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Original Research Article

Zinc-coated carbonate apatite bone substitute derived from avian eggshell for potential use in bone regeneration

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Abstract

Introduction: This study aimed to evaluates the feasibility of a novel zinc-coated carbonate apatite (ZnCHA) bone substitute, derived from avian eggshell, for potential application in bone defects and compares its properties with a commercial bone substitute in terms of bone regeneration.
Materials and Methods: Thirty rabbits divided into three groups (Groups 1, 2, and 3). Each underwent surgery to create three critical-size bone defects (CSDs) on their skulls, designated as Defects A, B, and C. Defect A served as the control, B was filled with Bio-Oss®, and C was filled with the ZnCHA. The rabbits were euthanized at 4, 8, and 12 weeks labeled under Groups 1, 2, and 3, respectively, and the specimens were analyzed in the laboratory to compare new bone regeneration.
Results: Fluorescence microscopic evaluation revealed new bone regeneration around the bone substitutes in both Defects B and C. A statistically significant difference in the mineral apposition rate was noted between the substitutes in Defects B and C for Groups 2 and 3. Histological analysis showed no significant difference in trabecular bone regeneration in Defect A across all groups ($P(a) > 0.05$). However, Defects B and C exhibited significantly increased bone regeneration at various stages of bone healing ($P(a) < 0.05$). A notable difference ($P(a) = 0.000$) was observed in the amount of new bone regeneration between Groups 2 and 3.
Conclusion: The results indicate that ZnCHA demonstrates favorable bone regeneration and could be a viable alternative bone substitute.

Keywords: Bone substitute, Avian eggshell, ZnCHA, Bovine bone.

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1. Introduction

Alveolar bone defects can be regenerated using a variety of bone substitutes. These substitutes are classified based on their origin, encompassing autografts, allografts, xenografts, and alloplastic grafts.[1] The properties and efficacy of different bone substitute types have been extensively investigated and reported in the literature.[1,2,3,4]

Autogenous bone is regarded as the gold standard in bone regeneration surgeries. Nevertheless, due to the potential complications arising from the surgical harvesting of autogenous bone, there is a growing demand for reliable and safer alternative bone substitutes in both dental and orthopedic surgeries.[5,6,7,8]

Alternative bone substitutes, including allografts and synthetic alloplastic grafts, are increasingly accepted and popular for use in human patients.[8,9,10] These alternatives have demonstrated promising clinical outcomes in various applications, such as guided bone regeneration for augmenting atrophic alveolar ridges, sinus lifts, and socket preservation.[8,9,10]

The high-quality of new formed bone helps to create improved sites for dental implant placement, potentially

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leading to increased implant survival rates.[11] The preliminary in vivo research on zinc-coated carbonate apatite (ZnCHA) derived from avian eggshell has been successfully developed, showing encouraging results in bone healing and maturation.[12]

Bone substitutes that exhibit osteoconductive properties, optimal degradation rates during bone formation, and antibacterial effects are likely to enhance bone regeneration.[12,13] However, as Chou et al.[13] have noted, the exposure of bone substitutes during the healing period can impede bone healing and adversely affect overall bone formation. While most commercially available synthetic bone substitutes are primarily composed of calcium and phosphates.

Several studies have highlighted that human bone minerals also include zinc, magnesium, fluoride, among others, which may play significant roles in bone regeneration.[14,15,16,17,18,19]

The aim of this in vivo study was to assess the feasibility of employing the newly developed ZnCHA bone substitute from avian eggshell in bone regenerative surgeries and to compare its properties and efficacy with those of commonly used commercial bovine bone substitutes.

2. Materials and Methods

2.1. Study design

The animal study was conducted following approval from the Institutional Animal Ethical Committee of Shanghai Second Medical School, Shanghai Jiaotong University, China (Approval Number: 2014027). Thirty six-month-old male New Zealand rabbits, each weighing approximately 3.0±0.25 kg, were obtained from Shanghai CheDun Experimental Animal Farm, China. A sample size calculation determined that 30 rabbits were sufficient to achieve statistical significance. Blinding was performed by assigning random numerical labels to the rabbits and treatment groups, which were known only to the laboratory personnel responsible for processing the specimens.

2.2. Preparation of zinc-coated carbonate apatite (ZnCHA)

Avian eggshells were cleaned in a 3% sodium hypochlorite solution (NaClO; Shanghai KeCheng Fine Chemical Plant, Shanghai, China) to remove impurities and organic proteins. The cleaned eggshells were ground into powders (300–500 μm) and immersed in a 0.1 mol/L sodium phosphate solution (Na_2HPO_4; Shanghai WenMin Biochemical Science and Technology Ltd., Shanghai, China). The mixture was microwaved (Carousel; Sharp, Shanghai, China) at medium power for 60 minutes, rinsed with double-distilled water, and air-dried. The powders were then soaked in a 500 ml 0.2 mol/L zinc acetate solution (Zn-Ac; GuoYao Group Chemical Reagent Ltd., China) and microwaved until complete evaporation of the solution occurred. After rinsing and air-drying, the powders were analyzed using scanning electron microscopy (SEM; SEM515, Philips, The Netherlands), X-ray diffraction analysis (XRD; D8 Advance, Bruker, Germany), Fourier-transform infrared spectroscopy (FT-IR; Avatar 360, Nicolet, Waltham, MA, USA), and energy-dispersive X-ray analysis (EDXA; SEM515, Philips, The Netherlands).[12,13]

2.3. Surgical procedure of placement

Anesthesia was induced via intravenous injection of ketamine hydrochloride (0.1 ml/kg; Jiangsu Hen Rui Medicine Co., Ltd., China). The surgical area was shaved and disinfected with povidone-iodine solution. Under sterile conditions, a midline sagittal incision was made to expose the calvarial bone. Using a surgical trephine bur (Dentium®, South Korea) under copious saline irrigation, three critical-size bone defects (CSDs), each 6 mm in diameter and 2 mm in depth, were surgically created on the skull of each rabbit. The defects were designated as Defect A (left unfilled, control), Defect B (filled with Bio-Oss®; Geistlich Pharma AG, Wolhusen, Switzerland), and Defect C (filled with ZnCHA). After placement of the bone substitutes, the defects were covered with the pericranium, and the scalp was closed in layers using resorbable sutures (Vicryl® 4-0; Ethicon, Johnson & Johnson, USA). Hemostasis was achieved, and care was taken to ensure proper closure.

2.4. Postoperative care

Postoperatively, each rabbit received an intramuscular injection of penicillin G potassium (300,000 units; Shanghai Gong Yi Vet Medicine Co., Ltd., Shanghai, China) once daily for 7 days. For analgesia, buprenorphine (0.05 mg/kg, subcutaneously) was administered twice daily for 3 days.

2.5. Fluorochrome labeling

To assess new bone formation, fluorochrome labeling was performed. Calcein (5 mg/kg) was administered intramuscularly on days 14 and 13 before euthanasia, and tetracycline (30 mg/kg) was administered on days 4 and 3 before euthanasia.

2.6. Euthanasia and specimen collection

Rabbits were euthanized at their respective time points using an overdose of sodium pentobarbital (100 mg/kg, intravenous). Calvarial specimens, including the defect areas and surrounding bone (extending 2 mm beyond each defect), were harvested.

2.7. Histological preparation

Specimens were fixed in 4% formaldehyde (pH 7.1) for 24 hours, dehydrated, embedded in paraffin, and sectioned into slices of 8 μm and 20 μm thickness. The 20 μm sections were used for fluorescence microscopy to evaluate new bone formation and calculate Mineral Apposition Rate (MAR). Since these injections were administered 10 days apart, MAR was determined by measuring the average distance between

the calcein and tetracycline bands. The 8 μm sections were stained with Goldner's trichrome for light microscopic evaluation. For Calculation of Trabecular Bone and Residual Bone Substitutes, the region of interest (ROI) was selected in each sectioned slide from the defect border towards the center of the defect area. The ratio of trabecular bone area (Tb-Ar) to the whole tissue area (T-Ar) within the ROI, indicative of the amount of new bone formation, was calculated. The analysis aimed to quantify the total tissue area (T-Ar), the area of newly formed trabecular bone (Tb-Ar), and the area of residual graft material (R-Ar). The proportion of new bone regeneration was calculated based on the ratio of Tb-Ar to T-Ar, and the degree of bone graft absorption was determined by the ratio of R-Ar to T-Ar.[20,21,22]

2.8. Assessments

Scanning Electron Microscopy (SEM), Energy-Dispersive X-ray Analysis (EDXA), Fourier-Transform Infrared Spectroscopy (FT-IR), and X-Ray Diffraction Analysis (XRD) demonstrated the chemical properties of Zinc-Coated Carbonate Apatite (ZnCHA) derived from avian eggshells. This was illustrated in **Figure 1**, **Figure 2**, **Figure 3**, **Figure 4**, respectively.[12] Fluorescence microscopic evaluation, light microscopic evaluation, mineral apposition rate (MAR) calculation, and quantification of trabecular bone and residual bone substitutes were conducted to assess bone regeneration.

2.9. Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA tests were employed to compare measurements across different time intervals and between groups. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of ZnCHA

SEM, EDXA, FT-IR, and XRD analyses confirmed the chemical properties of ZnCHA derived from avian eggshells (Figures 1–4).[12]

3.2. Sample characteristics

All 30 rabbits completed the study without complications. The average age was 6 months, and the average weight was 3.0 ± 0.25 kg.

3.3. Fluorescence microscopic evaluation

Fluorescence microscopy revealed new bone regeneration around both Bio-Oss® and ZnCHA. Green fluorescence (calcein) and yellow fluorescence (tetracycline) indicated sites of mineralization (**Figure 5**). In both Defect B (filled with Bio-Oss®) and Defect C (filled with ZnCHA), the formation of new bone was evident, with mesh-like fluorescence bands observable in both defects in Group 3 (B-3 and C-3), as illustrated in Figure 5. In Group 1 (4 weeks), new bone formation was primarily adjacent to defect margins. In Groups 2 and 3 (8 and 12 weeks), extensive bone regeneration within defects was observed, more pronounced in ZnCHA (Defect C).

3.4. Mineral apposition rate (MAR)

Mean MAR values (μm/day), standard deviations, and P-values are presented in **Table 1**. Defect A was excluded from the calculations as it did not contain any bone substitute. In Group 1, there was no significant difference between Defects B and C ($P > 0.05$). However, in Groups 2 and 3, ZnCHA showed significantly higher MAR than Bio-Oss® ($P < 0.05$).

3.5. Light microscopic evaluation

Goldner's trichrome staining showed that Defect A, serving as the control group, exhibited uncalcified bone (UB) characterized by orange-stained areas using the Goldner's trichrome stain. The green or light blue-stained areas indicated calcified bone (CB), as illustrated in **Figure 6**. In Group 3, Defects B and C demonstrated more trabecular bone (TB) formation compared to Defect A, as shown in **Figure 7**, **Figure 8**. Dense and evenly distributed calcified new bone was predominantly observed in Defect C, which was filled with ZnCHA, rather than in Defect B, which was filled with Bio-Oss®, across all three groups. Under the light microscope, Defects B and C showed relatively distinct differences in Group 3 (12 weeks). Specifically, trabecular bone formation in Defect C of Group 3 (12 weeks) was greater than in Defect B of the same group. Among the three defects, Defect A consistently showed the least amount of calcified bone across all groups.

3.6. Calculation of trabecular bone and residual bone substitutes

Percentages of trabecular bone area (Tb-Ar/T-Ar) and residual graft material area (R-Ar/T-Ar) are shown in **Table 2**, **Table 3** respectively. For trabecular bone formation (**Table 2**), Defect A showed no significant difference across groups ($P > 0.05$). Defects B and C showed significant increases over time ($P < 0.05$). ZnCHA had higher percentages than Bio-Oss® in Groups 2 and 3 ($P < 0.05$).

For residual bone substitute (**Table 3**), Defect B showed no significant change over time ($P > 0.05$). Defect C showed a significant decrease over time ($P < 0.05$). ZnCHA had less residual material than Bio-Oss® at 8 and 12 weeks ($P < 0.05$).

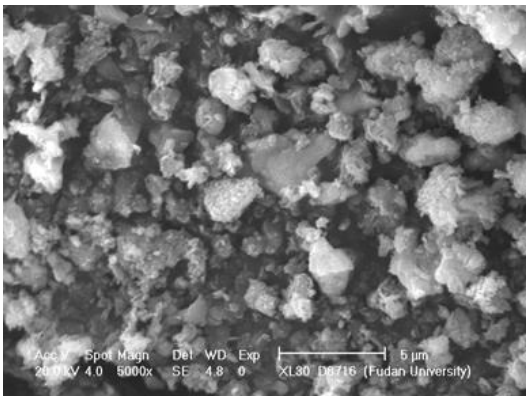

Figure 1: SEM analysis (magnification x 5,000) of avian eggshells.

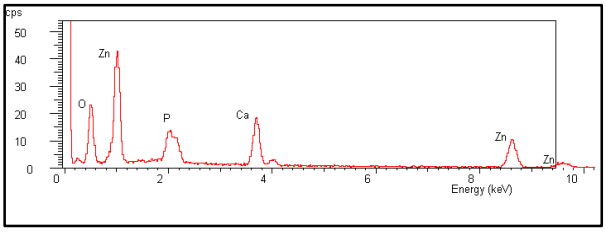

Figure 2: EDXA analysis of treated avian eggshells showing the compositions of O, Zn, P, and Ca elements.

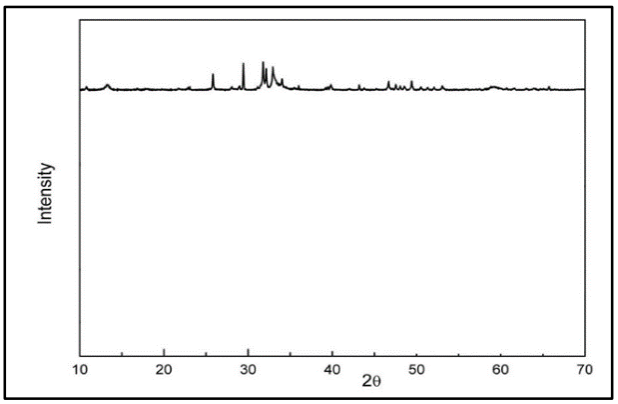

Figure 3: XRD analysis of treated avian eggshell.

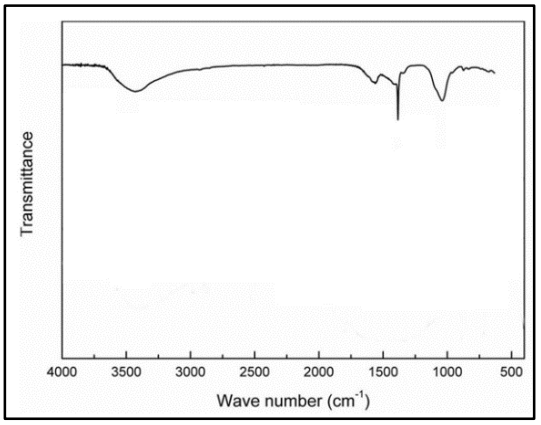

Figure 4: FT-IR analysis of treated avian eggshell.

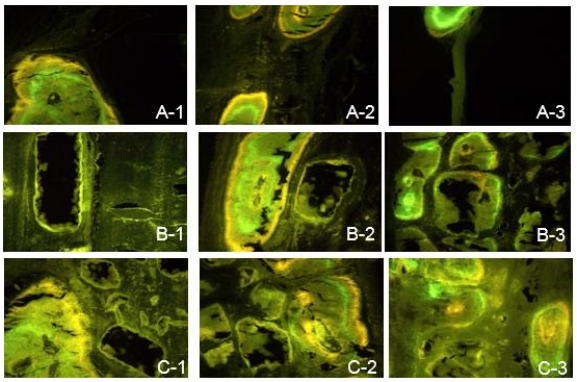

Figure 5: Fluorescence microscopic analysis.

Defect A (Control): A-1 (4 weeks), A-2 (8 weeks), A-3 (12 weeks); the photo in A-3 showed newly formed calcified bone in most areas of the defect.

Defect B (filled with Bio-Oss®): B-1 (4 weeks), B-2 (8 weeks), B-3 (12 weeks); the photo in B-3 showed newly formed calcified bone in many areas of the defects, with some areas remaining empty and not filled with newly formed calcified bone.

Defect C (filled with ZnCHA): C-1 (4 weeks), C-2 (8 weeks), C-3 (12 weeks); the photo in C-3 showed newly formed calcified bone in all areas, while the photos in C-1 and C-2 showed dense and evenly distributed calcified new bone with a small amount of uncalcified bone in between.

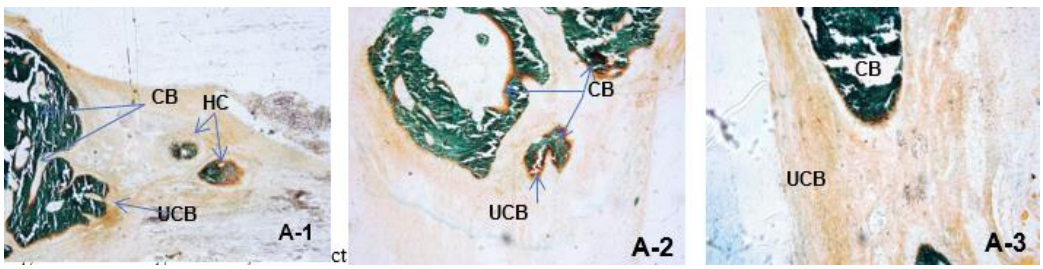

Figure 6: Histological Analysis: Defect A (control without bone substitutes).

Photomicrographs (x100) of Groups A1 (4 weeks), A2 (8 weeks), and A3 (12 weeks).

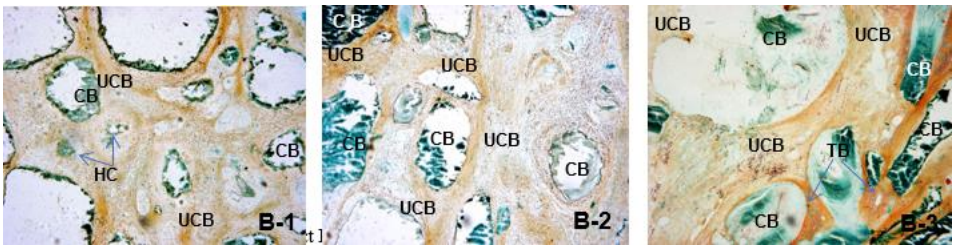

Figure 7: Histological Analysis: Defect B (defect filled with Bio-Oss®).
Photomicrographs (x100) of Groups B1 (4 weeks), B2 (8 weeks), and B3 (12 weeks).

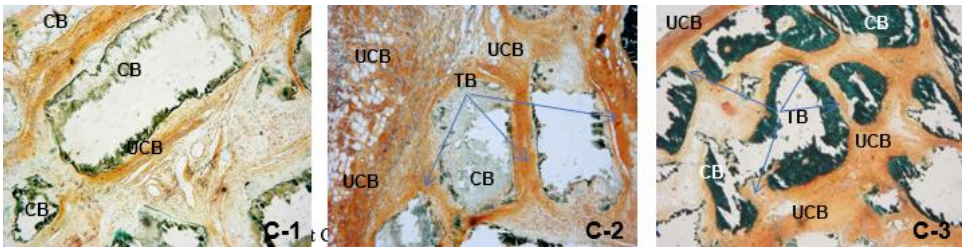

Figure 8: Histological Analysis: Defect C (defect filled with ZnCHA).
Photomicrographs (x100) of Groups C1 (4 weeks), C2 (8 weeks), and C3 (12 weeks).

Figure 6, **Figure 7**, **Figure 8**; CB – Calcified Bone; HC – Haversian Canal; TB – Trabecular Bone and UCB – Uncalcified Bone.

Table 1: Mean and standard deviation values of mineral apposition rate (MAR).

Table 1	4 weeks	8 weeks	12 weeks	P(a)
Bio-Oss®(B)	3.66±1.19	4.36±0.86	4.21±0.98	0.401
ZnCHA(C)	4.05±1.86	4.50±1.43	4.48±0.50	0.087
P(b)	0.634	0.001(*)	0.002(*)	

P(a): Comparison of the same material at different time periods.
P(b): Comparison of different materials in the same time period.
* P value < 0.05 indicates statistical significance.

Table 2: Mean and standard deviation values of the ratio of trabecular bone area (Tb-Ar) to the whole tissue area (T-Ar) within the region of interest (ROI).

Table 2	4 weeks	8 weeks	12 weeks	P(a)
Control (A)	0.029±0.033	0.030±0.036	0.034±0.008	1.000
Bio-Oss®(B)	0.085±0.014	0.128±0.017	0.210±0.033	0.000(*)
ZnCHA(C)	0.095±0.015	0.128±0.016	0.229±0.028	0.000(*)
P(b)	0.011(*)	0.001(*)	0.001(*)	

P(a): Comparison of the same material at different time periods.
P(b): Comparison of different materials in the same time period.
* P value < 0.05 indicates statistical significance.

Table 3: Mean and standard deviation values of the ratio of residual bone graft material area (R-Ar) to the whole tissue area (T-Ar) within the region of interest (ROI).

Table 3	4 weeks	8 weeks	12 weeks	P(a)
Bio-Oss®(B)	0.294±0.051	0.292±0.041	0.291±0.032	0.953
ZnCHA(C)	0.270±0.026	0.201±0.020	0.113±0.016	0.000(*)
P(b)	0.565	0.001(*)	0.000(*)	

P(a): Comparison of the same material at different time periods.
P(b): Comparison of different materials in the same time period.
* P value < 0.05 indicates statistical significance.

4. Discussion

Achieving optimal bone regeneration is contingent upon the use of an appropriate bone substitute with excellent osteoconductive properties.[23,24,25] Commercial allografts have demonstrated promising bone regeneration in clinical studies, with most being derived from bovine or porcine bone.[26,27,28,29,30,31,32,33] This research aimed to evaluate whether avian eggshell could be modified and used as an alternative bone substitute in bone regeneration. Our preliminary in-vivo study on bone substitutes derived from modified avian eggshells showed promising new bone regeneration.[12] Kattimani et al.[34,35,36,37] reported that eggshell-derived nano-hydroxyapatite exhibited superior bone regeneration properties without infection or negative side effects.

Avian eggshells from hens, primarily composed of calcium carbonate (CaCO3), were converted to carbonated apatite (CHA) through chemical and heat modifications.[4,12] This conversion aimed to create a bone substitute that facilitates bone regeneration in humans. Human bone composition is closer to CHA than hydroxyapatite, suggesting that a bone substitute with similar chemical characteristics to human bone could enhance new bone regeneration.

Additionally, zinc coating on the bone substitute may prevent or reduce bacterial colonization during wound healing. Chou et al.[13] reported that zinc-coated commercial resorbable collagen membranes had antibacterial effects and aided in new bone regeneration. In this research, zinc-coated carbonate apatite (ZnCHA) derived from avian eggshells was tested and compared with a commonly used commercial bovine bone substitute, Bio-Oss®. New Zealand rabbits were used, and critical-size bone defects (CSD) were created on their skulls, where either Bio-Oss® or ZnCHA bone substitutes were placed for comparison during bone healing.

The study aimed to determine if the ZnCHA bone substitute would provide similar bone healing properties to commercial bone grafts. Schmitz et al.[38] defined a CSD as the minimal bone defect that could not heal by itself in an animal's lifetime. Three CSDs (6 mm in diameter) were created on each rabbit's skull, based on our preliminary animal study.[12] Groups of ten rabbits were euthanized at 4, 8, and 12 weeks after CSD operation to observe and evaluate the efficacy of bone substitutes in new bone regeneration and their degradation patterns over time.

Fluorescence microscopic evaluation (Figure 5) showed that both CSDs filled with ZnCHA (Defect C) and commercial bovine bone substitute (Defect B) developed new bone randomly at 4 weeks (Group 1). The defects without any bone substitute (Defect A, control) showed bone regeneration mainly adjacent to the natural bone (defect margins) across all three groups at different time periods. Defects B and C displayed diffused fluorescence labels at the inner layer of the bone substitutes with yellow bands (stained with tetracycline), indicating new bone regeneration on the outer layer of the bone substitutes, calcifying toward the inner layer with bone cell apposition and degradation. Our result agreed with a clinical study concluded that eggshell-derived hydroxyapatite becomes a viable choice as regenerative material because of its biocompatibility, lack of disease transfer risks, ease of use, and versatile novel bone graft substitute material.[35]

The degradation rate of bone substitutes is crucial in determining the new bone regeneration rate.[39,40,41] Sartori al et.[40] Stated that the degradation process of the tested bone substitutes, both magnesium-doped bone substitutes clearly induced bone formation, but they should be ideally resorbed and thoroughly substituted by bone tissue at the same time.

Histological analysis (Figures 6, 7, & 8) demonstrated that Defects B and C showed higher bone regeneration across all three groups compared to Defect A (control). The increased bone regeneration in Defects B and C likely resulted from enhanced bone cell activities.[42,43] Sader et al.[43] mentioned that β-TCMP stimulated adhesion and proliferation of human osteoblast cells. Both Bio-Oss® and ZnCHA bone substitutes may have facilitated bone regeneration due to their excellent osteoconductive properties, promoting osteoblast and osteoclast activities. Ali et al.[44] reported increased newly formed blood vessels and osteoid tissue regeneration in all BCP+i-PRF (biphasic calcium phosphate and injectable platelet-rich fibrin), correlating with increased bone regeneration in a sheep animal model.

In **Table 1**, Defect C (filled with ZnCHA) showed significantly higher MAR values than Defect B (filled with Bio-Oss®) in both Group 2 (8 weeks) and Group 3 (12 weeks), indicating ZnCHA's comparable osteoconductive property in bone regeneration to the commercial bovine bone graft. A randomized controlled clinical study using of eggshell-derived nano-hydroxyapatite as novel bone graft substitute reported that almost complete disappearance of lamina dura (91.67%) was observed in grafted sites at the end of sixth-month follow-up and these changes are attributed to the osteoconductive property of the material and remodeling of bone.[34]

Table 2 showed high percentages of new bone regeneration for both Bio-Oss and ZnCHA across all three groups (groups 1, 2, 3), demonstrating having bone substitutes was crucial to bone healing. Kattimani et al.[36] in a clinical study concluded that eggshell-derived hydroxyapatite showed enhancement of bone regeneration, and healing was complete by the end of 12 weeks with a trabecular pattern in all patients irrespective of the size of the lesion involved.

Table 3 indicated that the residual amount of ZnCHA decreased gradually from 4 to 12 weeks, suggesting ZnCHA's

favorable degradation rate for bone deposition. Hence, ZnCHA may serve as a potential bone substitute with sound osteoconductive ability. The degradation velocity is an essential factor for bone regeneration. In eggshell-derived hydroxyapatite, the calcium is released rapidly from the surface because of rapid degradation which increased local calcium essential for bone regeneration.[45]

Throughout this study, ZnCHA bone substitute demonstrated promising bone regeneration, excellent osteoconductive properties, and biocompatibility. Therefore, it may be considered a viable alternative bone substitute for bone regenerative surgeries.

5. Conclusion

The novel ZnCHA bone substitute developed from avian eggshell, which consists of chemical properties similar to human bone, may be considered as an alternative bone substitute. In this animal study, the ZnCHA bone substitute has demonstrated excellent characteristics and properties conducive to favorable bone regeneration.

6. Source of Funding

None.

7. Conflict of Interest

None.

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