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ORIGINAL ARTICLE

Histomorphometric evaluation of a nano-sized eggshell-containing supplement as a natural alloplast: An animal study

Rania Salama [a,*], Mohammed Khashaba [b], Dalia El Rouby [c]

[a] Biomaterials Department, Faculty of Dentistry, Cairo University, Egypt
[b] Oral & Maxillofacial Surgery, Faculty of Dentistry, Cairo University, Egypt
[c] Oral & Maxillofacial Pathology, Faculty of Dentistry, Cairo University, Egypt

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KEYWORDS

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Abstract *Background:* Limitations of autogenous grafts have dictated the need for development of new biomaterials that can serve as allografts. A paradigm shift directed manufacturers to revert to nature in the search for such allografts. This study aimed to evaluate an eggshell-based supplement, Membrell's® BONEhealth Plus D_3 & K_2, indicated to support bone mineral density, as a natural bone graft material.

Methods: Twelve 5 × 10 × 1 mm full-thickness cranial bone defects were created in six adult male New Zealand rabbits. Six defects were filled with Membrell's® BONEhealth™ Plus D_3 & K_2, and the others were left empty as control. The animals were sacrificed 14 days postoperatively. The defects were dissected and prepared for histological assessment. Bone formation was compared both qualitatively and quantitatively. The area percent of newly-formed bone was evaluated in five successive regions using image analysis. Statistical analysis was performed using unpaired *t*-test. Differences between the two groups were considered significant at $p \leq 0.05$.

Results: Cranial bone defects filled with the nano-sized eggshell powder "Membrell's® BONEhealth™ Plus D_3 & K_2" revealed significantly higher levels of osteoid, newly-formed,

* Corresponding author at: Biomaterials Department, Faculty of Dentistry, Cairo University, 11 El-Saraya Street, Manial, Cairo, Egypt.
E-mail address: Rania.salama@dentistry.cu.edu.eg (R. Salama).

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regularly-arranged bone trabeculae in the center of the defects (47.37% ± 1.12) compared to the control defects (21.6% ± 4.92), which revealed no bone formation. A rapid rate of resorption of the nano-sized eggshell powder and consequently a rapid osteogenic effect was evident.

Conclusions: The eggshell-based graft powder, Membrell's® BONEhealth™ Plus D_3 & K_2, is a biocompatible material which has the potential to enhance new bone formation.

1. Introduction

Since environmental waste has become a critical issue for humans, animals, and vegetation, waste management via recycling has become a priority. Waste management will help protect the environment, maintain health quality, and reduce financial burdens. Recycling utilizes wastes to achieve sustainable products, such as biomaterials. The use of waste products as biomaterials utilized in the therapeutic field has added great value to the field of waste recycling (Abdulrahman et al., 2014). Corals (Murugan and Rao, 2002), cuttlefish shells (Rocha et al., 2005), bovine bone (Herliansyah et al., 2009), and sea snail shells (Sahin et al., 2015) are examples of materials that have been successfully reused for biomaterial synthesis. Among the widely bioavailable wastes are the eggshells, which are widely-produced in the egg industry and which represent approximately 11% of the eggs' total weight (Stadelman, 2000). The United Nations Food and Agricultural Organization (FAO) reported that, in 2004, the United States of America produced around 600,000 tons of eggshells (Padmanabhan et al., 2009). According to the FAO (2009), egg production worldwide in 2008 was almost 62 million tons, with China having the greatest production (Oliveira et al., 2013). Therefore, the need for alternative uses of eggshells seemed beneficial. Not only does eggshell calcium provide the best natural calcium source—better than that of limestone or coral sources (Lee and Oh, 2003; Ruff et al., 2012; Uraz et al., 2013)—it is also better absorbed by the body (Dolińska et al., 2016). Eggshell powder has been reported as safe for use by several European and Asian animal and human studies (Ruff et al., 2012; Uraz et al., 2013).

Bone tissue damage, due to disease and/or improper healing after accidents or traumatic injuries, remains a common surgical problem. Bone grafts have been used to augment osseous defects (Moore et al., 2001). Despite the fact that autogenous bone grafts are considered the gold standard, they suffer from limitations, including inadequacy of supply, donor site morbidity, prolonged operation time, and high cost (Damien and Parsons, 1991). Moreover, post-transplantation graft resorption remains a problem (Zins and Whitaker, 1983). Alloplasts have been introduced as an alternative to autogenous grafts (Damien and Parsons, 1991). They are used as space fillers and scaffolds to facilitate bone formation and promote wound healing (Kumar et al., 2013). Such graft materials aim to engineer/regenerate bone. Graft materials should be bioresorbable, elicit no antigen-antibody reaction and provide a reservoir for minerals that induce formation of new bone (Moore et al., 2001). It has become well-accepted that natural products are safer than synthetic alternatives (Yu and Deming, 1998). Natural coral skeleton, composed of calcium carbonates, has been reported as an effective substitute when treating osseous defects. Coralline calcium carbonate is biocompatible, fully-resorbable and results in rapid bone formation because no surface transformation to a carbonate phase is required for the initiation of bone formation (Park et al., 2008; Uraz et al., 2013).

Eggshell has a mineral composition similar to coral, thus providing a promising material for synthesis of calcium phosphate ceramics (Lee and Oh, 2003; Park et al., 2008; Uraz et al., 2013). Eggshell is a biocompatible, bioresorbable, and osteoconductive biomaterial (Durmuş et al., 2008; Lee and Oh, 2003). It is composed of approximately 94% calcium carbonate, 1% calcium phosphate, 4% organic matter and 1% magnesium carbonate (Stadelman, 2000). Thus, it may act as a reservoir of raw materials needed for the biosynthesis of nanomaterials (Abdulrahman et al., 2014). The process of eggshell calcification has attracted attention since it is the most rapid biomineralization processes known (Lavelin et al., 2000). In addition, eggshell powder has a positive effect on bone metabolism (Rovenský et al., 2003). Commercial over-the-counter eggshell-containing supplements to support bone mineral health are available nowadays.

Although experimentally-prepared eggshell powder has been proven successful when used as an allograft (Dupoirieux, 1999; Dupoirieux et al., 1995; Durmuş et al., 2008; Uraz et al., 2013), currently to our knowledge, no data are available on the use of commercially available oral eggshell-based supplements as bone grafts. The aim of the current study was to evaluate, both histologically and histomorphometrically, an eggshell-based supplement, Membrell's® BONEhealth Plus D_3 & K_2, as a natural bone graft material in New Zealand rabbits. The null hypothesis of the current study was that the use of Membrell's® BONEhealth Plus D_3 & K_2 as a natural bone graft material in defects in New Zealand rabbits will yield no difference in bone formation compared to the untreated defects.

2. Materials and methods

2.1. Eggshell-based supplement

Membrell's® BONEhealth Plus D_3 & K_2, a commercially-available over-the-counter dietary supplement for bone health, was used in the present investigation as a natural bone graft material (Table 1). Contents of each capsule were emptied, weighed and equally-divided between two prepared cavities (50 g of powder per cavity).

2.2. Transmission electron microscopy analysis

The particle size of the powder within the capsule was measured using a transmission electron microscope (TEM) at

Table 1 Supplement facts provided with the Membrell's® BONEhealth Plus D_3 & K_2 box.

Supplement Facts Serving size 4 Capsules		
Amount per serving		% DV*
Vitamin C (ascorbic acid)	60 mg	100%
Vitamin D_3 (cholecalciferol)	800 IU	200%
Vitamin K_2 (menaquinone-7)	40 mcg	50%
Calcium (as Calcium from eggshells, ESC®)	1000 mg	100%
Magnesium	500 mg	125%
Zinc	7.5 mg	50%

* The Percent Daily Value (%DV).

80 kV, direct magnification of 12,000×, at accelerating voltage of 200 kV with magnetic imaging resolution of 22 Å. A weight of 0.2 g powder was added to 10 ml distilled water in a plastic tube. A drop of the suspension was placed on a 200-mesh carbon-coated copper grid and allowed to evaporate to allow sample examination.

2.3. Experimental animals and surgical technique

The current study was conducted following the protocol described by Wong and Rabie (2010, 2003, 1999). All animal experiments complied with the EC Directive 86/609/EEC guidelines and were approved by the Faculty of Dentistry, Cairo University Research Ethics Committee (REC #15-12-26). The procedure was conducted at the Faculty of Medicine, Cairo University Animal House. Six adult (five-month-old) New Zealand rabbits; from an inbred colony, weighing 3.5–4.0 kg were used. All animals were anesthetized with an intramuscular injection (0.59 ml/kg) of a combination of ketamine hydrochloride (Ketanes®, Alke, Turkey) and xylazine (Rompun®, Bayer, Leverkusen, Germany). The surgery was performed under sterile conditions. A midline skin incision was made on the skull, and both the periosteum and the temporalis muscle were laterally reflected using a periosteal elevator. Two full-thickness cranial bone defects measuring 5 mm × 10 mm × 1 mm were created in the parietal bones, one defect on each side of the midline (a total of 12 defects). To outline the defect, a stainless-steel wire template was bent to the required defect size to make full-thickness holes. The holes were then connected using surgical burs, under continuous copious irrigation with sterile saline. The rabbits were randomly divided into two groups by using software available online (www.researchrandomizer.org). Group 1 was comprised of three rabbits with six defects filled with the Membrell's® BONEhealth™ powder, where the content of one capsule (100 g) was equally divided among two defects. Group 2 included three rabbits with six defects that were left empty to serve as a negative control (Abdel-Ghany et al., 2017; Wong and Rabie, 2010, 2003, 1999). The eggshell-based supplement powder was directly loaded into the cavities prepared in Group 1 rabbits. Loading was done by means of a particulate graft applicator. All wounds were closed with interrupted 3/0 black silk sutures (Fig. 1). Postoperatively, both oxytetracycline hydrochloride and buprenorphine hydrochloride were administered daily (for 10 and 14 days, respectively). The animals were sacrificed with sodium pentobarbital two weeks after the surgery.

2.4. Specimen preparation for microscopic examination

Immediately after animal sacrifice, the parietal bones were dissected, fixed in 10% formalin, and decalcified in EDTA for four weeks. After decalcification, tissue blocks were processed and embedded in paraffin. Five-micron sections were cut perpendicular to the bone surface, mounted on glass slides, deparaffinized, hydrated, and stained with hematoxylin and eosin (H&E) stain for histological evaluation and histomorphometric analysis.

2.5. Measuring the area percent (%) of newly-formed bone (histomorphometric analysis)

The area percent (%) of the newly-formed bone was estimated by means of a Leica Qwin 500 analyzer computer system, (Leica Microsystems, Switzerland). The cursor was used to outline the areas of newly-formed bone trabeculae, where these areas became masked by a binary blue color measurable by the computer. The image analyzer was calibrated to automatically convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units (Fig. 2). In both groups, the area % of newly-formed bone was estimated in ten different fields in five successive regions of the defect, where region 3 represents the center of the defect (Wong and Rabie, 2010, 2003, 1999), using a magnification of 200×.

2.6. Statistical analysis

The data obtained from computer image analysis were represented as mean values and standard deviations (SD). Statistical analysis was performed using Statistical Package for Scientific Studies #18.0 (SPSS, Inc., Chicago, IL, USA). The unpaired *t*-test was used for statistical analysis of the differences between the groups. A value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Results of the transmission electron microscopy analysis

Transmission electron micrographs revealed that the size range of all the powder ingredients of the Membrell's® BONEhealth Plus D_3 & K_2 were smaller than 50 nm (Fig. 3).

3.2. Macroscopic examination

All six rabbits remained in good health throughout the course of the experiment, with no signs of infection or post-surgical complications and were, therefore, included in the histologic and histomorphometric evaluation.

3.3. Results of the microscopic examination

3.3.1. Control group

H&E-stained sections, obtained from the center of the bony defects, (Fig. 4A and B), revealed the presence of fibrous tissue

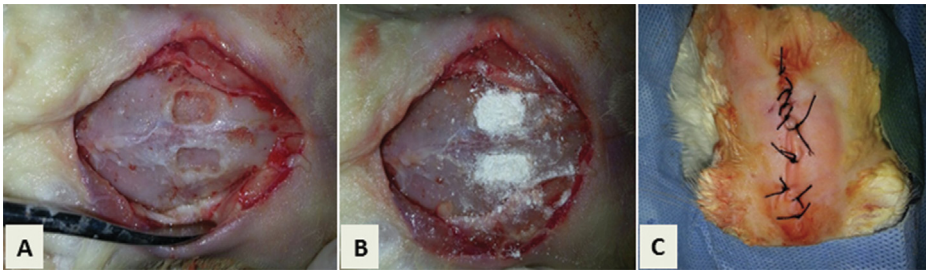

Fig. 1 Surgical procedure in the experimental rabbits showing: (A) the two bone defect beds, (B) the bone defects filled with Membrell's® BONEhealth Plus D_3 & K_2 and (C) the surgical wound after suturing.

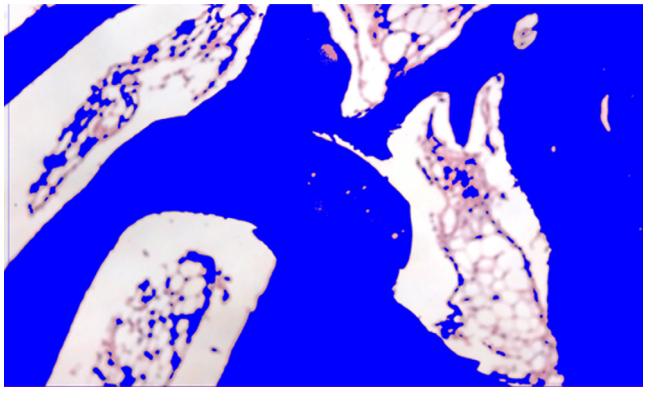

Fig. 2 The image displayed on the monitor of the image analyzer computer system (Leica Qwin 500). The area of bone trabeculae is masked by blue binary color to be measured by the computer system.

and thin irregular osteoid bone (B) growing in the site. Wide fibrovascular (FM) and fatty marrow (F) areas separating the scarce newly-formed bone trabeculae were observed. Lacunae-containing osteocytes were scattered within the marrow areas. Mild chronic inflammatory cell infiltration was noted within the fibrous tissue.

3.3.2. Experimental group (defects filled with Membrell's® BONEhealth Plus D3 & K2)

The H&E-stained photomicrographs of the samples from the experimental group are shown in Fig. 4C–F. Histological sections revealed deposition of osteoid newly-formed bone trabeculae (B) starting from the defect border and growing through the defect. The trabeculae were regularly arranged, separated by narrow bone marrow spaces, and surrounded by an osteoblastic layer. Lacunae containing osteocytes were detected within the trabeculae. Organized collagen fiber bundles (C) were observed at the periphery of the defects. The newly-formed bone consisted mainly of woven bone with some more mature lamellar bone apparent. No inflammatory cell infiltrate was present. The defects were free from any graft traces.

3.4. Histomorphometric analysis

Regions 2, 3, and 4 revealed a significantly larger mean area % of newly formed bone in the experimental group compared to those regions in the control group (Table 2).

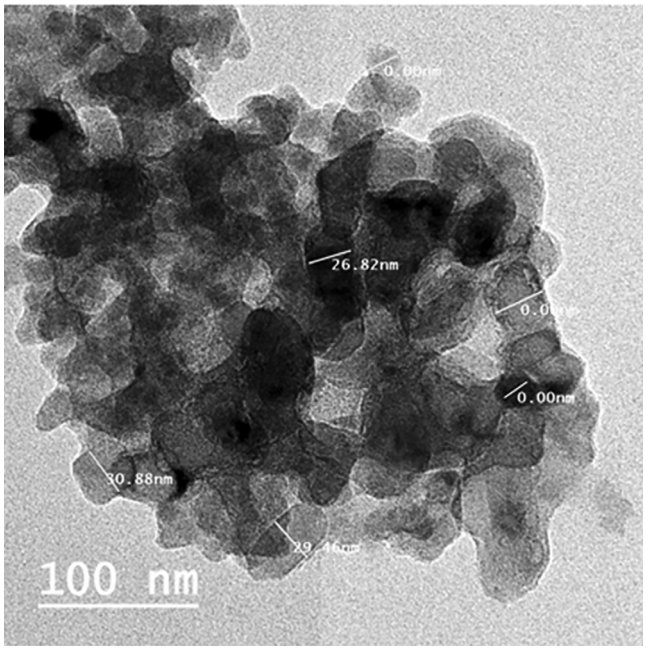

Fig. 3 Transmission electron micrograph of the Membrell's® BONEhealth Plus D_3 & K_2 capsule powder revealing particles of sizes 26.82, 29.46 and 30.88 nm.

4. Discussion

Following bone loss, osseous healing of a defect is one of the primary objectives of regenerative medicine. Bone tissue can regenerate completely, provided the space into which it has to grow is below the critical defect size. Otherwise, the use of a graft is necessary (Kumar et al., 2013). In reverting to natural graft materials, eggshell has been used as a potential bone substitute in maxillofacial reconstructive surgery (Park et al., 2008; Uraz et al., 2013). Therefore, Membrell's® BONEhealth™ Plus D_3 & K_2; an eggshell-based dietary supplement indicated to preserve bone health, was investigated for use as a graft material in the present study. In addition to its calcium content, it contains vitamin D_3 (cholecalciferol, which is necessary for calcium absorption, vitamin K_2 to help maintain bone mineralization, and vitamin C and zinc to help maintain the structural matrix of bones in healthy individuals.

In the present study, we assessed the biocompatibility and regenerative ability of an eggshell-containing supplement as a

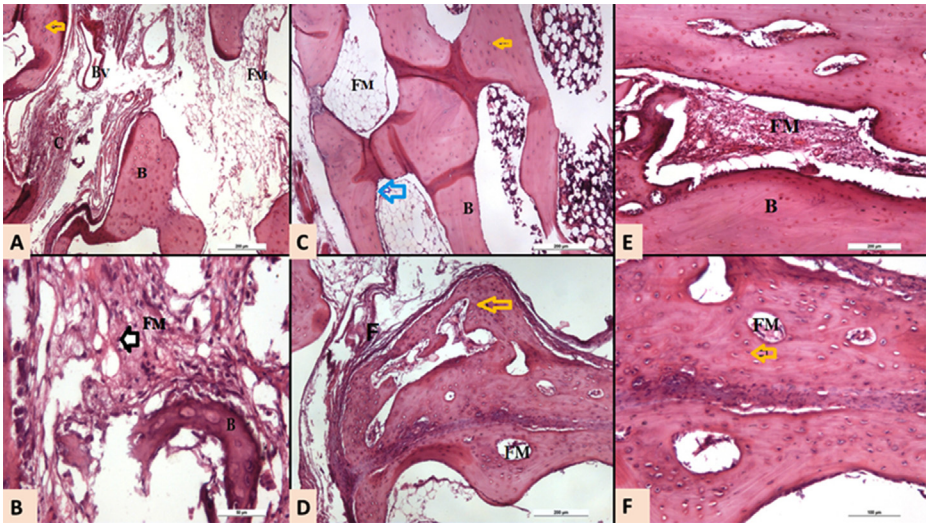

Fig. 4 Hematoxylin and Eosin-stained photomicrographs of the control group (A: 100× and B: 400×), and the experimental group (C–E: 100× F: 200×). The letters on the photomicrographs denote the following: *__B:__ osteoid bone, **FM:** fibrovascular marrow, **F:** fatty marrow, **BV:** blood vessels, **C:** collagen fibers. The yellow arrow is pointing at lacunae containing osteocytes, the black arrow at inflammatory cells and the blue arrow at osteoblasts.

Table 2 Mean ± SD* of the area % occupied by bone trabeculae in the different regions in both the control and experimental groups.

Group		Region 1	Region 2	Region 3 (center)	Region 4	Region 5
Control		82.3 ± 11.4	43.2 ± 7.1	21.6 ± 4.9	39.9 ± 5.0	80.4 ± 9.5
Experimental		81.8 ± 3.0	53.9 ± 4.0	47.4 ± 1.1	48.2 ± 50	81.0 ± 1.4
Significance of difference between groups	t value	0.1	4.2	16.2	4.5	0.2
	P value	0.8845 ns	0.0006**	0.0001**	0.0003**	0.8452 ns

* Mean and standard deviation of six defects.
** Statistically significant, ns = not significant at $p < 0.05$.

bone graft in-vivo prior to its use in humans. Mature rabbits were selected, as they represent approximately 35% of the animals utilized in musculoskeletal research. This is partly due to the ease of their handling and their size, as well as the minimal differences between human and rabbit bone composition and density. Compared to primates and some rodents, rabbits have faster skeletal change and bone turnover (Pearce et al., 2007). Moreover, osseous defects are more actively repaired in mature rabbits than in immature rabbits (Wong and Rabie, 1999). The protocol that was used resulted in minimal morbidity; as evidenced by the good health of all rabbits (Wong and Rabie, 2003).

The rabbits were sacrificed two weeks after surgery. This time interval allowed examination of bone formation during the early stages of healing and gave a better indication of the ability of new bone to grow across the bone defects (Wong and Rabie, 2010, 2003, 1999). The critical-sized bone defects that were created (5 mm × 10 mm × 1 mm) were big enough to ensure that no spontaneous healing would take place without leaving a hollow in the defect site. Non-grafted defects would represent non-unions that did not heal with new bone formation within fourteen days after their creation (Schmitz and Hollinger, 1986). To ensure that the periosteum did not act as a barrier during healing, no periosteum approximation was done.

After two weeks, no evident inflammation or necrotic bone tissue was observed in the defects grafted with the eggshell-based material. These results were similar to those reported by Dupoirieux, who attributed such biocompatibility findings to the calcium carbonate content being a natural component of bone (Dupoirieux, 1999; Dupoirieux et al., 1995). These findings were also consistent with the results by Uraz et al., (2013) who reported biocompatibility and improved bone formation with eggshell powder This may verify the manufacturer's claim, that, unlike other calcium sources, Membrell's® BONEhealth™ Plus D_3 & K_2 ESC® was compliant with California Prop 65 for all four heavy metals, with the full daily requirement for calcium of 1000 mg per day.

Significant levels of osteoid, newly-formed, regularly-arranged bone trabeculae were observed in the eggshell-grafted defects (Fig. 4C–F). Detection of lacunae-containing osteocytes within the trabeculae denoted bone vitality. The evident merging of woven and mature bone trabeculae indicated that the maturation process was in progress. Conversely, there was minimal bone healing across the defects of the control group, as evident in Fig. 4A and B. Therefore, the null hypothesis was rejected. Durmuş et al., (2008) reported that new bone formation in defect sites was observed following the use of eggshell powder of particle size 1000 μm. However, our findings

did not agree with those reported by Dupoirieux et al., (2000), who reported that no bone formation took place after 15 days of filling induced bony-defects in rats with eggshell powder particles ranging from 400 to 600 μm.

In the present study, the eggshell-based powder, Membrell's® BONEhealth™ Plus D_3 & K_2, used to fill the induced bony defects was within the nanometer range, as revealed by the TEM micrographs (Fig. 3). Compared to the control group, significantly higher bone formation was observed in both the center and in the surrounding zones of defects in the eggshell-treated group (Table 2). Since new bone forms in the space left by the resorption of the graft, the higher percentage of bone formed in the center of the eggshell-treated group defects may indicate successful graft degradation and replacement. This process is known as "creeping substitution" and takes place with successful graft materials (Gisep et al., 2003). Creeping substitution was also supported by the absence of any histologically observed residual graft material. Various particle sizes of ostrich eggshell were evaluated by Dupoirieux et al., (2003), who compared the resorption kinetics of four different eggshell powder particle sizes (50, 75, 150, and 300 μm in diameter) that were implanted in subcutaneous pouches of 30 rats. They concluded that resorption of smaller particles took place shortly after the operation, whereas the larger particles were resorbed more slowly. This was explained by the degradation hypothesis, which proposed that the particles initially undergo dissolution in the surrounding fluids and are then phagocyted when they reach a critical size (10–20 μm). Since the powder particle size in the current study was within the nano-size, pinocytosis rather than phagocytosis may have taken place, thereby accelerating the degradation process (Oh and Park, 2014). Accordingly, our findings revealed that the Membrell's® BONEhealth™ Plus D_3 & K_2, being nano-sized, had a rapid rate of resorption and an increased osteogenic effect. However, this may possibly impair the strength of the formed bone.

Our findings were inconsistent with the findings of Durmuş et al. (2008), who reported that there was a correlation between particle size, resorption rate, and osteogenic effect in terms of the amount of bone formation. They concluded that the smaller eggshell particles with rapid resorption resulted in a lower level of bone formation compared to that resulting from larger eggshell particles with relatively slower resorption rate. Therefore, further investigations are needed to evaluate the strength of the formed bone and to correlate strength with the rate of graft resorption and bone formation. These findings may be of clinical value, since such rapid bone healing may shorten the time needed for prosthetic placement following bone loss. The effects of the other constituents of the tested eggshell supplement on bone formation need to be investigated as well. Furthermore, eggshell powder should be investigated as a bioactive filler in polymeric scaffolds.

5. Conclusion

Eggshell powder is a safe, inexpensive, bioavailable, and biodegradable material that accelerates bone healing in surgical defects and may serve as a graft in maxillofacial reconstruction.

Ethical statement

All animal experiments complied with the EC Directive 86/609/EEC for animal experiments guidelines and had been approved by the Research Ethics Committee, Faculty of Dentistry, Cairo University.

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Conflict of interest

The authors have no financial conflicts of interest.

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