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Research Report

Expression of bone morphogenetic protein-2 after using chitosan gel with different molecular weight on wound healing process of dental extraction

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ABSTRACT

Background: *Bone morphogenetic protein-2 (BMP-2) is bone stimulator which capable of inducing differentiation of mesenchymal cells into osteoblast, stimulating bone formation in wound healing process of dental extraction. Chitosan is polymer composed N-acetyl-D-glucosamine unit that has been used in various applications in wound healing process and bone tissue engineering.* ***Purpose:*** *The objective of this research was to analyzed expressions of BMP-2 for 7,14 and 21 days after using chitosan gel with different molecular weight on wound healing process of dental extraction.* ***Method:*** *The research was an experimental laboratory study. Rattus nornegicus strain wistar male, aged 8-16 weeks, divided into 3 treatment groups namely group 1 and II which given chitosan gel 1 % with high and low molecular weight and group III as control which were not given chitosan gel. Chitosan gel were applied into the socket of dental extraction. Rat was decaputated 7,14 and 21 days after chitosan gel application and the jaw in the treated regions and control group were cut for immunohistochemical examination to observe BMP-2. Data were analyzed using ANOVA test.* ***Result:*** *The result of this research showed significant differences on BMP-2 for 7,14 and 21 days observation ($p<0,05$). The increasing of BMP-2 were found in the group which given chitosan gel with high molecular weight.* ***Conclusion:*** *It may be concluded that chitosan gel with high molecular weight can enhance the expresion of BMP-2 on wound healing process of dental extraction.*

Keywords: *Chitosan; Bone morphogenetic protein-2; wound healing*

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INTRODUCTION

Bone morphogenetic protein (BMP)-2 has been widely used as an effective growth factor in bone tissue engineering. It is a member of the transforming growth factor-β (TGF-β) superfamily of multifunctional cytokines which has remarkable ability to induce bone formation and bone tissue reconstruction. It plays critical roles in osteogenesis and bone metabolism.[1] The bone matrix is rich in growth factors, among which bone morphogenetic proteins (BMP_s), that are synthesized and secreted by osteoblast and incorporated into the matrix during bone formation. The BMP_s, released during osteoclastic bone resorption, are capable of inducing differentiation of mesenchymal cells into osteoblasts (osteinduction), stimulating bone formation in both remodeling and repairing process.[1,2]

Chitosan is carbohydrate copolymer composed of glucosamine and N-acetyl-D-glucosamine units. Chitosan has a number of properties including biocompatibility, biodegradability, mucoadhesiveness and wound healing capabilities that make it useful as a biomaterial.[3] Chitosan is very versatile and can be prepared as films, gels, sponges and other forms and has been used in various applications including wound healing and bone tissue engineering.[2,3] Biological activity of chitosan depend on their molecular weight and deacetylation degree. The deacetylation degree

and molecular weight are important parameter, which could influences the performance of chitosan in many its application. The deacetylation degree is related to the ability of chitosan to form isoelectric interaction with other molecules. The deacetylation degree more than 75% will make more amide group formed, so chitosan will become have high chemical reactivity, and be able to interact with proteins and other organic matrices, such as anionic glycosaminoglycans and proteoglycans, and extracellular matrix macromolecule. The molecular weight is the sum of the atomic masses of molecule.[4,5] It is one factor that determine the mucoadhesive properties. Chitosan with high molecular weight powder become chitosan gel with good physical characteristic, higher viscosity, neutral pH and have good mucoadhesive.[6] Chitosan is able to increase transforming growth factor beta 1 (TGF β1), platelets release transforming growth factor (PDGF), fibroblasts growth factor-2 (FGF2), BMP expression of mRNA on the seventh day.[6,7] In previous study, injectable chitosan gel has been found significantly enhance the osteoblastic differentiation of mouse osteoblast precursor cells. Chitosan gel formulation can release BMP-2 that enhance osteoblastic activity of bone cells by measuring alkaline phosphatase (ALP) specific activity of preosteoblast mouse bone marrow stromal cells.[8]

BMP-2 has been widely investigated for bone healing due to its properties. BMP-2 recruits stem cells to the bone healing, promotes angiogenesis and causes differentiation of stem cells into osteoblast.[3,6] In the wound healing process of dental extraction, it have different phases of alveolar healing were recognized by histological examination in socket. At the end of 1st week, the socket were filled granulation tissue and there was osteoid matrix deposition. At the end of 2nd week, there was progressive bone formation, and the end of 3rd week, most socket filled with thicker bone trabecula surrounding interconnecting space.[2] The increasing expression of BMP-2 on wound healing process of dental extraction could be achieved by using chitosan gel. The aim of this study was to analyzed expressions of BMP-2 for 7, 14 and 21 days after using chitosan gel with different molecular weight on wound healing process of dental extraction.

MATERIALS AND METHODS

The material in this experiment were chitosan powder purchased from Sigma chemical, St. Louis, USA. The degree of deacetylation was more than 75%. Chitosan with high molecular weight (Sigma, Product number: 419419, Lot number: MKBH5816V) and chitosan with low molecular weight (Sigma, Product number: 448869, Lot number: MKBH7256V), acetic acid 2% p.a (Merck, Germany), buffer formalin 4% and 10%, ketamin (Ketalar,Pfzer), xylazine (Merck, Germany), alkohol 80%, alkohol 95%, alkohol 100 % (absolute), xylene (Merck, Germany), buffer Parafin, EDTA 10 % (JT Baker, USA), NaSO4 2% (Merck, Germany), PBS, Tripsin 0,125%, H_20_2 0,5%, methanol (Merck, Germany), NaOH 1,25% (Merck, Germany) and monoclonal antibody bone morphogenetic protein-2 Rattus norvegicus.

Chitosan gel 1% (w/v) was made with diluted one gram of chitosan powder in 100 ml acetic acid 2%. It added with NaOH 1,25% solution to get neutral pH. The mixture was stirred until the gel was completely formed. After homogenization, the gels were stored in closed containers at ambient temperature until use. The characteristic of chitosan gel was evaluated includes solubility, pH, viscosity, physical characteristic, homogeneity, consistency, and duration of storage time. The homogeneity test of gel carried out using glass plates after the powder diluted in acetic acid 2%. It should be observed on optimized homogeneous. Consistency test could be done by using a penetrometer or mechanically sentrifugator. Gel without precipitation will produce a good consistency. Physical characteristic test or organoleptic analysis during the storage time includes change of colour, form of formulation gel and odorless. Measurement of viscosity using viscometer Ostwald (capillary method).[8,9]

The research was an experimental laboratory study. *Rattus nornegicus* strain wistar male, aged 8-16 weeks, divided into 3 treatment groups namely group 1 and II which given chitosan gel 1% with high and low molecular weight and group III as control which were not given chitosan gel. 0.1 ml chitosan gel were applied into the socket of dental extraction. Rat was decaputated 7, 14 and 21 days after chitosan gel application and the jaw in the treated regions and control group were cut for immunohistochemical examination to analyze expression of BMP-2. Fixation was performed using 10% buffer formalin and decalcification applying EDTA. Further process was dehydration and continued by clearance. The tissue could be cut using microtome in 4-6 μm thickness. Deparafin and rehydration were subsequently performed. BMP-2 monoclonal antibody was diluted by antibody diluents. It was washes by PBS. Streptavidin-biotin was dropped and incubated for 30 minutes, washed by PBS. Counterstained using haematoxyline and washed by flowing water and dried. It was given entelan and covered by cover glass. Light microscope was applied and the evaluation was done. The measuring result were analyzed using Anova test. It analyzed the comparison between chitosan treated with high molecular weight group, lower molecular weight group and the control groups ($p<0.05$).

RESULTS

Table 1 shown the evaluation of chitosan gel formulation. Chitosan gel considered as ideal formulation forms for wound healing dressing could have good characteristic and easy to applicate on dental socket. The expressions of BMP-2 for 7, 14 and 21 days observation after using chitosan gel with different molecular weight on wound healing process

of dental extraction shown in Figure 1, 2 and 3. The region which would be evaluated and observed in third apical socket. The expressions of BMP-2 in third apical socket was done 400 times magnification in all sub groups.

In our study, the expressions of BMP-2 on wound healing process of dental extraction using chitosan was more increasing compared to the control group. The expressions of BMP-2 in 7, 14 and 21 days observation after using chitosan gel with high molecular weight was more increasing than the other groups.

Table 2 and Figure 4 shown the mean and standard deviation of each group from immunohystochemistry study done in 7, 14 and 21 days after treatment. The expressions of BMP-2 for 7, 14 and 21 days observation after using chitosan gel with different molecular weight on wound healing process of dental extraction. The data

Table 1. Measurement solubility, pH, viscosity, homogeneity, consistency of chitosan gel

	pH	Viscosity	Homogeneity	consistency	solubility
Formula of chitosan					
High molecular weight	7,02	High	Excellent	excellent	good
Low molecular weight	6,78	Low	good	good	good

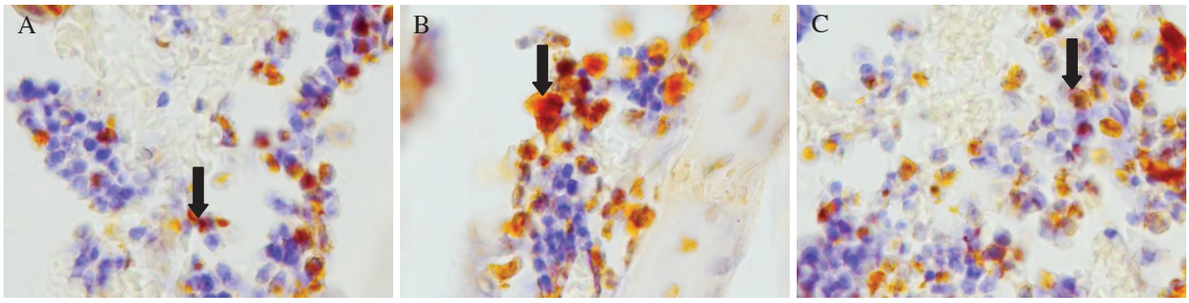

Figure 1. The expressions of BMP-2 at 7 days observation, magnification 400x. (A) Control group, without using chitosan; (B) Treatment group using chitosan gel with high molecular weight; (C) Treatment group using chitosan gel with low molecular weight.

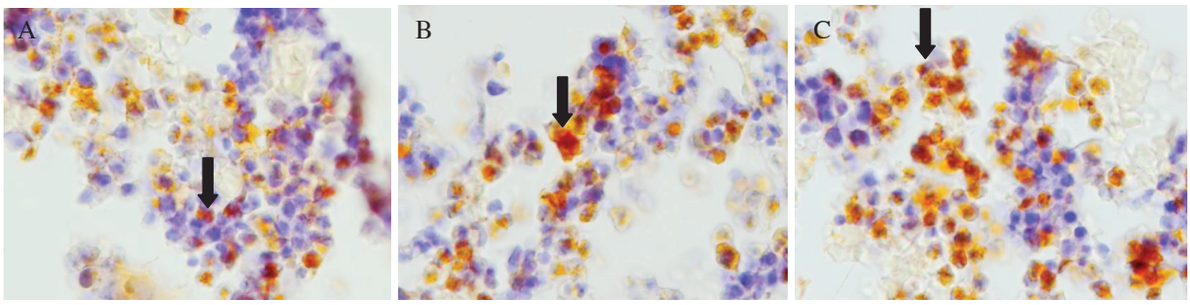

Figure 2. The expressions of BMP-2 at 14 days observation, magnification 400x. (A) Control group, without using chitosan; (B) Treatment group using chitosan gel with high molecular weight; (C) Treatment group using chitosan gel with low molecular weight.

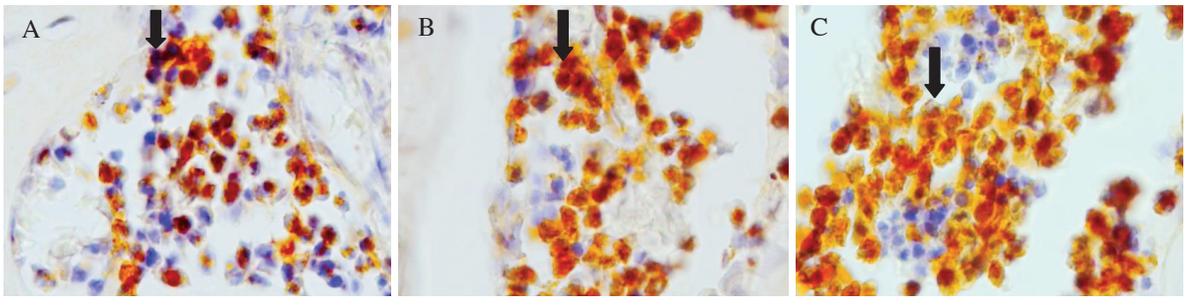

Figure 3. The expressions of BMP-2 at 21 days observation, magnification 400x. (A) Control group, without using chitosan; (B) Treatment group using chitosan gel with high molecular weight; (C) Treatment group using chitosan gel with low molecular weight.

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Table 2. The mean and standard deviation BMP-2 of each group at 7, 14 and 21 days after treatment

		7 days	14 days	21 days
Variable	Treatment	Mean± SD	Mean± SD	Mean± SD
The expressions of BMP-2	Control	11.17 ± 0.75	13.57 ± 1.81	11.50 ± 1.22
	Chitosan, high BM	18.25 ± 4.42	21.33 ± 2.67	19.29 ± 4.19
	Chitosan, low BM	11.80 ± 1.92	14.88 ± 1.45	14.00 ± 2.53

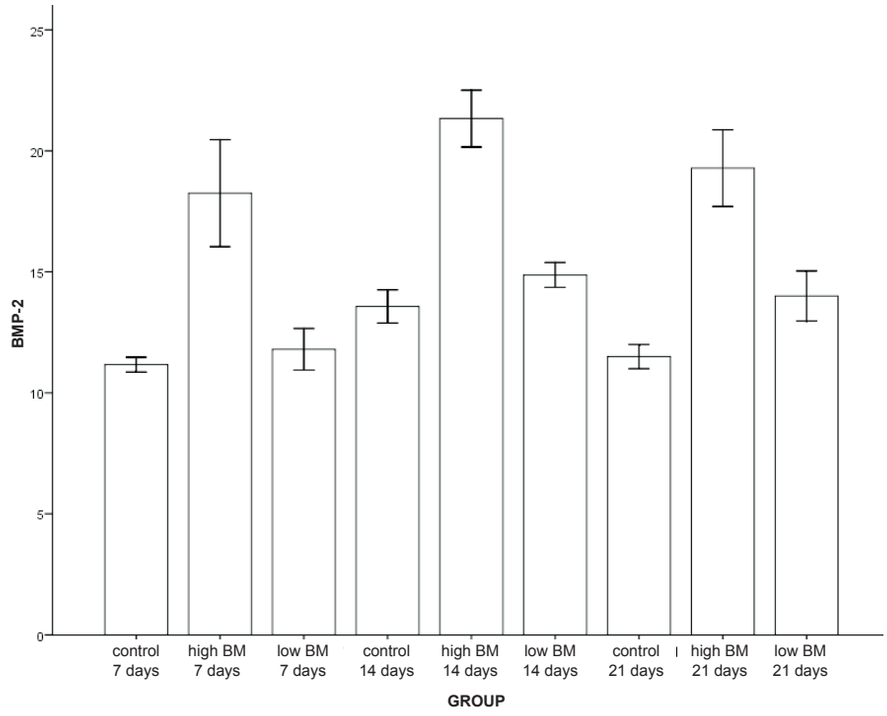

Figure 4. The graphic of expressions of BMP-2 on 7, 14 and 21 days using chitosan gel with high and low molecular weight group and control group.

was analyzed using Kolmogorov-Smirnov statistical test. It showed normal distribution ($p>0.05$) in which fulfiiling the requirement of parametric test. The Anova test showed, expressions of BMP-2 in treatment group using chitosan gel with high molecular weight were significant difference compared to the treatment group using chitosan gel with low molecular weight and control group ($p<0.05$).

DISCUSSION

The proliferation and differentiation of sufficient progenitor cells are critical for bone healing. These processes are regulated by growth factors such as TGF-β and BMP_S. BMP-2 can efficiency induce bone formation. Many materials, especially the natural polymers, have been researched for controlled release of BMP-2.[10,11] Chitosan is naturally derived polysaccharide. It has gained much attention as biomaterial in tissue engineering application, due to its antimicrobial activity and biocompatibility.[10]

Chitosan is reported to be biocompatible, biodegradable and non toxic, having some biomedical properties such as healing accelerator, antifungal, hemostatic, antimicrobial and analgesic. Several study have shown the activity of chitosan in healing process, where it enhanced the infiltration of inflamamatory cells in the injured area and release growth factor.[6,12] Chitosan is the partially deacetylated form poly-(1,4)-2-amino-2-deoxy-D-glucose) of chitin, and is a potential biomaterial for bone tissue engineering application.[1,10,12] In the present study, the chitosan powder was mixed with blood of each person and filled in dental socket, and it found that bone tissue regeneration will be faster in chitosan-filled socket than untreated dental socket.[13]

In our study it was used chitosan gel material with different molecular weight to analyzed expressions of BMP-2 *Rattus norvegicus* on wound healing process of dental extraction. It showed the expressions of BMP-2 in 7 and 14 days observation after using chitosan gel was increasing and decreasing at 21 days observation. Chitosan

is a polycationic complex carbohydrate with a structure similar to those of glycosaminoglycans specifically hyaluronic acid. Hyaluronic acid is thought to facilitate the migration and proliferation of progenitor cells. Previous studies have demonstared that chitosan might function as a substrate that enhances the migration and differentiation of osteoblast cells and conversely, it might inhibit the function of fibroblast and so indirectly facilitate osteogenesis.[14,15] Its biological properties of chitosan, including chitosan biodegradation by lysozim that can change chitosan polymer form (N-acetyl-D-glucosamine) to dimer active form. N-acetyl-D-glucosamine dimer active form cross-linked with matrix macromolecules extracellular as well as stimulate increased TGF-ß and BMP expression of mRNA.[6,15] Chitosan loading rhBMP-2 can facilitate bone regeneration of periodontal tissue. In the cavities filled with chitosan, not only the bone regeneration was faster, but also the density was similar to the density of the bone of the subject under study.[13]

According to the Figure 4, the expressions of BMP-2 in 7 and 14 days observation after using chitosan gel with high molecular weight was more increasing than the other groups. Chitosan was the material used to form gels due to its good gel forming property, biodegradability, biocompatibility and non toxic. Gels could be considered as ideal pharmaceutical forms to treat the dental socket. It have the ability to absorb exudates, which moisture on the wound surface. Besides, gels have high water vapor and oxygen permeability, as well as mechanical properties that resemble physiological soft tissue. Chitosan gel has a strong tissue-adhesive property.[12,17,18] The characteristic of chitosan gel is related with its molecular weight. The absorbtion mechanism also depend upon the particle size of chitosan powder. The molecular weight related to particle size of powder. Molecular weight and particle size has been linked with viscosity, low molecular powder which small particle size will result formulation with a low viscosity. In addition the molecular weight is also directly related to length of molecular chain, more longer the molecular chain, the viscosity more higher.[19-21] The viscosity of chitosan gel with high molecular weight more higher than formulation with low molecular weight. It have high viscosity and better mucoadhesive properties due to strong blood clot is formed in order to eliminate complications of dental extraction such as dry socket.[19,20,22] Chitosan with high molecular weight have more biodegradable induce N-acetyl-D-glucosamine dimer active of chitosan cross-linked with glycosaminoglycan and glycoprotein that part of matrix macromolecules extracellular as well as stimulate increased BMP-2 that is growth factor of bone healing. However, it can promote the accleleration of wound healing process on dental socket.[15,16,22]

BMP-2 can efficiently induce bone formation. It has been widely use as an effective growth factor in bone tissue engineering. Using C1C12 myoblast cells as in vitro models, the enhanced bioactivity of BMP-2 was attributed primarily to the stimulation of 6-O sulfated chitosan. A low dose of 6-O sulfated chitosan showed significant enhancement alkaline phosphatase activity and mineralization induced by BMP-2, as well as the expression of ALP and osteocalcin mRNA.[23] ALP is an enzyme regarded to be important in the process of mineralization, since high levels of ALP would promote hydrolysis of phosphate, produce orthophosphate and increase the calcium deposition. ALP activity and calcium deposition are widely used as marker for early and late differentiation of osteoblast cells, respectively.[15] Mature osteoblast produce mineralized bone matrix, and the component of this matrix, such as calcium deposition and osteogenic markers (osteocalcin and osteopontin), are usually used as markers for late stage of osteoblast differentiation. Osteocalcin and osteopontin expression marks the late stage of osteoblast differentiation, and BMP-2 is an important factor for osteoblast maturation and osteoblast activity.[2,3,15] In our study, the expressions of BMP-2 in 21 days observation after using chitosan gel with high molecular weight was decreasing. Probably, the activity of ALP has decreased. There was most socket filled with thicker bone trabecula surrounding interconnecting space in the end of the 3nd week. The major proportion of bone formation and the maximum mineral density has occurred in the end of the 2nd week. In the end of the 2nd week the activity of ALP has decreased [2] The using of chitosan gel with high molecular weight could increasing the expression of BMP-2. BMP-2 is major growth factor in bone healing. It may be concluded that chitosan gel with high molecular weight could accelerate bone healing process of dental extraction.

REFERENCES

1. Zhou H, Qian J, Wang J, Yao W, Liu C, Chen J, Cao X. Enhanced bioactivity of bone morphogenetic protein-2 with low dose of 2-N-6-O-sulfated chitosan in vitro and in vivo. Biomaterials 2009; 30(9): 1715-24.
2. Calixto RF, Teófilo JM, Brentegani LG, Lamano-Carvalho TL. Alveolar wound healing after implantation with a pool of commercially available bovine bone morphogenetic protein (BMP_S)–a histometric study in rats. Braz Dent J 2007; 18(1): 29-33.
3. Reves B, Jennings J. Osteoinductivity assessment of BMP-2 loaded composite chitosan-nano-hydroxyapatite scaffolds in a rat muscle pounch. Materias J 2011; 4: 1360-74.
4. Park JK, Chung MJ, Choi HN, Park YI. Effect of molecular weight and deacetylation degree of chitosan oligosaccharides on antitumor activity. Int J Mol Sci 2011; 12(1): 266-77.
5. Khan T, Peh K. Mechanical, bioadhesive strength and biological evaluation of chitosan films for wound dressing. J Pharm pharmaceut Sci 2000; 3(3): 303-11.
6. Sularsih, Wahjuningsih E, Yoshe Y. The characterization chitosan gel on wound healing process of dental extraction. Proceedings of 8th International Dental Scientific Meeting Dentistry Faculty of Hasanudin University, Makasar, 2014 Juni 20-22.
7. Kim S. Neuroprotective properties of chitosan and its derivatives. Marine Drug J 2010; 8(10): 2117-28.
8. Kim S, Kang Y, Kruegar C. Sequential delivery of BMP-2 and IGF-1 using chitosan gel with gelatin microspheres enhances early osteoblastic differentiation. Acta Biomater J 2012; 8(5): 17.

9. Matsunaga T, Yanagiguchi K, Yamada S, Ohara N, Ikeda T, Hayashi Y. Chitosan monomer promotes tissue regeneration on dental pulp wounds. J Biomed Mater Res A 2006; 76(4): 711-20.
10. Mappa T, Edy HJ, Kojong N. Formulation gel of extract sasaladahan leave (Peperomia pellucida (L.) H.B.K.) and effectivity test to wound healing process of burn wound. PHARMACON Jurnal Ilmiah Farmasi–UNSRAT 2013; 2(02): 49-55.
11. Anggraeni Y, Hendradi E, Purwanti T. The characteristic of formulation natrium diklofenak in system niosom with base gel of carbomer 940. Pharma Scientia 2012; 1(1): 1-15.
12. Yun Y, Kim S, Kang E. The effect of bone morphogenetic protein-2 (BMP-2) immobilizing heparinized-chitosan scaffolds for enhanced osteoblast activity. J Tissue Engineering And Regenerative Medicine 2013; 10(3): 1308-777.
13. Kim S, Kang Y, Kruegar C.Sequential delivery of BMP-2 and IGF-1 using chitosan gel with gelatin microspheres enhances early osteoblastic differentiation. Acta Biomater J 2012; 8(5): 17.
14. Nascimento EG. Evaluation of chitosan gel 1% silver sulfadiazine as an alternative for burn wound treatment in rats. J Acta Cirurgica Brasileira 2009; 24(6): 460-5.
15. Ezoddini A, Azam A, Yassei S. Effect chitosan on dental bone repair. J Health 2011; 3(4): 200-5.
16. Silberman M. Use of chitosan for stimulating bone healing and bone formation. Available from http://D:/CHITOSAN/BMP/BMP/Patente WO2006057011A2.2006. Accessed May 13, 2015.
17. Pang E, Paik J, Kim S, Jung U. Effect of chitosan on human periodontal ligament fibroblast in vitro and on bone formation in rate calvarial defect. J Periodontal 2005; 75: 1526-33.
18. Chin L, Halim AS. In vitro models in biocompability assessment for biomedical-grade chitosan [derivatives in wound management. J Molecular Science 2009; 10(3): 1300-13.
19. Karthikeyan G, Adsorbtion dynamics and equilibrium studies of Zn onto chitosan. J Chem Sci March 2004; 116(2): 119-27.
20. Zeng L. Absrobtion and distribution of chitosan in mice after oral administration. J Carbohydrat Polymer 2008; 71: 435-40.
21. Rochima E, Suhartono MT, Syah D, Sugiono. The viscosity and molecular weight chitosan from chitin deasetilate enzymatic reaction isolat Bacillus Papandayan. National Congress of Agricultural Assosiation (PATPI), Bandung 2007. P. 2-10
22. Sularsih. Type 1 collagen on wound healing process of dental extraction with different molecular weight of chitosan. Proceeding of International Congress Dentisphere 3, November 2013. p. 1-6
23. Kim S. Chitin chitosan oligosaccarides and their derivatives. USA: CRC Press; 2011. p. 233-8.