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The osteoinductive effect of chitosan–collagen composites around pure titanium implant surfaces in rats

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Background and Objective: The enhancing effects of chitosan on activation of platelets and differentiation of osteoprogenitor cells have been demonstrated *in vitro*. The purpose of this study was to evaluate the *in vivo* osteoinductive effect of chitosan–collagen composites around pure titanium implant surfaces.

Material and Methods: Chitosan–collagen composites containing chitosan of different molecular weights (450 and 750 kDa) were wrapped onto titanium implants and embedded into the subcutaneous area on the back of 15 Sprague–Dawley rats. The control consisted of implants wrapped with plain collagen type I membranes. Implants and surrounding tissues were retrieved 6 wks after surgery and identified by Alizarin red and Alcian blue whole mount staining. The newly formed structures in the test groups were further analyzed by Toluidine blue and Masson–Goldner trichrome staining, and immunohistochemical staining with osteopontin and alkaline phosphotase. The bone formation parameters of the new bone in the two test groups were measured and compared.

Results: New bone formed ectopically in both chitosan–collagen groups, whereas no bone induction occurred in the negative control group. These newly formed bone-like structures were further confirmed by immunohistochemical staining. Comparison of bone parameters of the newly induced bone revealed no statistically significant differences between the 450 and 750 kDa chitosan–collagen groups.

Conclusion: Our results demonstrated that chitosan–collagen composites might induce *in vivo* new bone formation around pure titanium implant surfaces. Different molecular weights of chitosan did not show significantly different effects on the osteoinductive potential of the test materials.

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Osseointegrated implant therapy has been widely and successfully applied in dental rehabilitation for more than a decade with predictable long-term results (1–4). Nevertheless, further research is necessary to identify biomaterials able to induce alveolar bone for the purpose of implant placement in defective alveolar ridges, as well as to enhance osseointegration in unfavorable conditions, such as patients with a heavy smoking habit or osteoporosis.

Alloplastic bone substitutes, such as inorganic bone powder, hard tissue replacement polymer, calcium phosphate ceramics, bioactive glass ceramics and modified forms of hydroxyapatites, which are composed of natural or synthetic materials, have demonstrated their various bone regenerative potencies in treatment of osseous and periodontal defects (5,6). However, these materials can have unpredictable clinical results due to a variable stimulation of bone formation.

Chitin (poly-*N*-acetyl-D-glucosamine) is one of the most common natural biopolymers and can be retrieved from shells of invertebrates, such as shrimps or crabs, cell walls of fungi/yeast, and endoskeletons of mollusks or exoskeletons of arthropods (7). Alkaline deacetylation of chitin produces chitosan (poly-D-glucosamine), which is structurally similar to glycosaminoglycans (8). The molecular weight may range from 50 to 2000 kDa, with a degree of deacetylation rate from 30 to 98%. *In vivo*, it is a biocompatible and biodegradable cationic compound that would be resorbed at a rate commensurate with new bone formation within a few weeks. Chitosan can be made into different forms, such as gels, membranes, powders, flakes or solutions, and its versatility has resulted in many commercial and biomedical applications (9).

Clinically, chitosan has been applied as a hemostatic agent (10,11) and as a controlled-release drug carrier (12). It has been reported to have effects such as an enhancement of wound healing and inhibition of bacterial growth and inflammation (13). Previous studies have shown that chitosan can be used as a scaffold material for osteoconduction (14,15). In an animal model, with surgically created bone defects, a modified chitosan carrying covalently linked imidazole groups was used to stimulate bone formation. The surgical defect was occluded with a trabecular bone structure in the peripheral area of the lesion and a mineralized nodule in the central part in association with a fibrous component (14). Histological evidence of periodontal regeneration has also been shown in surgically created one-wall intrabony defects in beagle dogs (15). However, whether chitosan has ability in osteoinduction is unclear. An *in vitro* study evaluating the effect of chitosan on bone formation at the cellular level has suggested that it may enhance the differentiation of osteoprogenitor cells and promote new bone formation (16). The results of subperiosteal placement of chitosan membranes demonstrated their ability to enhance osteogenesis at the site of their implantation with an early mild to severe inflammatory reaction (17). Other animal studies have reported beneficial results when chitosan-based biomaterials were used alone or in combination with tricalcium phosphate, osteoprogenitor cells or platelet-derived growth factors (18–20). In human studies, favorable clinical results were reported using chitosan in promoting the healing of periodontal pockets, palatal wounds and extraction sites (21–23). Implant-related *in vitro* studies have shown that placing chitosan on titanium implant surfaces decreased the wettability of the surfaces but increased the protein adsorption and osteoblast and mesenchymal cell attachment, and thus supported osseointegration of titanium implant fixtures (24–26). Our group has recently reported that chitosan not only enhanced platelet aggregation and activation, but also promoted subsequent release of growth factors, such as epidermal growth factor, platelet-derived growth factor (PDGF)-AB and transforming growth factor-β1 (TGF-β1; 27,28). Chitosan has proved to be a superior and versatile biomaterial with great potential in tissue regeneration.

Collagen is another commonly used biomaterial. A combination of chitosan and collagen may provide further advantages for tissue engineering (29–31). Type I collagen can form an ionic bond to chitosan and increase the structural strength and biophysiological stability of the materials in the form of chitosan–collagen composites (32,33). However, none of the previous studies has examined the effect of chitosan–collagen composites on *de novo* bone formation around titanium implant surfaces. In addition, molecular weight is one of the important factors in affecting not only the physicochemical properties of chitosan, such as tensile strength, chemical bonding, solubility and degradability, but also its biological properties, such as biodegradation and biocompatibility (34,35). Previous studies have investigated the influence of molecular weight on *in vitro* interaction of chitosan with cells and *in vivo* effectiveness of chitosan (36–38). The aim of the present study was therefore to evaluate the *in vivo* effects of chitosan–collagen composites on ectopic bone formation around titanium implant surfaces. In addition, further histomorphometric analyses were used to test whether the molecular weight of chitosan within the composites affected the amount of induced bone formation.

Material and methods

Two types of chitosan (Primex ingredients AS, Avaldenes, Norway) were used, with molecular weights of 450 and 750 kDa and with deacetylation degree > 90%,. The freshly prepared solvent consisted of a vitamin C solution (Merck, Darmsatadt, Germany) with a concentration of 20 mg/mL in de-ionized distilled water. Fifteen milligrams of chitosan powder was dissolved in 10 mL of the vitamin C solution to make a 0.15% chitosan solution. Ten microlitres of each chitosan solution were absorbed onto collagen type I (Col-I) membranes (BioMend®; Integra Life Sciences, Carlsbad, CA, USA) of approximate size 3 mm × 5mm, which were then wrapped around titanium mini-implants (1.6 mm diameter and 3 mm length; Biodent, Tokyo, Japan).

Each experimental group included 15 implants wrapped with Col-I membrane containing either 450 or 750 kDa chitosan. The negative control group consisted of 15 implants wrapped with plain Col-I membrane wet with vitamin C solution. These wrapped implants were immediately inserted into the subcutaneous area on the back of 15 5-wk-old male Sprague–Dawley rats in a nonrandomized positioning pattern under general anesthesia using an intramuscular injection of a combination of fentanyl citrate (0.315 mg/mL) and fluanisone (10 mg/mL) at a dose of 0.01 mL/100 g body weight.

Five rats were randomly selected from these 15 rats for whole mount staining for preliminarily identification of the possibility of new bone formation under the induction of test materials. All rats were killed with a CO_2 overdose 6 wks after implant insertion. Implants and surrounding tissues were identified, retrieved and fixed in 95% ethanol.

According to the developmental biology protocols, Alcian blue and Alizarin red stain were used to visualize the tissues undergoing chondrogenesis and osteogenesis around the titanium implant surfaces in all four groups. Once the bony structures were identified with whole mount stain in the two chitosan–collagen composite groups, further histomorphological verification of these induced bony structures was carried out in the other 10 animals. Bone markers, Alizarin red (0.2 mg/100 g body weight) and calcein (0.3 mg/100 g body weight), were injected intraperitoneally 4 and 1 d, respectively before the rats were killed. After the animals were killed 6 wks after implant insertion, the surrounding tissues were dissected from the implants, immediately fixed in 10% buffered formalin, and processed by paraffin embedding and serial sectioning at 5 μm thickness with a rotary microtome (MICROM HM310; Thermo Fisher Scientific, Waldolf, Germany). The sections were stained with either Toluidine blue or Masson-Goldner Trichrome or they underwent immunohistochemical staining for osteopontin and alkaline phosphatase, to evaluate bone formation. In detail, every fifth section was deparaffinized and rehydrated through graded ethanol to distilled water. Endogenous peroxidase was removed by incubation in 3% H_2O_2 for 15 min. The sections were rinsed in distilled water and processed for antigen retrieval by placing into 0.01 M citric acid in a water bath at 100°C for 40 min. After rinsing, sections were incubated with 3% bovine serum albumin for 10 min to reduce nonspecific antibody binding. Sections were then incubated overnight at 4°C with the primary antibody, anti-osteopontin or anti-alkaline phosphatase (Abcam, Cambridge, UK). Titers were checked, and final dilutions were 1:200 and 1:1000, respectively. Negative controls were run by substituting the primary antibody with bovine serum albumin. Biotinylated secondary antibody and subsequently streptavidin peroxidase conjugate were added onto the slides at room temperature for 20 min. The reaction was visualized using a streptavidin–biotin–immunoperoxidase system with 3-amino-9-ethylcarbizole as chromogen (Dako LSAB® 2 System; Dako, Carpinteria, CA, USA). All sections were counterstained with hematoxylin and mounted with aqueous mounting medium. After qualitative analysis of the two chitosan–collagen composite groups (450 and 750 kDa), we carried out a quantitative comparison of the osseoinductive ability by histomorphometric analyses with a microscope at ×200 magnification for the following parameters: (i) trabecular bone surface, measured by counting the number of cutting points (this is the area of trabecular surface per unit volume of bone tissue, Sv, in mm^2/mm^3); (ii) trabecular bone volume, measured by counting the number of hits (this is the volume occupied by trabecular bone expressed as a fraction of the volume occupied by marrow plus trabecular bone, BV/TV, in mm^3/mm^3); and (iii) mean wall thickness, determined by measuring the mean thickness of new bone formed at bone-forming sites when the formation phase was complete, or the mean distance between cement lines and the trabecular surfaces of completed structural units (MWT, in μm; 39,40).

A semicircular graticule/linear graticule was superimposed on the histological sections to calculate Sv, BV/TV and MWT (39,40). Mean and standard deviation within each group were calculated and were statistically analyzed using Student's paired *t*-test, with the significant difference level set at $p < 0.05$.

Results

Whole mount staining

The peri-implant tissues from five rats processed by whole mount staining showed a strong Alizarin red staining in both the 450 and the 750 kDa chitosan–collagen group (Fig. 1A,B). The surrounding tissues from the implant surfaces of the negative control group wrapped with plain Col-I membrane did not show any Alizarin red staining (Fig. 1C). These results strongly suggested that there were 'calcified structures' in the chitosan–collagen group. However, Alcian blue staining showed no sign of chondrogenesis at 6 wks after grafting in both chitosan–collagen groups and the negative control group. The numbers of implants showing positive staining in each group are listed in Table 1.

Histological analysis

While the results for the collagen control group showed negative findings, the nature of the calcified structures in the test groups was investigated using histomorphological approaches, including Toluidine blue stain, Masson–Goldner trichrome stain and immunohistochemical staining with osteopontin and alkaline phosphatase stain.

Toluidine blue stain and Masson–Goldner trichrome stain — Bone formation was evident in tissues from all rats treated with both forms of chitosan–collagen composites. Toluidine blue staining on all tissue sections showed a bony structure with osteocytes trapped within calcified bone and osteoblasts aligned on the surfaces of calcified bone for both the 450 (Fig. 2A,B) and the 750 kDa chitosan–collagen group (Fig. 2C,D). These bony structures were further confirmed by Masson–Goldner trichrome stain (blue) in both groups (Fig. 2E,F).

Immunohistochemical stain — The expression of osteoblast-related proteins (osteopontin and alkaline phosphatase) was verified on the histologically observed new bone. The osteopontin staining indicated early bone formation activity, while alkaline phosphatase staining represented the calcification process of bone formation. The results showed a strong osteopontin positive staining widely distributed on the sections from both the chitosan–collagen composite groups (Fig. 3A,B). Likewise, positive alkaline phosphatase staining

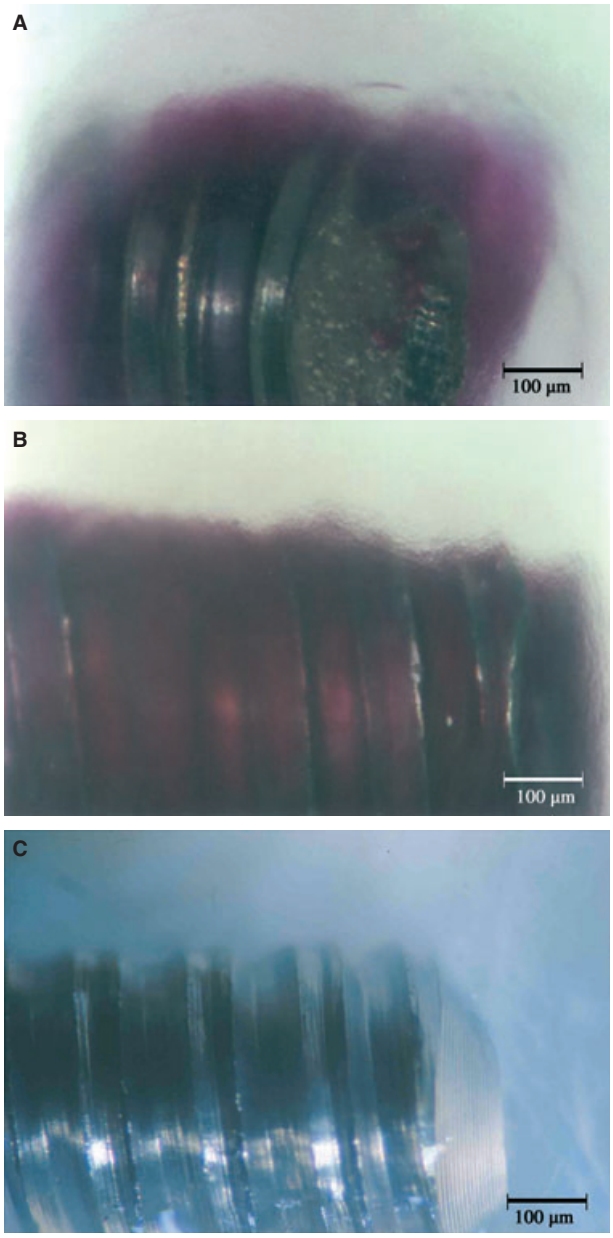

Fig. 1. Positive whole mount staining of calcified tissue on implant surfaces in 450(A) and 750 kDa chitosan–collagen composite group (B) and negative staining on implant surfaces in negative control group (C) with plain Col-I membrane (Alizarin red stain).

was widely distributed on the sections from both test groups (Fig. 3C,D). The presence of bone marker proteins (osteopontin and alkaline phosphatase) proved that the previously observed calcified structures were truly new bone.

Histomorphometric analysis of bone parameters

After confirmation of bone formation in the 450 and 750 kDa chitosan–collagen composite groups by the histomorphological analysis, a further quantitative evaluation was carried out by histomorphometrical measurement of the trabecular bone surface (Sv, in mm^2/mm^3), trabecular bone volume (BV/TV, in mm^3/mm^3) and mean wall thickness (MWT, in μm). The results of histomorphometric analysis showed that the mean values of all three bone parameters were slightly higher in the 750 compared with the 450 kDa chitosan–collagen composite group. Nevertheless, there were no statistically significant differences between these two groups in all parameters, including trabecular bone surface (Sv: 1.36 ± 0.39 vs. 1.41 ± 0.59 mm^2/mm^3), trabecular bone volume (BV/TV: 1.36 ± 0.39 vs. 8.34 ± 2.87 mm^3/mm^3) and mean (trabecular) wall thickness bone (MWT: 1.54 ± 0.60 vs. 1.72 ± 0.80 μm; Table 2).

Discussion

In the present study, chitosan–type I collagen composite showed the ability to enhance new bone formation on titanium implant surfaces, while the negative control group with collagen only showed negative results. The studies of Bumgardener *et al.* illustrated that chitosan-coated titanium increased albumin and fibronectin adsorption and cell attachment when using either an osteoblast precursor cell line or osteoblast cells. They showed that chitosan coating supported osseointegration of titanium implant devices (24,26). The present study further demonstrates heterotopic (extraskeletal) *de novo* bone formation induced by chitosan–collagen composite around titanium implants in the subcutaneous region of rats. This result demonstrates the osteoinductive potential of chitosan–collagen composite *in vivo* and supports previous *in vitro* cell culture research (16,41). Work by Klokkevold *et al.* (16) suggested that chitosan facilitated 'selective tissue regeneration' when applied to mesenchymal stem cells and therefore enhanced the potential for osteoblast differentiation and bone formation. Seol *et al.* (41) used chitosan sponges to support proliferation of rat calvarial osteoblasts in *in vitro* new bone formation. In the present *in vivo* animal experiment, chitosan–collagen composite was shown to be an osteoinductive material based on the following evidence: (i) the formation of calcified structures was verified by Alizarin red whole mount stain; (ii) the histomorphological tissue profile of the osseous structure was characterized by Toluidine blue staining; and (iii) osteoblast-secreted proteins,

Table 1. The fraction of samples showing positive whole mount staining results in each group

Group	Fraction of samples with positive staining results (positive/total)	
	Alizarin red	Alcian blue
Negative control	0/5	0/5
450 kDa chitosan–collagen	5/5	0/5
750 kDa chitosan–collagen	5/5	0/5

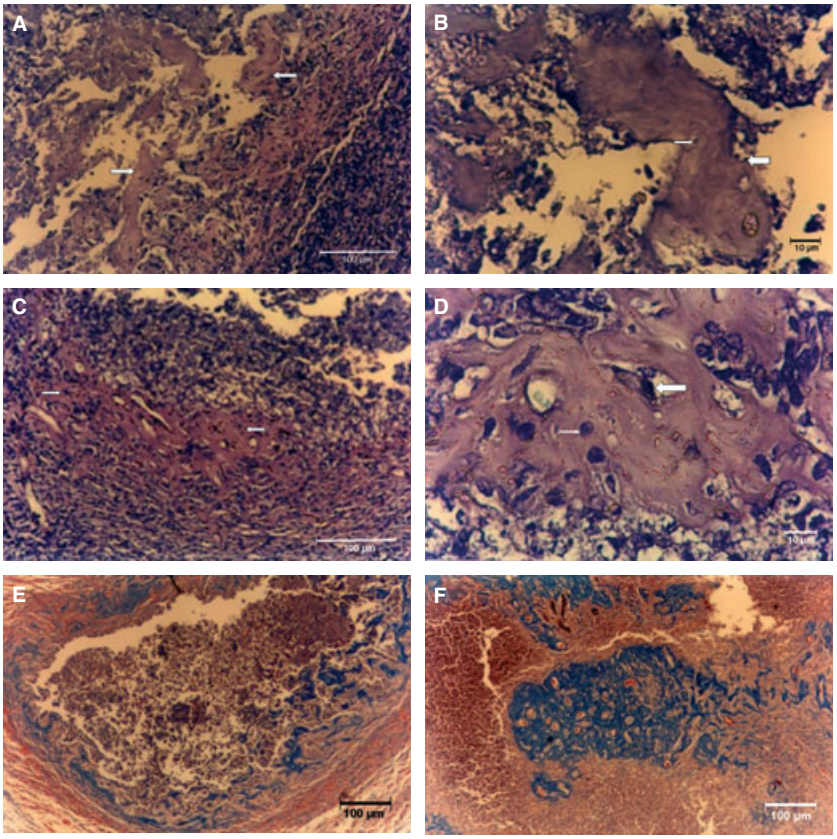

Fig. 2. (A) Tissue section from 450 kDa group showing calcified structures (arrows). (B) Higher magnification view of section shown in (A) illustrates the osteocytes (thin arrow) laid within the calcified bone and osteoblasts (thick arrow) aligned on the bone surfaces. (C)Tissue section from 750 kDa group showed calcified structures (arrows). (D) Higher magnification view of section shown in (C) illustrates the osteocytes (thin arrow) lying within the calcified bone and osteoblasts (thick arrow) aligned on the bone surfaces. Histological sections of the 450 (E) and the 750 kDa group (F) show calcified bony structures (stained blue) formed within surrounding connective tissue (A–D, Toluidine blue stain; E and F, Masson–Goldner trichrome stain).

osteopontin and alkaline phosphotase, were identified by immunohistochemical staining.

It is speculated that chitosan may be acting not only as a scaffold material, but may also involved in inducing new bone formation. By definition, osteoinduction is the process of transformation of local undifferentiated cells into bone-forming cells. Recombinant human bone morphogenic protein 2 (rhBMP2) is one of the most notable examples of an osteoinductive substance and has historically been assayed using subcutaneous or intramuscular implantation in animal models (42,43). In our study, chitosan was dissolved and adsorbed onto a collagen membrane. It was able to stimulate ectopic bone formation in a subcutaneous area, similar to the effect of rhBMP2. Therefore, the term osteoinduction was used. It has been postulated that chitosan, with its *N*-acetylglucosamine units, can bind to fibroblast growth factors and therefore stimulate angiogenesis and osteoblast-like cell proliferation (44). Moreover, it is known that chitosan can enhance platelet adhesion and aggregation, which is beneficial for blood coagulation (11). Platelets are enriched with PDGF and TGF-β. High concentrations of platelets or platelet-rich plasma release high levels of PDGF and TGF-β (45). Researchers in our laboratory have shown that chitosan can stimulate platelets to release these growth factors (28). These growth factors are capable of regulating osseous-related activities, such as fracture repair (46). Platelets can also stimulate the mitogenic activity of bone cells, thereby contributing to the regeneration of mineralized tissue (47,48). It is our hypothesis that chitosan can attract platelets and other osteoprogenitor cells from circulating blood in surrounding tissues. The subsequent activation of platelets in the graft sites promotes the release of platelet-derived growth factors, such as TGF-β, PDGF, insulin-like growth factor and endothelial cell growth factor, which are valuable for new bone formation. This in turn activates the cascade of wound healing and osteogenesis. It is possible that heterotopic bone formation involves differentiation of local mesenchymal cells in connective tissue cells into bone-forming cells under the influence of platelets and related growth hormones, is was enhanced by the presence of chitosan. In addition, collagen is a bioactive polymer, but did not induce any detectable ectopic bone formation by itself as a negative control in this study. However, it cannot be completely excluded that the chitosan–collagen composite may have synergistic effects on osteoinduction when the separate polymers are combined together. A combination of collagen and glycoaminoglycan-like

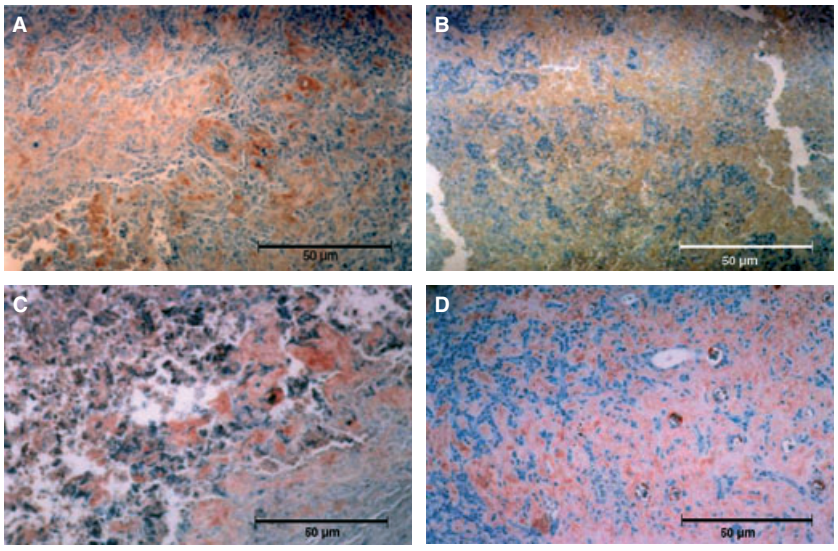

Fig. 3. Immunohistochemical staining of osteopontin showed strong positive staining (brown) widely distributed in the 450 (A) and 750 kDa groups (B). Immunohistochemical staining of alkaline phosphatase showed strong orange positive staining widely distributed in the 450 and (C) 750 kDa groups (D).

Table 2. The mean (standard deviation) of bone parameters in the two chitosan–collagen groups

	Molecular weight of chitosan		
Bone parameters	450 kDa	750 kDa	*p*-value
Trabecular bone surface (Sv; mm^2/mm^3)	1.36 (0.39)	1.41 (0.59)	Not significant
Mean wall thickness (MWT; μm)	7.87 (1.94)	8.34 (2.87)	Not significant
Trabecular bone volume (BV/TV; mm^3/mm^3)	1.54 (0.60)	1.72 (0.80)	Not significant

Student's paired *t*-test with a significant level of $p < 0.05$.

materials may provide a more advantageous environment, mimicking the natural extracellular matrix, for bone regeneration (29). Titanium implants have been used as vehicles to carry the chitosan–collagen composites because of the excellent mechanical properties and the bone compatibility of titanium (49). However, the lack of an active role of the titanium implants themselves in bone formation was excluded by the negative results of the control group. Its synergistic effect in the whole delivered material was not assessed in this study.

Osteoinductive biomaterials, usually containing families of bone initiator–bone morphogenic proteins and osteogenic proteins, can induce *de novo* endochondral bone formation *in vivo* (50,51). However, the concept of 'intrinsic osteoinduction' by the use of biomimetic biomaterials (smart materials) that can induce specific responses from the host tissues without the addition of exogenously applied human bone morphogenic proteins and osteogenic proteins was proposed by Ripamonti's group (52,53). They suggested that the initiation of bone formation was a secondary response, because a porous hydroxyapatite graft acted as a solid stratum for the adsorption, storage and controlled release of endogenously produced or circulating bone morphogenic proteins and osteogenic proteins (51). Furthermore, the intrinsic osteoinduction mechanism lacks a chondrongenic phase that is present in the process of bone formation induced by bone morphogenic proteins (52,54). Our study showed that at the sixth week, there was no sign of chondrogenesis after induction by chitosan–collagen composite. This might suggest that chitosan of different molecular weights carried by collagen induced new bone formation via a nonchondrogenic ossification process, possibly similar to the intrinsic osteoinduction mechanism of the porous hydroxyapatite. However, we will need to carry out further studies at earlier and different stages to confirm the lack of chondrogenesis throughout the process of osteoinduction.

Autogenous bone has proven to be the gold standard for bone augmentation materials (55). However, it poses specific problems, such as limited supply, donor site morbidity and occasional difficulties in shaping and adaptation (56). Other grafting materials, such as allografts, xenografts and synthetic materials, suffer from increased susceptibility to infection and immunological reactions, uncertain long-term host–graft interactions or undesirable slow resorption or nonresorption after implantation (57). Barrier membranes were shown in several studies to have a promising bone-promoting effect, and the guided bone regeneration technique has been used extensively for bone reconstructive procedures in implant dentistry (58). However, the complications with membrane exposure, infection and membrane collapse have also been reported to affect the final results of implant success (59,60). The material that we used in the present study, chitosan, is a nontoxic, nonimmunoreactive and nonmembranous material that would be resorbed at a rate commensurate with new bone formation within a few weeks (8). Likewise, the full resorption time for the collagen membrane used is about 6–8 wks. This study evaluated whether different molecular weights of chitosan in the chitosan–collagen composites may lead to different rates of bone formation. The histomorphometric analysis showed that the bone parameters in the 750 kDa chitosan–collagen composite group were slightly higher than those in the 450 kDa group. However, the differences were not statistically significant. This might be caused by low sample numbers or, indeed, it is possible that the high degree of deacetylation of the chitosan used in this

study played a more dominant effect than the molecular weight of chitosan on the results of new bone formation. One study has shown that the degree of deacetylation of chitosan rather than the molecular weight played a crucial role in cell morphology and activities of osteoblasts *in vitro* (61). Further studies will be concentrated on not only verifying the proposed mechanism of osteoinduction by chitosan–collagen composites, but also their clinical application for enhancing bone formation and osseointegration in compromised medical conditions or sites.

Conclusions

The results of the present study showed that chitosan–collagen composites might be capable of inducing new bone formation around pure titanium implants in the subcutaneous tissues of rats. There was no significant difference in bone parameters when results from two types of chitosan with molecular weights of 450 and 750 kDa were compared. Future application of chitosan–collagen composites for enhancing bone formation and osseointegration of implants in compromised conditions seems promising.

References

1. Adell R, Eriksson B, Lekholm U, Branemark PI, Jemt T. A long-term follow-up study of osseointegrated implants in the treatment of totally edentulous jaws. *Int J Oral Maxillofac Implants* 1990;**5:**347–359.
2. Albrektsson T. A multicenter report on osseointegrated oral implants. *J Prosthet Dent* 1988;**60:**75–84.
3. Buser D, Weber HP, Bragger U, Balsiger C. Tissue integration of one-stage implants: three-year results of a prospective longitudinal study with hollow cylinder and hollow screw implants. *Quintessence Int* 1994;**25:**679–686.
4. Weber HP, Crohin CC, Fiorellini JP. A 5-year prospective clinical and radiographic study of non-submerged dental implants. *Clin Oral Implants Res* 2000;**11:**144–153.
5. Misch CE, Dietsh F. Bone-grafting materials in implant dentistry. *Implant Dent* 1993;**2:**158–167.
6. Gross JS. Bone grafting materials for dental applications: a practical guide. *Compend Contin Educ Dent* 1997;**18:**1013–1036.
7. Muzzarelli RA. Chitin. In: Asinall GO, ed. *The Polysaccharides*. London: Academic Press, 1985:417–450.
8. Sanford PA. Chitosan: commercial uses and potential applications. In: Skjak-Braek G, Anthonsen T, Sanford PA, eds. *Chitin and Chitosan*. London: Elservier Applied Science, 1989:51–70.
9. Martino AD, Sittinger M, Risbud MV. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials* 2005;**26:**5984–5990.
10. Klokkevold PR, Lew DS, Ellis DG, Bertolami CN. Effect of chitosan on lingual hemostasis in rabbits. *J Oral Maxillofac Surg* 1991;**49:**858–863.
11. Thatte HS, Zagarins S, Khuri SF, Fischer TH. Mechanisms of poly-N-acetyl glucosamine polymer-mediated hemostasis: platelet interactions. *J Trauma* 2004;**57:**S13–S21.
12. Felt O, Buri P, Gurny R. Chitosan: a unique polysaccharide for drug delivery. *Drug Dev Ind Pharm* 1998;**24:**979–993.
13. Allen GG, Altman AC, Bensinger RE, Ghosh DK, Hirabayashi Y, Neogi AN. Biomedical applications of chitin and chitosan. In: Zikakis JP, ed. *Chitin, Chitosan and Related Enzymes*. New York: Academic Press, 1984:119–134.
14. Muzzarelli RA, Mattioli-Belmonte M, Tietz C *et al*. Stimulatory effect on bone formation exerted by a modified chitosan. *Biomaterials* 1994;**15:**1075–1081.
15. Park JS, Choi SH, Moon IS, Cho KS, Chai JK, Kim CK. Eight-week histological analysis on the effect of chitosan on surgically created one-wall intrabony defects in beagle dogs. *J Clin Periodontol* 2003;**30:**443–453.
16. Klokkevold PR, Vandemark L, Kenney EB, Bernard GW. Osteogenesis enhanced by chitosan (poly-N-acetyl glucosaminoglycan) in vitro. *J Periodontol* 1996;**67:**1170–1175.
17. Hidaka Y, Ito M, Mori K, Yagasaki H, Kafrawy AH. Histopathological and immunohistochemical studies of membranes of deacetylated chitin derivatives implanted over rat calvaria. *J Biomed Mater Res* 1999;**46:**418–423.
18. Lee YM, Park YJ, Lee SJ *et al*. Tissue engineered bone formation using chitosan/tricalcium phosphate sponges. *J Periodontol* 2000;**71:**410–417.
19. Park YJ, Lee YM, Park SN, Sheen SY, Chung CP, Lee SJ. Platelet derived growth factor releasing chitosan sponge for periodontal bone regeneration. *Biomaterials* 2000;**21:**153–159.
20. Lee JY, Nam SH, Im SY *et al*. Enhanced bone formation by controlled growth factor delivery from chitosan-based biomaterials. *J Control Release* 2002;**78:**187–197.
21. Pang EK, Paik JW, Kim SK *et al*. Effects of chitosan on human periodontal ligament fibroblasts in vitro and on bone formation in rat calvarial defects. *J Periodontol* 2005;**76:**1526–1533.
22. Sapelli PL, Baldassare V, Muzzarelli R, Emanuelli M. Chitosan in dentistry. In: Muzzarelli R, ed. *Chitin in Nature and Technology*. New York: Plenum, 1986:507–512.
23. Muzzarelli RA, Biagin G, Pugnaloni A *et al*. Reconstruction of parodontal tissue with chitosan. *Biomaterials* 1989;**10:**598–603.
24. Bumgardner JD, Wiser R, Elder SH, Jouett R, Yang Y, Ong JL. Contact angle, protein adsorption and osteoblast precursor cell attachment to chitosan coatings bonded to titanium. *J Biomater Sci Polym Ed* 2003;**14:**1401–1409.
25. Bumgardner JD, Wiser R, Gerard PD *et al*. Chitosan: potential use as a bioactive coating for orthopaedic and craniofacial/dental implants. *J Biomater Sci Polym Ed* 2003;**14:**423–438.
26. Bumgardner JD, Chesnutt BN, Yuan Y *et al*. The integration of chitosan-coated titanium in bone: an in vivo study in rabbits. *Implant Dent* 2007;**16:**66–79.
27. Chou TC, Fu E, Wu CJ, Yeh JH. Chitosan enhances platelet adhesion and aggregation. *Biochem Biophys Res Commun* 2003;**302:**480–483.
28. Shen EC, Chou TC, Gau CH, Tu HP, Chen YT, Fu E. Releasing growth factors from activated human platelets after chitosan stimulation: a possible bio-material for platelet-rich plasma preparation. *Clin Oral Implants Res* 2006;**17:**572–578.
29. Tan W, Krishnaraj R, Desai TA. Evaluation of nanostructured composite collagen-chitosan matrices for tissue engineering. *Tissue Eng* 2001;**7:**203–210.
30. Ma L, Gao C, Mao Z *et al*. Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. *Biomaterials* 2003;**24:**4833–4841.
31. Gingras M, Paradis I, Berthod F. Nerve regeneration in a collagen-chitosan tissue-engineered skin transplanted on nude mice. *Biomaterials* 2003;**24:**1653–1661.
32. Taravel MN, Domard A. Collagen and its interactions with chitosan. II. Influence of the physicochemical characteristics of collagen. *Biomaterials* 1995;**16:**865–871.
33. Taravel MN, Domard A. Collagen and its interactions with chitosan. III. Some biological and mechanical properties. *Biomaterials* 1995;**17:**451–455.
34. Chen RH, Hwa H-D. Effect of molecular weight of chitosan with the same degree of deacetylation on the thermal, mechanical, and permeability properties of the prepared membrane. *Carbohydr Polym* 1996;**29:**353–358.
35. Zhang H, Neau SH. In vitro degradation of chitosan by a commercial enzyme preparation: effect of molecular weight

and degree of deacetylation. *Biomaterials* 2001;**22:**1653–1658.
36. Yang J, Tian F, Wang Z, Wang Q, Zeng YJ, Chen SQ. Effect of chitosan molecular weight and deacetylation degree on hemostasis. *J Biomed Mater Res B Appl Biomater* 2008;**84:**131–137.
37. Prego C, Torres D, Alonso MJ. Chitosan nanocapsules as carriers for oral peptide delivery: effect of chitosan molecular weight and type of salt on the in vitro behaviour and in vivo effectiveness. *J Nanosci Nanotechnol* 2006;**6:**2921–2928.
38. Fang N, Chan V, Mao HQ, Leong KW. Interactions of phospholipid bilayer with chitosan: effect of molecular weight and pH. *Biomacromolecules* 2001;**2:**1161–1168.
39. Parfitt AM. The physiological and clinical significance of bone histomorphormetric data. In: Recker RR, ed. *Bone Histomorphometry: Techniques and Interpretation*. Boca Raton: CRC Press, 1983:143–223.
40. Parfitt AM, Drezner MK, Glorieux FH *et al*. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 1987;**2:**595–610.
41. Seol YJ, Lee JY, Park YJ *et al*. Chitosan sponges as tissue engineering scaffolds for bone formation. *Biotechnol Lett* 2004;**26:**1037–1041.
42. Urist MR. Bone: formation by autoinduction. *Science* 1965;**150:**893–899.
43. Sampath TK, Reddi AH. Dissociative extraction and reconstruction of extracellular matrix components involved in local bone differentiation. *Proc Natl Acad Sci U S A* 1981;**78:**7599–7603.
44. Muzzarelli RA, Zucchini C, Ilari P *et al*. Osteoconductive properties of methylpyrrolidinone chitosan in an animal model. *Biomaterials* 1993;**14:**925–929.
45. Okuda K, Kawase T, Momose M *et al*. Platelet-rich plasma contains high levels of PDGF& TGF-β and modulates the proliferation of periodontally related cells in vitro. *J Periodontol* 2003;**74:**849–857.
46. Barnes GL, Kostenuik PJ, Gerstenfeld LC, Einhorn TA. Growth factor regulation of fracture repair. *J Bone Miner Res* 1999;**14:**1805–1815.
47. Gruber R, Varga F, Fischer MB, Watzek G. Platelets stimulate proliferation of bone cells: involvement of platelet-derived growth factor, microparticles and membranes. *Clin Oral Implants Res* 2002;**13:**529–535.
48. Kawase T, Okuda K, Wolf L, Yoshie H. Platelet-rich plasma-derived fibrin clot formation stimulates collagen synthesis in periodontal ligament and osteoblastic cells in vitro. *J Periodontol* 2003;**74:**849–857.
49. Hartman HM, Vehofb J, Spauwena P, Jansenb JA. Ectopic bone formation in rats: the importance of the carrier. *Biomaterials* 2005;**26:**1829–1835.
50. Reddi AH. Symbiosis of biotechnology and biomaterials: applications in tissue engineering of bone and cartilage. *J Cell Biochem* 1994;**56:**192–195.
51. Ripamonti U, Duneas N. Tissue morphogenesis and regeneration by bone morphogenic proteins. *Plast Reconstr Surg* 1998;**101:**227–239.
52. Ripamonti U, Crooks J, Kikbride AN. Sintered porous hydroxyapatities with intrinsic osteoconductive activity: geometric induction of bone formation. *S Afr J Sci* 1999;**95:**335–343.
53. Ripamonti U. Smart materials with intrinsic osteoconductivity: geometric control of bone differentiation. In: Davies JE, ed. *Bone Engineering*. Toronto: Em Squared, 1999:215–221.
54. Ripamonti U. Osteoinduction in porous hydroxyapatite implanted in heterotopic sites of different animal models. *Biomaterials* 1996;**17:**31–35.
55. Buser D, Dula K, Hess D, Hirt HP, Belser UC. Localized ridge augmentation with autografts and barrier membranes. *Periodontol 2000* 1999;**19:**151–163.
56. Younger EM, Chapman MW. Morbidity at bone graft donor sites. *J Orthop Trauma* 1989;**3:**192–195.
57. Piattelli M, Favero GA, Scarano A, Orsini G, Piattelli A. Bone reactions to anorganic bovine (Bio-Oss) used in sinus augmentation procedure: a histologic long-term report of 20 cases in humans. *Int J Oral Maxillofac Implants* 1999;**14:**835–840.
58. Becker W, Dahlin C, Becker BE *et al*. The use of ePTFE barrier membranes for bone promotion around titanium implants place into extraction sockets: a prospective multicenter study. *Int J Oral Maxillofac Implants* 1994;**9:**31–40.
59. Arora BK, MacDonald W, Guttu RL, Laskin DM. Bone formation over partially exposed implants using guided tissue generation. *J Oral Maxillofac Surg* 1992;**50:**1060–1065.
60. Lorenzoni M, Pertl C, Polansky RA, Jakse N, Wegscheider WA. Evaluation of implants placed with barrier membranes. A restrospective follow-up study up to five years. *Clin Oral Implants Res* 2002;**13:**274–280.
61. Suphasiriroj W, Yotnuengnit P, Surarit R, Pitchyangkura R. The fundamental parameters of chitosan I polymer scaffolds affecting osteoblasts (MC3T3-E1). *J Mater Sci Mater Med* 2009;**20:**309–320.