Effect of chitosan on bone restoration in nasal bone defect: An experimental study

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Objective
The aim of this work was to study the effect of chitosan in restoration of bone defect (an experimental study).

Materials and methods
The study included 54 male guinea pigs. Nasal bone defect was done. The experimental animals were divided into a control group (group A), calcium sulfate group (group B), and chitosan-coated calcium sulfate group (group C). Three-dimensional computed tomography and histological examination were carried out at intervals of 1, 2, and 3 months for measuring the change in the size of the bone defect and confirmation of bone formation, respectively.

Results
The decrease in the size of the bone defect was significant in group C than in groups A and B. Also, histological results showed formation of woven bone after 1 month in groups B and C and formation of lamellar bone in group C in the second month, whereas the lamellar bone was formed in group B in the third month.

Conclusion
Radiological and histological studies showed that the new bone formation on defected nasal bone was more in group C. These findings suggest that chitosan is very effective in early bone formation.

Keywords:
bone formation, chitosan, guinea pigs

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Introduction

Autologous bone grafts, allografts, xenografts, and bone graft substitutes are all supposed to stimulate early bone formation. As the autograft resorbs and revascularizes, osteoprogenitor cells differentiate into osteogenic cells. This osteogenic cell activity results in new bone generation and healing of the bony defect. However, there is only a limited amount of autologous bone that can be harvested. In addition, the secondary surgery at the harvested site adds an additional degree of morbidity [1].

Calcium sulfate has been used in contained bone defects at sites without substantial compressive load [2,3]. Unlike hydroxylapatite or tricalcium phosphate, which is not completely absorbed and has a high residual rate, calcium sulfate is completely absorbed, and the rate of absorption and bone formation is relatively proportional [4].

Chitosan is a polysaccharide obtained by deacetylation of chitin, which is the major constituent of exoskeleton of crustaceous water animals [5–7]. It has been found to affect cellular migration and tissue organization during the wound-healing process; therefore, it may also enhance bone formation [5]. Muzzarelli *et al.* [8] and Klokkevold *et al.* [9] suggested that chitosan aids in the differentiation of the osteoprogenitor cells and thus may also facilitate bone formation.

The aim of this study was to study the effect of chitosan on restoration of segmental bone defect.

Materials and methods

Experimental group

The study was conducted after obtaining approval from Benha University Ethical Review Committee and was in accordance with the guidelines for animal experiments. This study was conducted on 54 male guinea pigs (weight 500–600 g) that were divided into three groups: group A (the control group), in this group nasal bone defect was restored without placement of any materials; group B, in this group nasal bone defect was closed by placement of calcium sulfate tablets; and group C, in this group nasal bone defect was closed by placement of calcium sulfate tablets coated by chitosan.

The animals were reared under similar conditions to exclude environmental and nutritional factors. The animals were male to obviate the impact of sex on speed and adequacy of bone formation and wound healing. The animals were of nearly similar age and weight. If any of the animals died during follow-up,

it was replaced by a new one to optimize the number according to the statistical rules.

Preparation of calcium sulfate tablets

Calcium sulfate tablets were prepared by mixing calcium sulfate hemihydrate powder in distilled water (Fig. 1). The water–calcium sulfate hemihydrate weight ratio was in the range of 0.27–0.30. Applying high pressure, the tablets were dried in a 60°C convection oven for 24 h (Fig. 2).

Preparation of calcium sulfate tablets coated with chitosan

Medium molecular weight (300 000 g/mol), 90% deacetylated chitosan (Sigma-Aldrich Co., Spruce st. Louis, MO, USA) was dissolved in 2–3% acetic acid solution (chitosan 3 g/2% acetic acid 100 ml) (Fig. 3). Calcium sulfate tablets were coated with chitosan twice through a machine (Fig. 4). The tablets were dried in a 40°C convection oven for 24 h (Fig. 5).

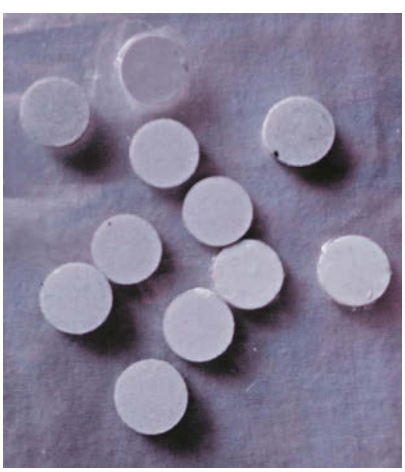
The operative procedure

Anesthesia with ketamine was given intramuscularly at a dose of 10 mg/kg. The hair on the maxilla, nose, and calvarium was shaved (Fig. 6). A volume of 1 ml of 1% lidocaine with adrenaline (1 : 100 000) was injected into the nasal dorsum subperiosteally. A vertical skin incision was made along the frontal calvarium and down the nasal dorsum just posterior to the nares and nasal tip (Fig. 7). The periosteum was incised in the midline, elevated carefully, and retracted laterally beyond the maxillonasalis suture lines. A diamond-shaped design was drawn on the bone using a foil template with long diagonal equal to 1.5 cm and short diagonal equal to 1 cm. Under the operating microscope, a drill was used to carefully burr off the nasal bones down to the

Figure 1

Calcium sulfate hemihydrate powder.

Figure 2



Calcium sulfate tablets.

Figure 3

Chitosan powder.

Figure 4

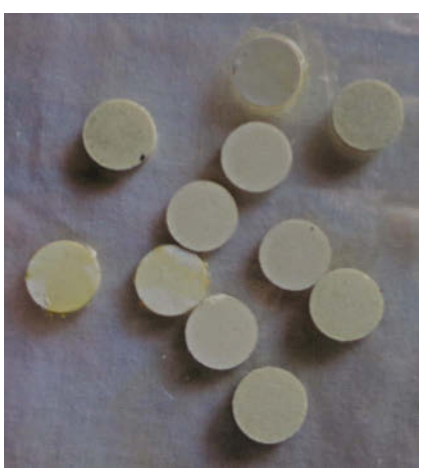
Spin XNG-m1 used for coating.

underlying mucosa; the depth of bone defect was about 0.2 cm (Fig. 8). After copious saline irrigation to remove bone dust and debris, the periosteum was closed as tight as possible with a running 10–0 nylon suture: in group A without placement of any material (Fig. 9), in group B after placement of calcium sulfate tablet (Fig. 10), and in group C after placement of calcium sulfate tablet coated with chitosan (Fig. 11). The skin was then closed using 4–0 proline sutures (Fig. 12).

Radiographic and histologic studies

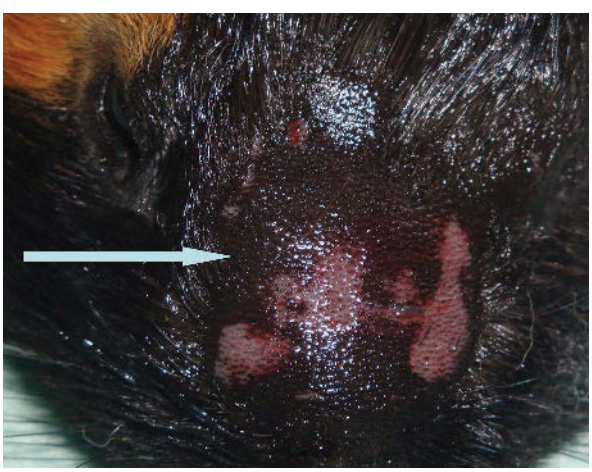
At intervals of 1, 2, and 3 months, six animals from each group were taken and computed tomography (CT) imaging was performed. The CT imaging of the skull was performed using coronal images. For CT technique using GE prospeed spiral CT scanner (Byunion, Europeen), the animal was put in a prone position with the head hyperextended resting on the chin after anesthesia similar to that in operative procedure. Axial images were obtained with spiral technique (supine position) and coronal reconstructions of the axial images were carried out. The gantry angle should be perpendicular to the hard palate to obtain direct coronal images. The cuts were taken as contagious 1 mm sections, 1 mm table feed, pitch 1 from the frontal bone down to involve the whole nose. Low exposure photography (120 kVp, 250 mA) can be used because of the bone algorithm technique at suitable windows to visualize both bone and soft tissues on a single set of images, and three-dimensional reconstruction was made using axial and coronal images for calculating residual defect size [surface area = (long diagonal´short diagonal)/2]. Animals were then killed and nasal bone specimens were taken and fixed in a 10% buffered formalin solution, decalcified with edetic acid and hydrochloric acid, cut in a coronal plane, embedded in paraffin sections, and stained with hematoxylin and eosin for histologic examination.

Figure 5



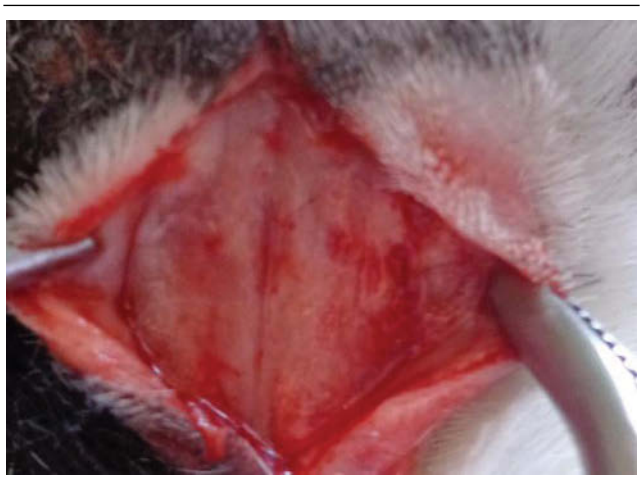
Chitosan-coated calcium sulfate tablets.

Figure 6



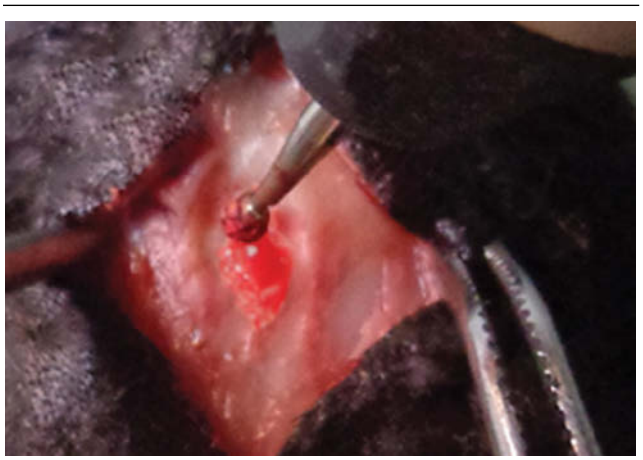
Shaving.

Figure 7



Skin incision.

Figure 8



Burring the defect.

Statistical analysis

The collected data were tabulated and analyzed using the suitable statistical methods. Paired *t*-test was used to compare between two means and one-way analysis of variance test was used to compare between values of more than two means and SDs of more than two groups. The SPSS program (version 11 in IBM compatible computer) was used. *P* values more than 0.05 were considered statistically nonsignificant, *P* values less than 0.05 were considered statistically significant, and *P* values less than 0.01 and 0.001 were considered statistically highly significant.

Results

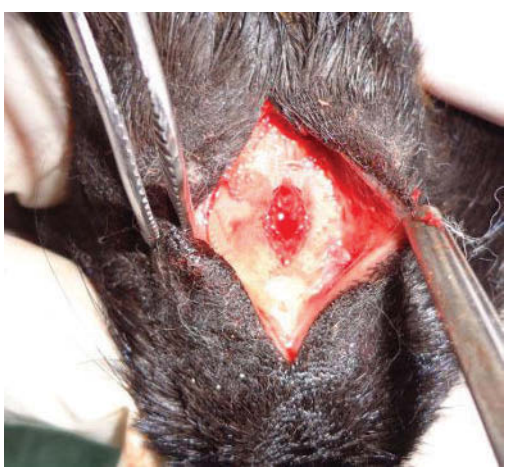
Bone growth and healing of the defect with respect to group A showed nonsignificant decrease in the surface area in the second and third months compared with the first month and also in the third month compared with the second month (Table 1 and Fig. 13). However, groups B and C showed significant decrease in the surface area in the second and third months compared with the first month and also in the third month compared with the second month (Tables 2 and 3 and Fig. 13).

Table 1 Means (χ) ± SD of surface area (cm²) among group A at different times

Times	Surface area (cm²)			
	χ ± SD	χ of difference	Paired *t*	*P*
0	0.75 ± 0	–	–	–
First month (M1)	0.54 ± 0.05	0.21 ± 0.26	t_1=1.98	>0.05
Second month (M2)	0.53 ± 0.04	0.22 ± 0.27	t_2=1.99	>0.05
Third month (M3)	0.54 ± 0.04	0.21 ± 0.26	t_3=1.98	>0.05

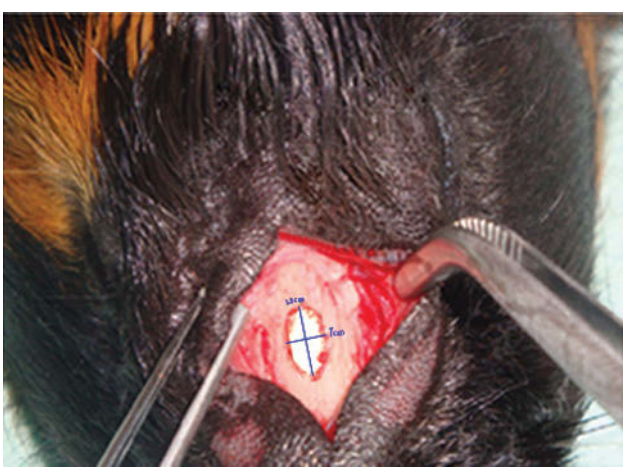
t_1=0 vs. M1, t_2=0 vs. M2, t_3=0 vs. M3.

Figure 9



Diamond-shaped defect in group A.

Figure 10

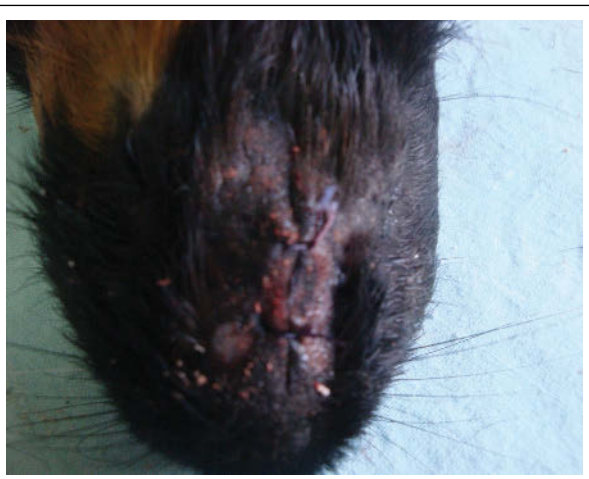


Calcium sulfate tablet.

Figure 11

Chitosan-coated tablet in the defect.

Figure 12



Skin closure in the defect in group B.

With respect to the mean decrease in the surface area in the first month, there was nonsignificant result in group B compared with group A (P = 0.067), significantly higher result in group C compared with group A (P = 0.001), and significantly higher result in group C compared with group B (P = 0.003) (Table 4).

With respect to the mean decrease in the surface area in the second month, there was significantly higher result in group B compared with group A (P = 0.021), significantly higher result in group C compared with group A (P = 0.001), and significantly higher result in group C compared with group B (P = 0.013) (Table 5).

With respect to the mean decrease in the surface area in the 3rd month, there was significantly higher result in group B compared with group A (P < 0.001), significantly higher result in group C compared with group A (P < 0.001), and significantly higher result in group C compared with group B (P = 0.002) (Table 6).

Figures 14–18 showed examples of CT results of the study groups.

Histologic results showed formation of woven bone after 1 month in groups B and C, formation of lamellar bone in group C in the second month, and formation of the lamellar bone in group B in the third month (Figs 19–22).

Discussion

Because of the limitations in the value of biological grafts (infection, vascular necrosis, atrophy, resorption, limited amount of material supply, occurrence of immunologic response because of genetic differences, and the risk of induction of transmissible diseases), considerable attention has been directed toward the use of synthetic materials [10–14]. The aim of using

Table 2 Means (χ) ± SD of surface area (cm²) among group B at different times

Times	Surface area (cm²)			
	χ ± SD	χ of difference	Paired t	P
0	0.75 ± 0	–	–	–
M1	0.46 ± 0.03	0.29 ± 0.03	t_1=22.87	0.000
M2	0.35 ± 0.03	0.4 ± 0.03	t_2=32.67	0.000
M3	0.21 ± 0.4	0.54 ± 0.03	t_3=44.1	0.000

t_1=0 vs. M1, t_2=0 vs. M2, t_3=0 vs. M3.

Table 3 Means (χ) ± SD of surface area (cm²) among group C at different times

Times	Surface area (cm²)			
	χ ± SD	χ of difference	Paired t	P
0	0.75 ± 0	–	–	–
M1	0.37 ± 0.04	0.38 ± 0.04	t_1=23.27	0.000
M2	0.25 ± 0.03	0.5 ± 0.03	t_2=40.83	0.000
M3	0.16 ± 0.03	0.59 ± 0.03	t_3=48.18	0.000

t_1=0 vs. M1, t_2=0 vs. M2, t_3=0 vs. M3.

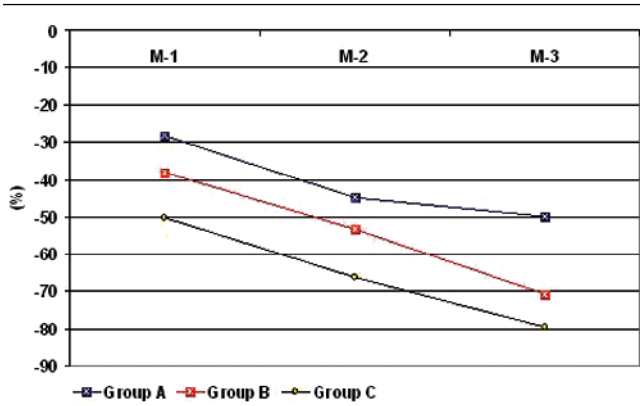
Table 4 Mean ± SD decrease in wound surface area estimated at the end of the first month with respect to the baseline surface area

	Group A	Group B	Group C
% of surface area decrease			
Value	29.6 ± 7.4	38.2 ± 4.6	50.7 ± 5.3
P_1 (A vs. B)		0.067	
P_2 (A vs. C)			0.001
P_3 (B vs. C)			0.003

Table 5 Mean ± SD decrease in wound surface area estimated at the end of the second month

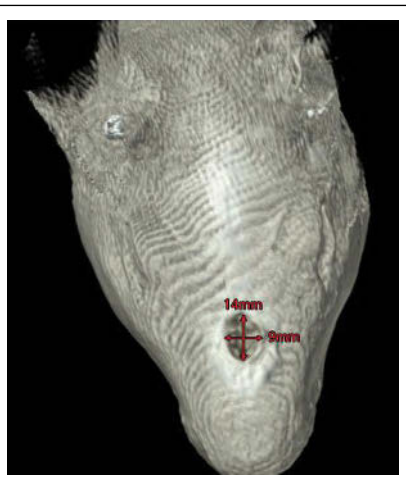
	Group A	Group B	Group C
% of surface area change			
Value	46.2 ± 4.2	54 ± 3.9	66.4 ± 4.1
P_1 (A vs. B)		0.021	
P_2 (A vs. C)			0.001
P_3 (B vs. C)			0.013

Figure 13



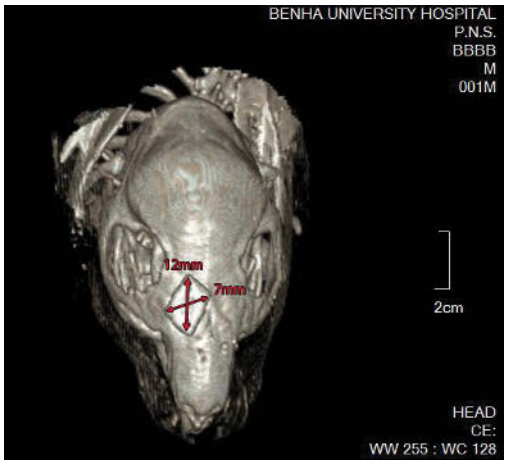
Mean change in wound surface area estimated throughout the study period in the three groups.

Figure 14



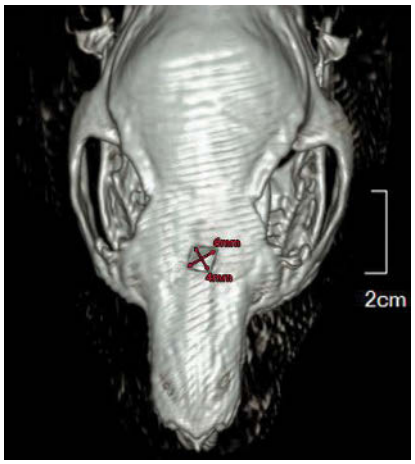
Example of group A after 1 month.

Figure 15



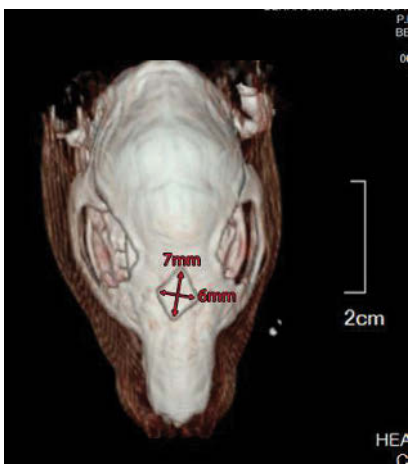
Example of group B after 1 month.

Figure 16



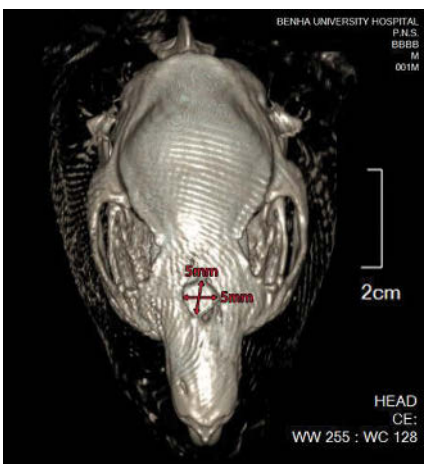
Example of group B after 3 months.

Figure 17



Example of group C after 2 months.

Figure 18



Example of group C after 3 months.

Figure 19

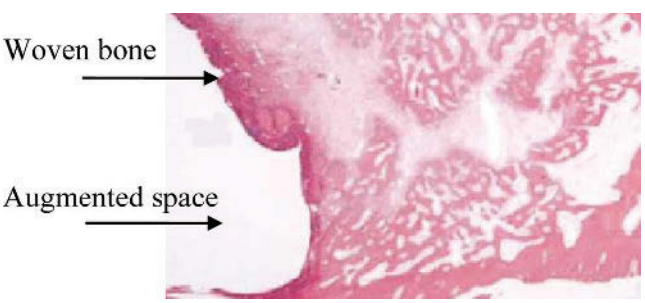
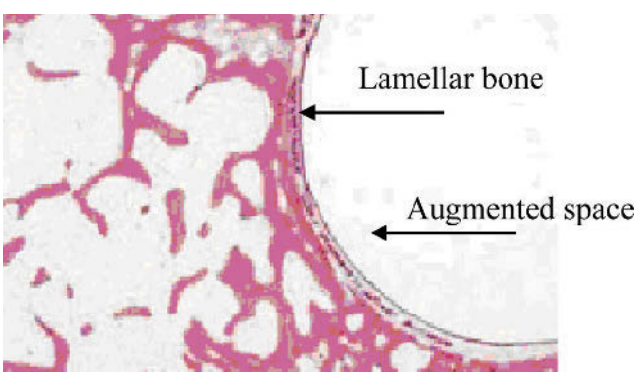


Figure 19 Group B after first month of implantation showing newly formed woven bone at the periphery of the augmented space.

Figure 20

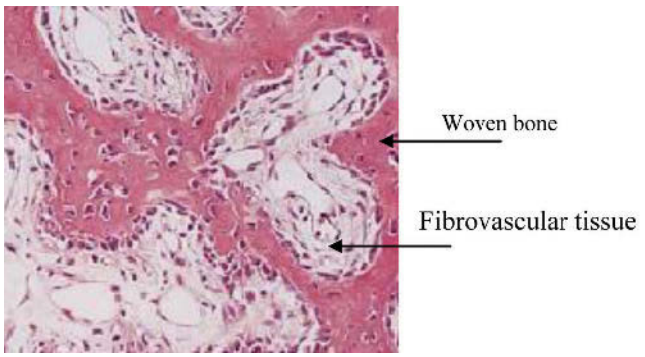


Group B after third month of implantation showing the augmented space deeply concave because of resorption of calcium sulfate.

such material as graft is to promote adequate bone regeneration at the defective site by acting as a scaffold for osseous growth. Dense and porous hydroxylapatite and tricalcium phosphate ceramics are the materials most widely utilized [15,16].

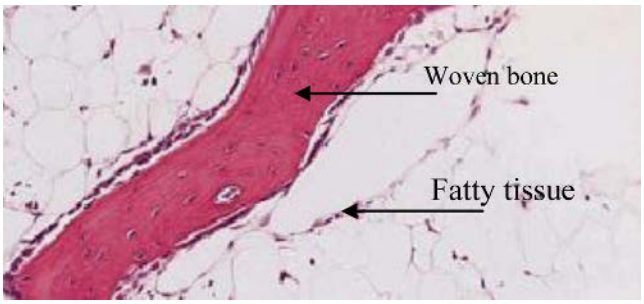
Peltier and Jones [17] proved that the bone graft substitute should be resorbed at a rate that was balanced with new bone growth. Ceramic substitutes

Figure 21



Group C after first month of implantation showing many trabeculae of woven bone embedded in the cell-rich fibrovascular tissue.

Figure 22



Group C after second month of implantation showing some newly formed lamellar bone embedded in the fatty tissue.

Table 6 Mean ± SD decrease in wound surface area estimated at the end of the third month

	Group A	Group B	Group C
% of surface area change			
Value	50.1 ± 8.9	70.8 ± 4.6	79.5 ± 3.4
P_1 (A vs. B)		<0.001	
P_1 (A vs. C)			<0.001
P_2 (B vs. C)			0.002

such as tricalcium phosphate and hydroxylapatite may inhibit bone formation and weaken the intensity of newly formed bones because they typically exhibit a biological residence time greater than that required for new bone formation [4].

Calcium sulphate is an osteoconductive substance, which, unlike tricalcium phosphate and hydroxylapatite, is completely absorbed. Calcium sulphate induces angiogenesis, causes the transfer to the bone graft area of the osteoprogenitor cells, and facilitates new bone formation [1,18,19].

Chitosan is a carbohydrate biopolymer extracted from *N*-acetylated chitin, a structural ingredient in the skeletons of crustaceans (such as lobster, crab, crawfish) and the cell wall of fungi [20–22]. Chitosan has biocompatibility and biodegradability as well as osteoinduction [9]. Normally, chitosan is combined with a growth factor such as fibroblast growth factor, which is in the trabecular bone, and helps in the mitosis of various kinds of matrix cells [6]. Chitosan activates macrophages and mononuclear cells and induces the production of fibroblast growth factor and platelet-derived growth factor [7]. Malette *et al.* [23] have reported that, in an experiment with dogs, the injection of chitosan into the bone defect area causes an increase in bone regeneration.

Our results showed that mean decrease in the surface area was significant in groups B and C but significantly higher in group C than in group B. In addition, the remodeling of woven bone into lamellar bone was early in group C than in group B. This means that bone formation occur in groups B and C but growth rate of new bone is more in group C. In agreement with our study, Cui *et al.* [4], who studied the effect of chitosan-coated pressed calcium sulfate pellets combined with recombinant human bone morphogenetic protein 2 on restoration of segmental bone defect of 12-mm of radius in rabbits, found that chitosan-coated pressed calcium sulfate pellets showed relatively higher density and slightly slower resorption that closely coincides with the growth rate of new bone. This made it possible to restore segmental bone defect; particularly when combined with recombinant human bone morphogenetic protein 2, coated pellet would enhance its osteogenesis.

Canter *et al.* [24] studied the effect of slow release of bone morphogenetic protein 2 and transforming growth factor β-2 in a chitosan gel matrix on cranial bone graft survival in an experimental cranial critical size defect model. The bone formation became apparent at the time point of eighth postoperative week and still persisted at 14th postoperative week. This study is in agreement with our results, as lamellar bone formation occurred in the second month and persisted at the end of third month.

Kim *et al.* [1] studied the role of bone morphogenic protein, transforming growth factor β-induced gene h3 (ig-h3), and chitosan in early bone consolidation in distraction osteogenesis in a dog model. They found that new bone was generated in all groups. The amount of new bone generation in the distracted zone was in the following order: highest in the bone morphogenic protein group (the ig-h3 group) followed by the chitosan group, and then the control group. The difference between our results and their results was because of the use of different materials in their study.

In our study, there were no patients with extrusion of materials or infection. This is supported by the study by Rao and Sharma [25] who found that acute systemic toxicity tests in mice did not show any significant toxic effects of chitosan and eye irritation tests in rabbits and skin irritation tests in guinea pigs did not reveal any undesirable toxic effects of chitosan. Pyrogen-free status could be noticed with chitosan films on animal pyrogen testing; samples retrieved after 3 and 7 days of intramuscular implantation did not reveal identifiable untoward changes.

Conclusion

The findings of this study indicate that chitosan-coated pressed calcium sulfate tablets showed higher growth rate of new bone than calcium sulfate tablets. This indicates that chitosan enhances the process of osteogenesis.

Acknowledgements

Conflicts of interest
None declared.

References

1 Kim I-S, Park JW, Kwon IC, Baik BS, Cho BC. Role of BMP, βig-h3, and chitosan in early bony consolidation in distraction osteogenesis in a dog model. Plast Reconstr Surg 2002; 109:1966–1977.

2 Turner TM, Urban RM, Gitelis S, Kuo KN, Andersson GBJ. Radiographic and histologic assessment of calcium sulfate in experimental animal models and clinical use as a resorbable bone-graft substitute, a bone-graft expander, and a method for local antibiotic delivery. One institution's experience. J Bone Joint Surg A 2001; 83:8–18.

3 Alexander DI, Manson NA, Mitchell MJ. Efficacy of calcium sulfate plus decompression bone in lumbar and lumbosacral spinal fusion: preliminary results in 40 patients. Can J Surg 2001; 44:262–266.

4 Cui X, Zhang B, Wang Y, Gao Y. Effects of chitosan-coated pressed calcium sulfate pellet combined with recombinant human bone morphogenetic protein 2 on restoration of segmental bone defect. J Craniofacial Surg 2008; 19:459–465.

5 Kind GM, Bines SD, Staren ED, Templeton AJ, Economou SG. Chitosan: evaluation of a new hemostatic agent. Curr Surg 1990; 47:37–39.

6 Hauschks PV. In: Hall BK, (editor). Growth factor effect in bone. *Bone*. London: CRC Press; 1990. 103–113.

7 Cunningham NS, Paralkar V, Reddi AH. Osteogenin and recombinant bone morphogenetic protein 2B are chemotactic for human monocytes and stimulate transforming growth factor β1 mRNA expression. Proc Natl Acad Sci USA 1992; 89:11740–11744.

8 Muzzarelli RAA, Mattioli-Belmonte M, Tietz C, Biagini R, Ferioli G, Brunelli MA, *et al.* Stimulatory effect on bone formation exerted by a modified chitosan. Biomaterials 1994; 15:1075–1081.

9 Klokkevold PR, Vandemark L, Kenney EB, Bernard GW. Osteogenesis enhanced by chitosan (poly-*N*-acetyl glucosaminoglycan) in vitro. J Periodontol 1996; 67:1170–1175.

10 Heppenstall, RB, Fracture healing, in fracture treatment and healing (R. B. Heppenstall, ed.) WB. Saunders, company, Philadelphia, 1980;97-112.

11 Buck BE, Malinin TI, Brown MD. Bone transplantation and human immunodeficiency virus: an estimate of risk of acquired immunodeficiency syndrome (AIDS). Clin Orthop Relat Res 1989; 240:129–136.

12 Binderman I, Fin N. In: T Yamamuro, L Hench, J Wilson (editors). Bone substitutes – organic, inorganic and polymeric; cell material interactions. *CRC handbook of bioactive ceramics*. Boca Raton: CRC Press; 1990. 45–51.

13 Ripamonti U. In: T Yamamuro, LL Hench, J Wilson (editors). Inductive bone matrix and porous hydroxyapatite composites in rodents and non-human primates. *CRC handbook of bioactive ceramics*. Boca Raton: CRC Press; 1990. 245–253.

14 Damien CJ, Parsons JR. Bone graft and bone graft substitutes: a review of current technology and applications. J Appl Biomater 1991; 2:187–208.

15 Ricci JL, Blumenthal NC, Spivak JM, Alexander H. Evaluation of a low-temperature calcium phosphate particulate implant material: physical–chemical properties and in vivo bone response. J Oral Maxillofac Surg 1992; 50:969–978.

16 De Groot, K. In: DF Williams, (editor). Degradable ceramics. *Biocompatibility of clinical implants materials, vol. I*. Boca Raton, FL: CRC Press; 1981. 19:9–224.

17 Peltier LF, Jones RH. Treatment of unicameral bone cysts by curettage and packing with plaster-of-Paris pellets. J Bone Joint Surg A 1978; 60A:820–822.

18 Tay Vikas BKB, Patel V, Bradford DS. Calcium sulfate- and calcium phosphate-based bone substitutes mimicry of the mineral phase of bone. Orthop Clin North Am 1999; 30:615–623.

19 Cho BC, Park JW, Baik BS, Kim IS. Clinical application of injectable calcium sulfate on early bony consolidation in distraction osteogenesis for the treatment of craniofacial microsomia. J Craniofacial Surg 2002; 13:465–475.

20 Amano K, Ito E. The action of lysozyme on partially deacetylated chitin. Eur J Biochem 1978; 85:97–104.

21 Pangburn SH, Trescony PV, Heller J. Lysozyme degradation of partially deacetylated chitin, its films and hydrogels. Biomaterials 1982; 3:105–108.

22 Shigemasa Y, Saito K, Sashiwa H, Saimoto H. Enzymatic degradation of chitins and partially deacetylated chitins. Int J Biol Macromol 1994; 16:43–49.

23 Malette WG, Quigley HJ, Adickes ED. *Chitosan effect in nature and technology*. New York: Plenum Press; 1986; 435–457.

24 Canter HI, Vargel I, Korkusuz P, Oner F, Gungorduk DB, Cil B, *et al.* Effect of use of slow release of bone morphogenetic protein-2 and transforming growth factor-beta-2 in a chitosan gel matrix on cranial bone graft survival in experimental cranial critical size defect model. Ann Plast Surg 2010; 64:342–350.

25 Rao SB, Sharma CP. Use of chitosan as a biomaterial: studies on its safety and hemostatic potential. J Biomed Mater Res 1997; 34:21–28.