research. Animals were acquired from Alexandria University's Medical Research Institute's animal home. In the experimental animal home, they were housed in identical environmental settings. All animals' procedures followed the National Research Council Guidelines for the care and use of laboratory animals (14). The results were obtained from this study by histological and histomorphometric analysis.

Rabbits were randomly assigned into 3 equal groups;

Group A: Negative control group (empty bone defect)

Group B: Defects filled with chitosan hydroxyapatite composite hydrogel

Group C: Defects filled with chitosan hydroxyapatite vitamin D3 hydrogel.

Sample size calculation

The previous study, which examined the impact of sustained-release vitamin D3 loaded in poly-lactic acid (PLA) nanoparticles and applied to grafting materials around titanium dental implants, served as the basis for the calculation of the lowest sample size. The production of PLA nanoparticles and encapsulation efficiency (EE) on vitamin D3 was determined by Mutlu et al. (2021) (15). PLA nanoparticles were effective delivery vehicles for vitamin D3's prolonged release. In sheep with box-shaped iliac bone defects around dental implants, sustained release of vitamin D3 infused into the PLA system enhanced the new bone formation (NBF%) and BIC% values in the early and late healing groups. To find the difference in Bone Implant Contact (BIC) between the groups under study, the sample size was computed. The minimum needed sample size was found to be quite small (2 defects per group) based on their results, using a power of 90% (b=0.20) to detect a standardized effect size in BIC (primary outcome) of 7.014, and a level of significance 5% (α error allowed =0.05). Therefore, it is advised to have a minimum sample size of six defects per group (three groups, two sacrifice times, and a total sample size of six x two x three = 36 defects) (16).

To maintain the sample size, each sample that left the research for whatever reason was replaced (17).

Eligibility Criteria

Inclusion criteria

Healthy male rabbits weighing 2500-3500 grams (approximately aged 6 months) were applied in this research.

Exclusion criteria

Rabbits involved in any prior experimental investigation, as well as those with wounds or illnesses, were specifically eliminated.

Allocation concealment

Method of randomization

The block size was variable (18), and the allocation sequence was produced using the permuted block randomization approach. Using sealed opaque envelopes, the allocation sequence or code was kept secret from the individual assigning participants to the intervention arms (19).

Material

Chitosan (alpha chemical company).

Hydroxyapatite (Bio Gap) Egypt Trade company.

Vitamin D3 (Sigma Aldrich Chemie)

Poloxamer.

Poloxamer 407 (PLX) was purchased from (Sigma Aldrich Chemie, ST. Louis USA.

Trephine Bur diameter 9 mm (JDENTALCARE)

Preparation of thermosensitive Chitosan hydrogel

2.5% w/v Chitosan solution was prepared by dissolving the polymer in acidified water (0.5% v/w acetic acid) under magnetic stirring (at 80 rpm) for 2 h then the solution was left in a refrigerator for 24 h.

To develop the thermosensitive chitosan hydrogel, 15% w/v Poloxamer(plx) dispersion was employed in the current study as previously reported (20). In brief, Poloxamer(plx) was dissolved in the prepared Chitosan solution under sonication for 15 min then refrigerated for 24 h to accomplish complete PLX's dissolution.

Afterwards, HA (50% w/w) was mixed with the hydrogel then the blend was poured into a mold and allowed to solidify at 4 ° for 24 h (20).

Vit D3-loaded hydrogel was prepared following the above-mentioned method while mixing Vitamin D (0.0014 w/w of chitosan) prior to pouring the solutions into the molds. Any processing of Vit D3 was performed in the dark due to its light sensitivity. Each Scaffold contained50 µg (Equivalent to 2000 IU) of Vitamin D3 (15).

Thermosensitive sol-gel transition

The phrase "gelation time" describes the amount of time needed to see the sol-gel transition in a water bath at 37°C. In brief, a beaker containing the hydrogel was placed in the water bath at 37 °C and the time needed for the hydrogel to stop flowing after the beaker was inverted was recorded.

Chitosan thermosensitive hydrogel exhibited altered consistency at 4 °C and 37 °C. At 4 °C, the preparation had a low viscosity fluid nature however, a high viscous gel was obtained at 37 °C. Such phenomena can be explained by the thermosensitive nature of PLX, where the crosslinking degree of PLX entanglements increases by elevating the temperature. The gelling time was recorded to be 35 seconds which is convenient in the current study as it decreased the bleeding at the wound site.

Surgical instruments

Blade, periosteal elevator, tissue retractor, scissor, tweezer forceps.

Handpiece, trephine bur, motor, Needle holder, and black silk suture were used.

Operative phase

Anesthesia

General anesthesia was administered to the rabbits using a combination of 33 mg/kg of ketamine base and 13 mg/kg of 2% xylazine hydrochloride (21).

Preparation before surgery

The animals were put in a supine position. The leg of the animal was shaved at the surgical site before transferring to the Animal Operating Room for surgery. Then, the leg was disinfected with povidone-iodine and draped with a sterile towel. All surgical procedures required the surgeon and the assistants to perform thorough surgical hand scrub and wear sterile gowns, gloves, face masks, and caps to avoid infection.

Surgical Approach Fig1 A-F

The tibia tuberosity was palpated and a 3 cm vertical skin incision was done along the bone (fig 1a) exposing the subcutaneous tissue to the deep fascia we chose the site of surgery for better accessibility. The fascia was sharply dissected with a sharp blade exposing the muscle of the tibialis anterior at the upper part of the tibia. Gently the periosteal elevator was introduced to elevate and strip the muscle of the tibialis anterior on the medial side of the proximal tibia and expose the bone (fig 1b).

A critical size defect 9 mm in diameter was created using the trephine bur (fig 1c). The hydrogel was applied to the defect according to the specific allocated group (fig1d). Wound closure was achieved using a double-layer closure where the muscle was closed using interrupted suturing technique(fig1e) also interrupted sutures were used for the skin incision (fig1f). Povidone-soaked gauze and bandage were used for wound dressing.

Post-operative phase

The animals were housed in the Medical Research Institute's animal house under comparable circumstances. The animals were raised in separate cages, free to move around, and fed regular granulated food. Following surgery, each animal got an identical course of ampicillin (25 mg/kg body weight) every eight hours for five days.

The animals received Cataflam (IM) painkiller every eight hours for the first two days. Throughout the first week, the animals were monitored daily to

see whether or not any inflammation or infection-related symptoms were present.

Animal sacrification

At the conclusion of the fourth and sixth week following surgery, six animals from each group were slaughtered.

Animal euthanasia

The rabbits were euthanized with an overdose of ketamine at 4 and 6 weeks postoperatively, the tibia specimens were taken. The tibia specimens were removed and prepared for histomorphometric analysis and light microscopic inspection (22).

Histological examination

The specimens underwent decalcification, washing, and fixing in 10% neutral buffered formalin. Following washing, the samples were cleaned in xylene, dehydrated in increasing alcohol grades, and then infiltrated and embedded in paraffin wax. Hematoxylin and eosin were used to stain serial slices of 5 m thickness that were cut from paraffin blocks using a rotary microtome. A light microscope (optika microscope B – 190TB) was used to view the dyed slices to check for the formation of new calcified bone (23).

Using ImageJ 1.46r software, a histomorphometric assessment of the newly created bone was carried out to compare the proportion of the newly formed bone's surface area in various groups (24).

Statistical analysis of the data

The computer was given data, and IBM SPSS software package version 20.0 was used for analysis. (IBM Corp., Armonk, NY) To confirm that the distribution was normal, the Shapiro-Wilk test was performed. The terms range (minimum and maximum), mean, and standard deviation were used to characterize quantitative data. The results were deemed significant at the 5% level.

The used tests were

Student t-test

For normally distributed quantitative variables, to compare between two studied weeks.

One-way ANOVA test

For normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) for pairwise comparisons the p-value was considered significant as the following

$P > 0.05$ = non -significant (NS).

$P < 0.05$ = significant (S).

$P < 0.001$ = highly significant (HS).

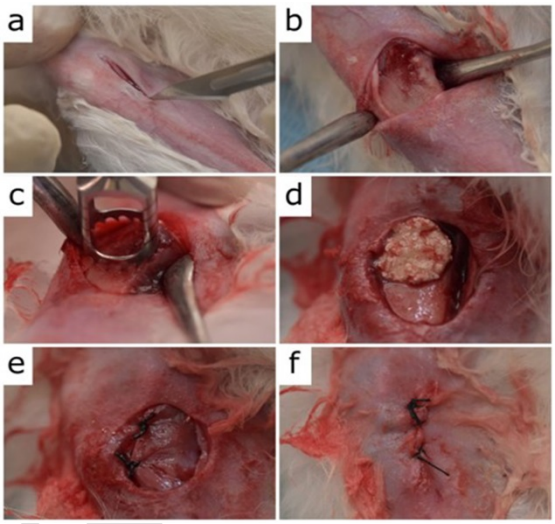

Figure (1): (a-f) Showing the steps of surgical procedure of the critical size defect (a) Skin incision, **(b)** Exposing bone, **(c)** Creating the defect using the trephine bur, **(d)** Application of the scaffold, **(e)** Muscle closure, **(f)** Skin closure.

RESULT

In the present study 36 adult male rabbits 6 months old (2500- 3500) were used and surgical defects measured 9mm were performed in their tibias.

Samples were collected and evaluated histologically and histomorphometrically.

Rabbits were sacrificed at 4 and 6 weeks intervals to compare healing patterns in defects.

Histological evaluation of the H&E stained sections.

Histological findings

Group A

Group A (control group) After 4 weeks samples from the control group revealed a defect filled with granulation tissue and inflammatory reaction and weak osteointegration, extravasated RBC osteocyte with wide lacunae. Fine and immature bone spicules are observed. (Fig 2A).

Group A (control group) after 6 weeks Observation of the H&E stained sections revealed developing fine bone trabeculae and wide marrow spaces (Fig 2B)

Group B

Group B (Defects filled with chitosan hydroxyapatite composite hydrogel) After 4 weeks, the group showed defects filled with Scaffold fragments surrounded by thicker bone trabeculae compared to group A (Fig 3A).

Group B (group B Defects filled with chitosan hydroxyapatite composite hydrogel) after 6 weeks mature bone trabeculae Resting and reversal lines were observed indicating activation of bone remodeling, Scaffold inside the bone, Primary osteon (Fig 3 B).

Group C

Group C (defects filled with chitosan hydroxyapatite vitamin D composite hydrogel)

after 4 weeks Good osteointegration represented by osteocytes and osteoblast and Reversal lines. Resting and reversal lines were observed indicating bone maturation and remodeling (Fig 4A).

Group C (group c defect filled with chitosan hydroxyapatite vitamin D) after 6 weeks Good osteointegration represented by osteocytes and osteoblast, Reversal line Resting and reversal lines were observed indicating bone maturation and remodeling, Scaffold inside bone (Fig 4 B).

Histomorphometric analysis

Table (1) demonstrates that there was a statistically significant difference between the empty bone defect control group and the other group. Group II (Defects filled with chitosan hydroxyapatite composite hydrogel) and group III (Defects filled with chitosan hydroxyapatite vitamin D3 hydrogel).

In 4 weeks

Table (2) demonstrates that, after six weeks, there was a statistically significant difference between the control group, groups II and III.

Over four weeks, group III's mean bone surface area was larger than that of groups II and control, as shown in (Fig 5)

In the six weeks shown in (Fig 6), the mean value of bone surface area in group III was larger than that of groups II and control group.

In 4 WEEKS, a statistically significant difference was detected between the control group, group II, and group III, as indicated by the previous table.

(OR) Means with completely different letters (a–c) are significant; Means with any little common letter (a–c) are not significant).

The above table indicates that, after six weeks, there was a statistically significant difference between the control group, group II, and group III.

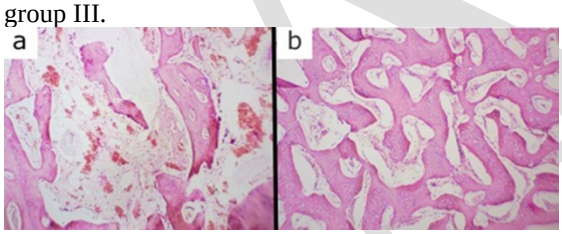

Figure (2):Control group Microphotograph (a) after 4 weeks viewss a defect filled with granulation tissue and an inflammatory reaction. Microphotograph **(b)** after 6 weeks shows a defect filled with fine bone trabeculae and wide marrow spaces.

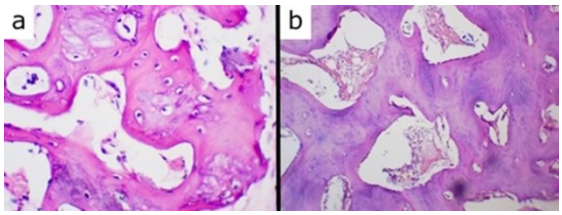

Figure (3): Group B Microphotograph (a) after 4 weeks shows a large amount of thicker bone trabeculae. Scaffold fragments surrounded by bone trabeculae. microphotograph **(b)** after 6 weeks shows Resting and reversal lines were observed, indicating bone maturation and remodelling. Scaffold is showen inside bone.

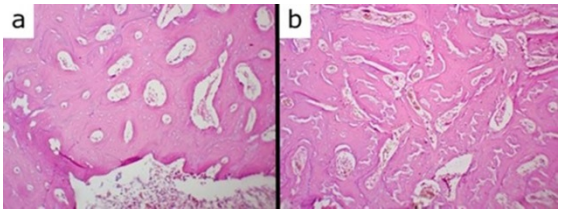

Figure (4): Group C Microphotograph (a) after 4 weeks views Good osteointegration represented by osteocytes and osteoblast and reversal lines. Microphotograph **(b)** shows Good osteointegration represented by osteocytes and osteoblast. Resting and reversal lines were observed indicating bone maturation and remodeling, Scaffold is shown inside bone.

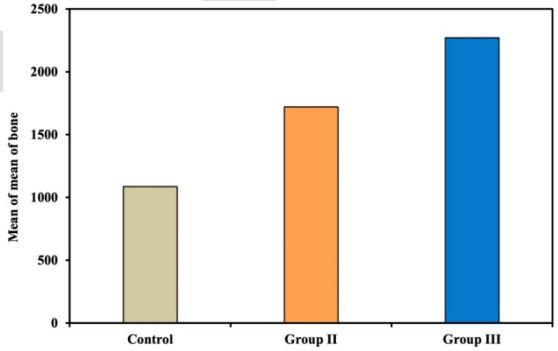

Figure (5): Comparison of the bone surface area means in the fourth week across the three groups under study.

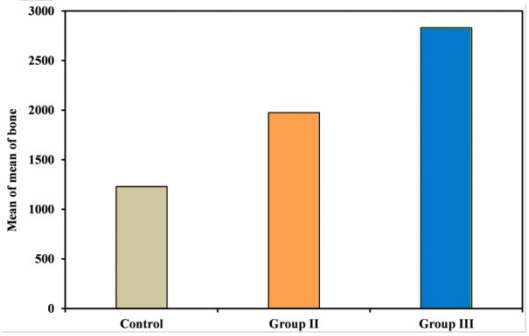

Figure (6): Comparison of the three research groups based on the sixth-week mean bone surface area.

DISCUSSION

Through the activation of osteoclasts and osteoblasts, vitamin D3 plays a critical role in the metabolism of calcium and phosphorus, including intestinal absorption, renal excretion, reabsorption, and use for bone turnover and mineralization (25).

The skin is now the sole tissue that is understood to go through the UV-B-induced physiological conversion of 7-dehydrocholesterol to hormonally

active calcitriol (1alpha,25-dihydroxy vitamin D3) (10).

Two pro-vitamins, ergocalciferol (Vit. D2) and cholecalciferol (Vit. D3) are naturally generated when UV light activates two inactive forms of vitamin D, ergosterol (found in plants) and 7-dehydrocholesterol (found in animals). The pro-vitamin is absorbed by the body and then hydroxylated in the kidney (1,25-dihydroxycholecalciferol and 1,25-hydroxyergocalciferol) and liver (25-hydroxyergocalciferol and 25 hydroxycholecalciferol). This process creates the pro-vitamins active forms (26).

Most experts agree that a vitamin D of less than 20 ng/mL indicates a vitamin D deficiency, while a vitamin D of 21 to 29 ng/mL is considered insufficient. To get the full range of health benefits that vitamin D3 provides, it is recommended that adults and children maintain levels >30 ng/mL (27).

Numerous animal models confirmed the beneficial relationship between systemic vitamin D3 supplementation and bone healing, whereby the metabolites of vitamin D3 aided in the repair of shattered bones (28).

In an investigation on the impact of vitamin D on canine alveolar sockets, local calcitriol application was found to potentially accelerate New Bone Formation (NBF), increase bone density, and enhance the stability of implants for both non-Vitamin D/Ca and Vitamin D/Ca groups in surgically produced alveolar sockets treated with 40% HA/60% b-TCP alloplast. It may be demonstrated that, at the given dose, systemic vitamin D3 can have a stronger positive impact on ridge preservation, NBF, and bone density than local calcitriol treatment by comparing the Calcitriol/Alloplast subgroups of the Non-VitaminD/Ca and VitaminD/Ca groups (28).

Based on data from the ISQ (Implant Stability Quotient), Hong, Yen's study comparing systemically and locally given vitamin D in a dog model with grafted mandibular extraction sockets they revealed improved main implant stability. Despite not using the push-out test or BIC% to measure osseointegration, the researchers observed that systemic vitamin D treatment generally produced positive results (28).

Although vitamin D2 and vitamin D3 in their 1,25-dihydroxy forms may both activate the vitamin D receptor, vitamin D3 has a greater potency and is more commonly referred to as calcitriol (1,25-dihydroxycholecalciferol) (26).

Osteoblasts contain the vitamin D receptor (VDR), which alters the expression of genes linked to osteoblast development and mineralization, including alkaline phosphatase (ALPL), osteocalcin (BGLAP), and osteopontin (SPP1). It has been demonstrated that 1,25D3 stimulates mineralization via actions in the pre-mineralization stage, which comes before mineralization. 1,25D3 is known to stimulate mineralization of human osteoblasts in vitro (29).

Chitosan is widely utilized in the pharmaceutical and medical industries since it is biodegradable, biocompatible, and less harmful. Additionally, it has a wide range of applications, including medication transport, packaging, antibacterial agents, tissue engineering, antiaging agents, antibody response enhancers, and the treatment of illnesses (including cancer treatment) (30).

The mineral hydroxyapatite is a ceramic-based bone transplant alternative. This category's principal materials, in addition to hydroxyapatite, are tricalcium phosphate (TCP), bioactive glass ceramics (Bio glass), calcium phosphate cement (CPC), and calcium sulphate. The benefits of hydroxyapatite crystal osteoconduction and bioresorption are as follows: Nonetheless, its brittleness makes it difficult to shape due to its inherent rigidity, brittleness, and inflexibility, limiting its utility as a load-bearing implant material

Razouki et al found that vitamin D increases bone formation and osteogenesis, these findings concurred with the current research's findings which showed better bone formation in the group containing vitamin D (31).

Khairallah et al. used uremic rabbit models to study the impact of vitamin D3 applied locally on bone growth. They found improvement in bone regenerative capacity by increasing osteoblastic activity when comparing the study group to the control group. Their findings are in agreement with our result (32).

Tancan Uysal et al. demonstrated that locally given active forms of vitamin D can promote bone regeneration in the stages of growth and retention of an orthopedically expanded mid-palatal suture. This finding corroborated the findings of our investigation. It has been shown that vitamin D3 improves the creation and osteogenesis of bone. The increased bone area and osteoblast count in mice treated with the active form of vitamin D indicated that to promote bone production, vitamin D increases osteoblast differentiation and/or proliferation (33).

Fugl et al. did not find any significant effects on bone formation by local administration of calcitriol due to the brief trial period (1-3) weeks and a single application of vitamin D treatment (34).

Topical vitamin D administration on dental implants reduces crestal bone loss and increases the bone-to-implant ratio by 10%, according to research by Oscar Salomo-Coll (35).

Maria Satue et al. discovered that implants coated with vitamin D3 precursor demonstrated an appositive influence on osteoblast development and proliferation as well as an increase in bone

production, which is consistent with our results (36).

According to Hongrui Liu et al., local calcitriol injection has a beneficial effect on bone maturation and remodeling during the repair of mandibular bone abnormalities in rats. According to our study, calcitriol had great potential for both enhancing osteogenic differentiation and suppressing osteoclastogenesis (37).

In the current study, osteoblasts were found on the bone surface near the scaffold with an area of remodeling lines and dilated blood capillary engorged with RBCs, this indicates the osteoinductivity of the scaffold. These results were explained by Hu etal)who showed that HA was more conducive to proliferation, and osteogenic differentiation (38).

In our study chitosan hydroxyapatite group was higher in bone formation than the control group This was in accordance with Marcovi etal who showed that the increase in bone density was a result of local calcium deposition and mineralization originating from the degraded CS/nHA scaffold (39).

Table (1): Comparison between the three studied groups according to Mean of bone surface area in 4th week.

	Control	Group II	Group III	F	p
Mean of bone Min. – Max.	794.3 –1484.1	1375.8 –2348.5	1768.7 –2692.1	16.563*	<0.001*
Mean ± SD.	1086.6c ± 285.9	1722.0b ± 398.8	2271.7a ± 376.2		
Sig. bet. grps.	p1=0.020*,p2<0.001*,p3=0.044*				

6 replica for each group SD: **Standard deviation**

F: **F for One way ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

p: p value for comparing between the three studied groups

p_1: p value for comparing between **Control** and **Group II**

p_2: p value for comparing between **Control** and **Group III**

p_3: p value for comparing between **Group II** and **Group III**

*: Statistically significant at $p \leq 0.05$

In 4 WEEKS, a statistically significant difference was detected between the control group, group II, and group III, as indicated by the previous table.

Table (2): Comparison of the three research groups based on the sixth-week mean bone surface area.

	Control	Group II	Group III	F	P
Mean of bone Min. – Max.	649.2 –1921.9	1723.6 –2967.3	2347.1 –3564.6	16.471*	<0.001*
Mean ± SD.	1231.1c ± 435.6	1976.5b ± 490.2	2832.3a ± 521.0		
Sig. bet. grps.	p1=0.044*,p2<0.001*,p3=0.020*				

6 replica for each group SD: **Standard deviation**

F: **F for One way ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

p: p value for comparing between the three studied groups

p_1: p value for comparing between **Control** and **Group II**

p_2: p value for comparing between **Control** and **Group III**

p_3: p value for comparing between **Group II** and **Group III**

*: Statistically significant at $p \leq 0.05$

CONCLUSION

According to our findings, applying vitamin D 3 (calcitriol) locally speeded up the production of new bone as shown by histological and histomorphometric analysis. The mean value of bone surface area according to histological and histomorphometric

analysis in group III (chitosan hydroxyapatite vitamin D hydrogel) was higher than group II (chitosan hydroxyapatite composite hydrogel.) and control group (empty bone defect) at 4 weeks and 6 weeks.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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