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Effect of Chitosan on Expression of Osteogenic Genes during the Healing of Rat Extraction Socket

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Purpose: This study was performed to evaluate the effect of chitosan combined with absorbable gelatin compressed sponge on the expression of osteoblastic differentiation marker genes during the healing of rat extraction socket.
Materials and Methods: Twenty-four male Wistar rats were used. In control group, the extraction socket was closed with suture. In chitosan group, the socket was filled with chitosan combined with Gelfoam (Pharmacia & Upjohn Co.) and closed with suture. In each group, the animals were sacrificed at 3 days, 1 week, 2 weeks, and 4 weeks postoperatively. The expression of osteoblastic differentiation marker genes, including *BSP*, *OCN*, *Runx2*, and *Col1* were quantified by real-time polymerase chain reaction.
Result: Compared to control group, the mRNA level of *BSP* in chitosan group increased significantly at 2 weeks after extraction and the level of *OCN* decreased significantly at 3 days and 4 weeks after extraction ($P<0.05$). The mRNA levels of *OCN*, *Runx2*, and *Col1* in chitosan group increased slightly at 2 weeks after extraction, but there was no statistical difference between groups.
Conclusion: The results indicate that chitosan has some effects on the expression of osteogenic genes during the healing of extraction sockets.

Key Words: Chitosan; Extraction socket; Osteoblastic differentiation

Introduction

The alteration of the alveolar ridge occurs concomitantly with the healing of the soft and hard tissue but the process of remodeling may continue also after the termination of bone formation in

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the extraction sockets[1]. The healing processes of extraction socket have been evaluated in many animal and human models[2,3].

Bone has a remarkable regenerative ability, but the inherent osseous processes are unable to repair the large defect during the healing time[4]. Tissue engineering has been emerged as a possible solution for these clinical conditions. Several strategies can be employed to develop new bone tissue. Those strategies may involve the use of an extracellular matrix (ECM)—like structure (scaffold), cells, and growth factors. These 3 basic components need to be well synchronized to achieve a successful tissue engineering therapy[5]. The cells would synthesize the ECM of new bone tissue, whereas the scaffold would provide an adequate three-dimensional (3D) environments for the cells[6]. Mammalian cells require interactions with their microenvironment to survive, proliferate, and function. In tissue physiology, these 3D environments are largely composed of ECM proteins. In tissue engineering, these 3D structures are initially provided to the cells through the use of biodegradable and biocompatible scaffolds. They provide an environment that allows for the adhesion of cells and their proliferation, migration, and differentiation until these cells and the host cells begin to secrete and shape their own microenvironment. Therefore, scaffolds are considered a critical component of tissue engineering[7]. To accomplish the goals of tissue engineering, scaffolds should satisfy the following requirements; should be porous to allow placement of cells and growth factors, should allow effective transport of nutrients, oxygen, and waste, should be biodegradable, leaving no toxic byproducts, should be replaced by regenerative tissue while retaining the shape and form of the final tissue structure, should be biocompatible and should have adequate physical and mechanical strength[8].

In recent studies, naturally derived polymers have been proposed for the scaffold such as starch, gellan gum, soy, silk and chitosan[9]. In particular, chitosan has shown an excellent combination of properties and it has been demonstrated that it is suitable biomaterial for the development of scaffolds for bone tissue engineering. Chitosan can be used either alone, or in combination with other biodegradable polymers such as starch or silk, or with ceramics such as hydroxyapatite[9].

This study was performed to evaluate the effect of chitosan combined with absorbable gelatin compressed sponge on the expression of osteoblastic differentiation marker genes during the healing of rat extraction socket.

Materials and Methods

1. Preparation of Chitosan Gel

Twenty grams of chitosan powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 99.5% acetic acid (1 ml) and distilled water (30 ml).

2. Surgical Procedures

Twenty-four maleWistar rats (Damool Science, Daejeon, Korea) weighing about 400 g were used in this study. Animals were housed individually in standard rats cages with an ambient temperature of 24°C to 26°C and 12/12 hour light/dark cycle. Animals had free access to drinking water and standard laboratory pellets.

The rats were anesthetized by intramuscular ketamine HCl (60 mg/kg, Ketalar; Yuhan, Seoul, Korea) and Xylazine (3 mg/kg, Rumpun; Bayer, Seoul, Korea) and operated on under sterile condition. Two percents lidocaine HCl with 1 : 100,000 epinephrine (HUONS Co. Ltd., Seongnam, Korea) was infiltrated in the maxillary vestibule. We used the split-mouth design in this study[10]. Briefly, right and left maxillary molars were extracted with dental elevators and forceps. In control group (n=24) the right maxillary first molar of each animal was extracted and the extraction socket was closed with 5-0 nylon (Ailee Co. Ltd., Busan, Korea). In chitosan group (n=24) the left maxillary first molar was

extracted and the socket was filled with chitosan combined with Gelfoam (Pharmacia and Upjohn Company, Kalamazoo, MI, USA) and closed with 5-0 nylon. Animals were given penicillin (Augmentin; Ilsung, Seoul, Korea) for 3 days to control the postoperative infection.

Animals were sacrificed with 0.9% normal saline and 4% KCl solution at 3 days, 1 week, 2 weeks, and 4 weeks after extraction. The maxilla was dissected, and the extraction sockets were surgically removed from the surrounding tissue. The extraction sockets were frozen immediately in liquid nitrogen, crushed and placed in tubes containing RNA isolation buffer. The experimental protocol was approved by the Ethical Committee for Animal Experiment in Chonnam National University.

3. Real-Time Polymerase Chain Reaction (PCR)

The total RNA was isolated from the tissue using a QIAzol RNA Lysis reagent (QIAGEN Sciences Inc., Germantown, MD, USA) according to the manufacturer's instructions. Purified RNA was measured with a spectrophotometer (Nanodrop 100; Thermo Fisher Scientific, Waltham, MA, USA). One milligram RNA was used to synthesize cDNA with the PrimeScript RT reagent kit (Takara Korea Biomedical Inc., Seoul, Korea) according to manufacturer's recommedation. The quantitative real-time PCR was conducted using the SYBR Premix Ex Taq kit (Takara Korea Biomedical Inc.) in triplicate in the Applied Biosystems 7300 Real-Time PCR System (Life Technologies Korea, Seoul, Korea). The thermal cycling conditions were as follows: 15 minutes at 95°C, followed by 40 cycles at 95°C for 10 seconds, 58°C for 15 seconds, and 72°C for 20 seconds. All quantitation was normalized to an endogenous control *β-actin*. The data for gene expression were analyzed by the ΔΔCt method as described previously[11]. Amplication efficiency of different genes was determined relative to *β-actin* ($\Delta Ct=Ct_{gene}-Ct_{\beta\text{-}actin}$) the mRNA in each sample was calculated by comparative ($\Delta\Delta Ct=\Delta Ct_{gene}-\Delta Ct_{\beta\text{-}actin}$) value method. The fold change in gene expression relative to the control was calculated by $2^{-\Delta\Delta Ct}$. The primer sequences were shown in Table 1.

4. Statistical Analysis

Each experiment, containing triplicate independent samples, was repeated at least twice, and qualitatively identical results were obtained. Independent sample t-test was used to determine any statistically significant difference between the control and experimental groups and one-way analysis of variance followed by Tukey's *post*

Table 1. Sequences of the primers used for real-time polymerase chain reaction

Gene	Product size	Primer sequence	NCBI reference sequence
BSP	80 bp	Forward: GAAAGAGCAGCACGGTTGAGTA	NM_012587.2
		Reverse: GTCATAGGTTTCATACGCAGTGTTG	
OCN	105 bp	Forward: CAGACAAGTCCCACACAGCAA	NM_013414.1
		Reverse: CAGGTCAGAGAGGCAGAATGC	
Runx2	100 bp	Forward: AGAATGATGGTGTTGACGCTGAT	NM_053470.2
		Reverse: CTTCAATAGGGTCGCCAGACA	
Col1	113 bp	Forward: TGAGCCAGCAGATTGAGAACA	NM_053304.1
		Reverse: CCAGTACTCTCCGCTCTTCCA	
β-Actin	81 bp	Forward: TCCTGTGGCATCCATGAAACTA	NM_031144.3
		Reverse: TGTGTTGGCATAGAGGTCTTTACG	
		Reverse: CTTCAATAGGGTCGCCAGACA	

NCBI: National Center for Biotechnology Information.

hoc test was used to determine any statistically significant differences according to the time with the use of PASW Statistics version 18.0 software program (IBM Co., Armonk, NY, USA). P-values less than 0.05 were considered significant.

Result

In the results of real-time PCR, the mRNA level of *BSP* increased significantly in chitosan group (87% increase) at 2 weeks after extraction compared to the control group (P<0.05; Fig. 1A). The mRNA level of *BSP* at 2 weeks after extraction increased significantly in chitosan group compared to other healing time points (P<0.05; Fig. 1A). The mRNA level of *OCN* decreased significantly in chitosan group (47% decrease) at 3 days and 4 weeks after extraction compared to the control group (P<0.05). The mRNA level of *OCN* increased slightly in chitosan group (25% increase) at 2 weeks after extraction compared to the control group. However, there was no statistical difference at 2 weeks after extraction compared to the control (Fig. 1B). The mRNA level of *OCN* at 1 and 2 weeks after extraction increased significantly in chitosan group compared to other healing time points (P<0.05; Fig. 1B). The mRNA level of *Runx2* in chitosan group decreased slightly at 3 days, 1 and 4 weeks after extraction and increased slightly at 2 weeks compared to the control group. However, there were no statistical differences compared to the control group (Fig. 1C). The mRNA level of *Runx2* in chitosan group increased significantly at 2 weeks after extraction compared to other healing time

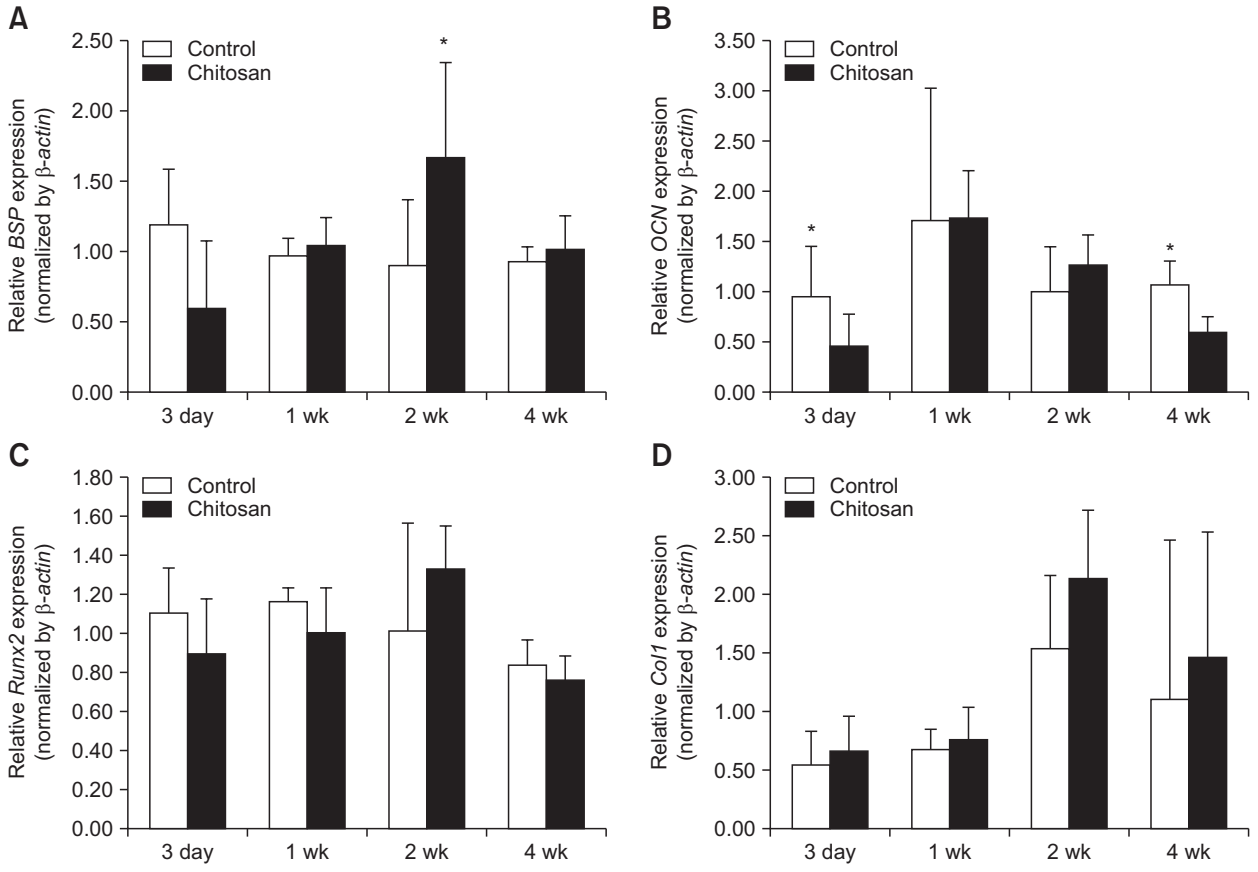

Fig. 1. Expression profiles of *BSP* (A), *OCN* (B), *Runx2* (C), and *Col1* (D) during healing of rat extraction socket without or with chitosan by quantitative real-time polymerase chain reaction. The relative expression of *BSP*, *OCN*, *Runx2*, and *Col1* gene normalized against a housekeeping gene (*β-actin*). *P<0.05.

points (P<0.05; Fig. 1C). The mRNA level of *Col1* in chitosan group was slightly higher than control group at all time points after extraction. However, there was no statistical difference compared to the control (Fig. 1D). The mRNA level of *Col1* in chitosan group increased at 2 weeks after extraction compared to other healing time points (P<0.05; Fig. 1D).

Discussion

Bones are developed by two main processes: intramembranous and endochondral ossification[12]. Five different cell types are involved in bone maintenance and remodeling; mesenchymal stem cells, bone-lining cells, osteoblasts, osteocytes, and osteoclast. Osteoblasts can be derived from mesenchymal stem cells that synthesize the organic matrix of the bone, osteoid[13]. The osteoid is non-mineralized organic matrix including proteins such as *BSP*, *OCN*, *Col1*, osteopontin, and bone morphogenetic proteins.

An ideal material for bone regeneration should be biocompatible, biodegradable, easy to apply, and effective on bone repair. Since chitosan has been shown by many researchers to possess these properties[14,15], this study was performed to evaluate its effectiveness on the healing of extraction socket.

Chitin is the second most abundant natural polymer after cellulose. Chitosan is a linear polysaccharide, obtained from the deacetylation of chitin, the primary structural polymer of the exoskelecton of crustaceans, cuticles of insects, and cell wall of fungi[16]. Chitosan is the common name for the family of deacetylated chitins, with different degrees of deacetylation. By definition, when the number of N-acetyl glucosamine units is higher than 50%, the polymer is considered chitin. On the other hand, when the number of N-acetyl glucosamine units is superior, its name is chitosan[17].

Chitosan is a semi-crystalline polymer and its crystallinity is dependent of the degree of deacetylation[16]. The solubility of chitosan depends on the free amino and N-acetyl groups, which are soluble in acidic pH[18]. Chitosan, used in this study, presents a wide range of properties that make it appropriate for tissue engineering applications because of its biodegradability, biocompatibility, antibacterial activity, wound healing properties, and bioadhesive character[9]. One of the most important characteristic of chitosan, for tissue engineering applications, is its ability to be shaped into various form, such as microspheres, paste, membranes, sponges, fibers and porous scaffolds[19]. It is important for bone regeneration materials to be highly adaptable to the extraction socket. In this study the chitosan was used in the form of gel combined with Gelfoam to fill in the extraction sockets and remained there for local absorption. This application method was different from the others in terms of using chitosan gel combined with Gelfoam to achieve good adaptation to bone defects. The osteogenic gene expressions in chitosan group showed to be higher than control group. However, this study has a limitation that we did not consider the effect of Gelfoam to healing on extraction socket. Gelfoam is a one of the surgical coagulants and often used in clinical situation[20]. This material is un-inflammatory in tissue and does not cause the infiltration of cells and scar formation[21]. Therefore, it is thought that there is few effect of Gelfoam to healing process in this study.

The differentiation of progenitor cells into secreting cells or osteoblast-like cells is critical in the healing process, and inducing differentiation is required for a biomaterial to be considered a tissue engineering material.

OCN is non-collagenous protein found in bone and dentin and secreted solely by osteoblasts and thought to play a role in the body's metabolic regulation[22]. *Runx2* is a key transcription factor associated with osteoblast differentiation. *Col1* is the most abundant collagen of the human body and presents in scar tissue, tendon, bone, dermis

and dentin[23]. *BSP* is a highly post-translationally modified acidic phosphoprotein normally expressed in mineralized tissues such as bone and dentin[24]. *BSP* belongs to the SIBLING (small integrin-binding ligand N-linked glycoprotein) gene family[25] and displays several characteristics typical of these proteins. *BSP* is expressed by several cell types associated with mineralized tissues but is expressed in abundance by osteoblasts. As well, *BSP* has been found to be expressed at pathological sites of mineralization such as microcalcifications in breast, thyroid and prostate neoplasms[26] and atherosclerotic plaques[27]. Even though there is ample evidence to suggest that *BSP* is a marker of osteoblast differentiation and the onset of mineral formation, there is no direct evidence suggesting the protein has an effect on either of these processes in vivo. Especially, this study revealed that *BSP* in chitosan group were expressed higher than the control at 2 weeks after extraction. It can be explained that higher cell proliferation and differentiation on chitosan were attributed to the high density of positive charges on chitosan at physiological pH that attracted negatively-charged proteins and cells[28].

Cell response to the presence of chitosan has been extensively investigated[29,30]. Up-regulation of various types of biologic markers has been reported in the presence of chitosan in several cell culture studies and animal studies[29-32]. Waltregny et al.[26] reported that *BSP* is an osteoblastic differentiation marker and has functions as a hydroxyapatite nucleator. Lin and Chen[33] assayed the biological responses of osteoblast including cell proliferation, viability, morphology, type 1 collagen expression, alkaline phosphatase activity, and mineralization. They concluded that cells on chitosan coating had higher proliferation, type 1 collagen deposition, and degree of mineralization compared to the control. Chevrier et al.[34] reported that chitosan increased vascularization and induced granulation tissue generation. At 2 weeks after operation, a mixture of woven bone and fibrous tissue was seen in treated defects, whereas in untreated ones, the fibrous tissue was dominant. In this study, the peak expression of mRNA including *BSP*, *OCN*, *Runx2*, and *Col1* were observed at 2 weeks after extraction. These results seemed to be also consistent with previous studies[34,35]. Liu et al.[35] reported that osteoblastic differentiation marker such as *TGF-β1*, *BMP-2* and *VEGF* had peak expression level after 2 weeks.

The results of this study support the hypothesis that chitosan can induce the differentiation of osteoblasts. According to these results, chitosan can be useful to preserve the remaining alveolar bone after extraction for the future implant installation. However, further studies such as histological evaluation and immunohistochemistry are needed to clarify the detailed mechanism of chitosan how it induces osteoblastic differentiation of mesenchymal stem cells or osteoblasts in extraction socket. Moreover, this study has the other limitation that rat extraction socket is too small to estimate the precise effect of chitosan. Therefore, clinical evaluations are also required to apply this technique in clinical situations.

Conclusion

This study was performed to evaluate the effect of chitosan combined with absorbable gelatin compressed sponge on the expression of osteoblastic differentiation marker genes during the healing of rat extraction sockets. In conclusion, these results indicate that chitosan has some effects on the expression of osteoblastic differentiation marker genes during the healing of extraction sockets and it can be used for preservation of alveolar bone after extraction.

Conflict of Interest

No potential conflict of interest relevant to this

article was reported.

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