

The Prospect of Chitosan on The Osteogenesis of Periodontal Ligament Stem Cells

Yuniarti Soeroso^{*1,6}, Endang Winiati Bachtiar^{2,6}, Bachtiar Boy M^{3,6},
Benso Sulijaya⁴, Siti Wuryan Prayitno⁵

1. BDS, DDS, Specialist in Periodontology, PhD; Lecturer at Department of Periodontology Faculty of Dentistry, Universitas Indonesia.
2. BDS, DDS, M.Biomed, PhD; Lecturer at Department of Oral Biology Faculty of Dentistry, Universitas Indonesia.
3. BDS, DDS, M.S, PhD; Professor at Department of Oral Biology Faculty of Dentistry, Universitas Indonesia.
4. BDS, DDS, Specialist in Periodontology; Lecturer at Department of Periodontology Faculty of Dentistry, Universitas Indonesia.
5. BDS, DDS, MPH, MSc.D, Specialist in Periodontology, PhD; Professor at Department of Periodontology Faculty of Dentistry, Universitas Indonesia.
6. Oral Science Research Center, Faculty of Dentistry, Universitas Indonesia.

Abstract

Biomaterial innovation is needed for bone substitute which can help the osteogenesis process on periodontal tissue regeneration. This study is an experimental in-vitro study of different concentration of chitosan and aimed to analyze the osteogenesis of human periodontal ligament stem cells (PDLsc) cultured in chitosan.

Forty eight PDLsc were collected from healthy and periodontitis third lower molar. There were 24 healthy teeth-PDLsc and 24 periodontitis teeth-PDLsc. Each groups were divided into three experimental groups and were layered by 200 µl concentration of chitosan (0.15%, 0.2%; 0.25%) and diluted in 0,5 % citric acid. Cell culture without chitosan was used as a control group. The levels of alkaline phosphatase (ALP), osteocalcin and calcium deposit in the cell culture were measured on day 3, day 7, day 14 and day 21.

Chitosan at concentration of 0.25 % shows a potential impact of PDLsc in both periodontitis and healthy teeth samples, especially in the level of ALP and calcium deposit.

Chitosan has potential effect in increasing of osteogenesis process in periodontal ligament cells for regeneration of periodontal tissues.

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Introduction

Bone regeneration is one of the most important issues on periodontal therapy. Biomaterial innovation is needed for bone substitution which can help the osteogenesis process on periodontal tissue regeneration. Autogenous bone graft is considered as gold standard for bone regeneration; however, the process is complex and costly. Furthermore, histological studies in both humans and animals have demonstrated that grafting procedures often result in healing with a long junctional epithelium

rather than a new connective tissue attachment.¹

Therefore we need to produce new materials that may overcome this issue. Osteopontin, bonesialoprotein, osteocalcin, type 1 and type 2 collagen, and alkaline phosphatase (ALP), type 1 procollagen are all important components in the formation of cementum and periodontal ligament that can be considered as potential bone substitution.^{2,3,4,5,6} Expression of osteocalcin in organisms is confined to the bone, dentin and cementum.⁷

Chitosan is a material from invertebrata like shrimp skin, crab and lobster which is known as chitin. Chitosan has been tested as bone graft material and it has the characteristic of nondigestibility, high viscosity, water binding properties, biocompatible, biodegradable and low cytotoxicity.^{8,9,10,11} In other study showed that treatment in Periodontitis patients, found Chitosan alone was and its combination with metronidazole was effective due to its antimicrobial properties.¹²

*Corresponding author:

Yuniarti Soeroso,
BDS, DDS, Specialist in Periodontology, PhD
Department of Periodontology, Building B, 2nd Floor.
Faculty of Dentistry, Universitas Indonesia.

E-mail: yuniarti_22@yahoo.co.id

Natural poliaminosacaride has been proven as an antitumor, antirheumatic and biocompatible material in bone regeneration. It also has an osteoconductive ability and provides a physical effect by which the matrix of the graft forms as a scaffold that favors outside cells to penetrate graft and form new bone.¹³

Bone formation can be seen by measuring the total serum of alkaline phosphatase activity, osteocalcin and type 1 collagen, immunoassay of human osteocalcin and alkaline phosphatase are the most sensitive marker in bone formation.¹⁴ Recently using osteoblast progenitor cells, chitosan has been shown to increase Alkaline phosphatase.¹⁵

The current in-vitro study aim to observe the effect of wasted prawn skin derived chitosan on the osteogenic process of human PDL stem cells, by measuring the level of osteocalcin, collagen, alkaline phosphatase (ALP) and calcium deposit. Stem cells which play a role in the regeneration of periodontal tissue progenitor cells can be derived from the periodontal ligament.¹⁶

Materials and methods

Preparation of chitosan

We extracted Chitosan derived from 25 grams of shrimp mixture containing of white shrimp (*Penaeus merguensis*), krosok shrimp (*Metapenaeus* sp.) and striped shrimp (*Parapneosis* sp.). Chitosan was processed acetylated, chipping and irradiated for sterilization at the National Atomic Energy Agency BATAN in Jakarta, Indonesia. Preparation of chitosan started by cleaned of skin shrimp from other materials, washed with water, dried naturally without sunlight, demineralized using 1N hydrochloric acid for 24 hours and eliminated of residual hydrochloric acid with distilled water at pH of the solution between 6-7. The processed was continued to deproteinized with 1N sodium hydroxide for 24 hours and elimination of residual sodium hydroxide until the pH level reached between 6-7. Finally, we dried the products called chitin and acetylated it with sodium hydroxide (1:1) at a temperature of 80 degrees Celsius. At the end, it washed with distilled water and dried (now this product is called chitosan). The extract was then made as concentrate of chitosan 0.15%, 0.2% and 0.25%.

Preparation of stem cells culture

Harvested human periodontal ligament stem cells (PDLsc) with tissue culture techniques, isolated from apex scrapings from healthy and periodontitis of lower third molar teeth. Identification and confirmation of stem cells was done by using magnetic beads and anti stro-1 (MagnaBind™ Protein A and Protein G Beads, Pierce, Rockford, IL), a marker of mesenchymal stem cells.^{17,18} Stem cell culture was done in α -MEM that contained 10% fetal bovine serum (FBS), and 250 μ g/ml fungizone/Amphotericin B, in 37° (5% CO₂). The differentiation into osteoblast was evaluated by bone formation colony through VonKossa staining.¹⁹

Exposure of chitosan on the stem cells.

Forty eight samples divided into 2 groups of periodontal ligament stem cells (PDLsc) from healthy and periodontitis teeth. Each group were divided into 3 experimental groups, layered by 200 μ l concentration of chitosan (0.15%, 0.2%, 0.25%) and then diluted in 0,5 % citric acid. Cell culture without chitosan was used as a control group. On day 3, day 7, day 14 and day 21 the levels of ALP (calorimetry), osteocalcin (ELISA) were measured. Von Kossa staining technique was used to evaluate calcium deposition in the cell culture. The level of ALP was evaluated by measuring the reaction of the supernatant of osteoblast cell culture with a specific substrate for ALP using a spectrophotometer (405nm Stem cell culture, bone forming unit), triplicate in two times. The level of osteocalcin was evaluated by ELISA in the Laboratory of Oral Biology, Faculty of Dentistry, Universitas Indonesia 2011.

A number of 10.000 stem cells were incubated and spread in tissue culture plate, with and without layered by 200 ml of chitosan - diluted in 0,5 % citric acid (2mg/ml in 0,25 acetate acid). Evaluation of osteogenesis was done after 72hr. The experiment was evaluated in day 3, day 7, 14 and day 21 to measure ALP, osteocalcin.

Viability cell test with MTT assay in PDL stem cell after chitosan layering

MTT assay test calorimetri is performed to measure the enzym activity and to evaluate the viability of stem cell after chitosan layering. MTT assay test also can be done for cytotoxicity test of potential medicine or toxic material which may irritated or provoked of cell life time and growing of cell.²⁰

Results

Totallu we had 24 healthy and 24 periodontitis teeth samples which divided into 4 different types of therapy. The mean difference (and standard deviation) of all biomarkers in periodontitis group can be found in table 1, while in table 2 data were shown for the healthy group. In periodontitis patients, Chitosan with 0.15% concentration had a significant difference with the control group for osteocalcin at day 3 and 14; ALP at day 21; and calcium deposit at day 14 and 21.

Chitosan with 0.20% concentration showed significant difference with the control group for collagen at day 3, ALP at day 7, 14 and 21; and calcium deposit at day 14. Significant difference was found for chitosan with 0.25% concentration for both ALP and calcium deposit at day 7, 14 and 21.

In addition at chitosan concentration of 0.25%, the increase of ALP at day 7 was significantly different ($p < 0.05$) compared to day 3, and the increase at day 21 was also significantly different compared to day 14.

Among healthy subjects, Chitosan with 0.15% concentration significantly difference with the control group for ALP at day 7, 14 and 21; and calcium deposit at day 14 and 21. Chitosan with 0.20% concentration significantly difference with the control group for ALP at day 14 and 21; and calcium deposit at day 3, 7 and 14. Significant difference was found for chitosan with 0.25% concentration for both collagen and ALP at day 14; and calcium deposit at day 14 and 21. In addition chitosan concentration of 0.15% the increase of ALP at day 7 was significantly different ($p < 0.05$) compared to day 3; while at concentration of 0.20% and the changes of ALP were significantly different between day 14 and 7 and between day 14 and 21.

Since changes in ALP and calcium deposits were shown significantly difference among different groups, we plot the mean differences of those biomarkers between days of observation. In figure 1a we can see that concentration of 0.20% and 0.25% gave a lower ALP than the control group in both periodontitis and healthy group subjects at all days. In addition in figure 1b, chitosan at concentration of 0.25% gave a higher calcium deposit than the control group in all subjects at all days.

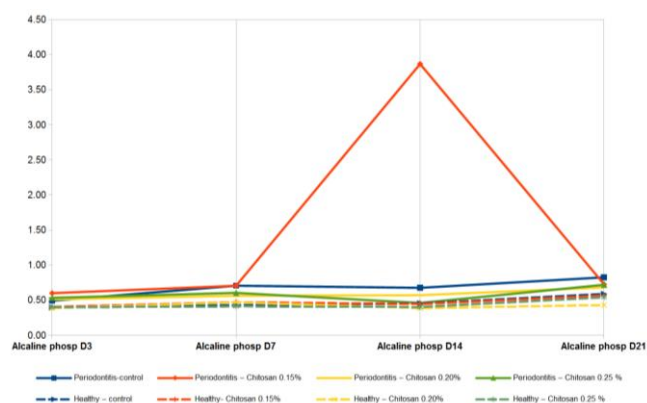


Figure 1a. Mean of alkaline phosphatase between groups and days.

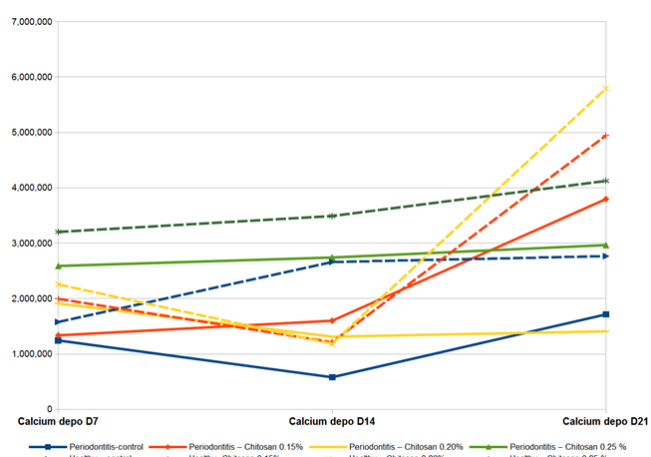


Figure 1b. Mean of calcium deposit between groups and days.

Discussion

This in-vitro study, we use wasted prawn skin derived chitosan to observe the osteogenic process of human PDL stem cells. Its potential benefit has been widely explored in many researches. The use of Hydroxyapatite (HA) / Tri Calcium Phosphate (TCP) as a carrier modified with human PDLsc already been transplanted into the dorsal surfaces of immunocompromised mice and showed the ability of cementum/PDL-like structures forming. PDLsc has showed the capacity to regenerate periodontal ligament attachment by forming collagen fibers connected to the cementum.²¹⁻²³

Related with previous studies, in this study we want to explore the potential effect of chitosan on the osteogenic process of human PDL stem cells (PDLsc), based on the level of osteocalcin, collagen, alkaline phosphatase (ALP) and calcium deposit on day 3, 7, 14, and

21. Chitosan at concentration of 0.25% shows impact of PDLsc in both periodontitis and healthy tooth, especially in the level of ALP and calcium deposit. In this study, we use a fresh PDLsc scraped from both of healthy and periodontitis teeth. In contrast with Tatullo et al (2015), he proved that the cryo-preserved PDLsc from extracted teeth could be useful for clinical therapeutic application.²⁴ In our opinion, both fresh and cryo-preserved PDLsc samples show almost no significant difference. This study showed a similar result with a previous study in which chitosan increased the expression of ALP in the osteoblast pregenerator cell.¹⁴ In this condition, chitosan may have osteo-conductive character which accelerate the secretion of osteocalcin that only needs 3 days. Chitosan has an effect as blood and tissue interphase in healing process and proved as a biocompatible material in bone regeneration, also osteoconduction provides a physical effect as a scaffold in forming new bone.¹² Osteoconduction causes differentiation of mesenchymal cell into bone cell and stimulation of bone development.⁸ Significant difference showed in this study in calcium deposition of PDL derived stem cell between chitosan and control group indicated that PDL derived stem cell cultured with chitosan increased osteogenesis and can be used as a scaffold to guidance bone development. Prior studies by Muzzarelli Boudrant et al., 2012; Muzzarelli, Greco, Busilacchi, Sollazzo & Gigante, 2012; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa & Domb, 2004 revealed that chitosan has an chemo-attract and an ability in activating macrophages and neutrophils in initiate healing process; promote granulation tissue and re-epithelialization.²⁵

Conclusions

Chitosan concentration of 0.25 % has a potential effect in increasing osteogenesis process in periodontal ligament cells for regeneration of periodontal tissues. Further study is needed to confirm the result of this preliminary study.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

References

1. Christoph A.R, Giulio R, Salvatore B, William V.G. Advanced Reconstructive Technology in Periodontal Tissue Repair. *J Periodontol* 2000, 2012, 59: 185-202.
2. Bhongade M, L, , Tiwari I, R, . A Comparative Evaluation of the Effectiveness of an Anorganic Bone Matrix/Cell Binding Peptide with an Open Flap Debridement in Human Infrabony Defects: A Clinical and Radiographic Study. *J of Contemp Dental Pract* 2007;8:1-13.
3. Lekic P, Sodek J, McCulloh CAG. Osteopontin and bonesialoprotein expression in regenerating rat periodontal ligament and alveolar bone *Anat Rec* 1996;244:50-58.
4. Mac Neil R L, Berry J E, D'Errico J A, Strayhorn C L, Piotrowski B, Somerman M J. Role of two mineral-associated adhesion molecules, osteopontin and bone sialoprotein, during cementogenesis. *Connect Tissue Res* 1995;33:1-7.
5. Matsuura M, Herr Y, Han K Y, Lin W L, Genco R J, Cho M I. Immunohistochemical expression of extracellular matrix components of normal and healing periodontal tissue in the beagle dog. *J Periodontol* 1995; 66:579-93.
6. Talwar SA GG, Aloia JF, Gambert SR , Talavera F. Bone Markers in Osteoporosis *Mar* 29, 2011.
7. Romi Barat, Anegundha Srinatha, Jayanta K.Pandit, Shampa Anupurba, Mittal N. Chitosan insert for periodontitis: Influence of drug loading, plasticizer and crosslinking on in vitro metronidazole release. *Acta Pharm* 2007;57:469-77.
8. Tianhong Dai, Masamitsu Tanaka, Ying-Ying Huang, Michael R, Hamblin. Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects. *July 2011, Vol. 9,(7):857-879 (doi:10.1586/eri.11.59)*
9. Fwu-Long Mi, Chin-Tsung Huang, Hsiang-Fa Liang, Mei-Chin Chen, Ya-Ling Chiu, Chun-Hung Chen, et al. Physicochemical, antimicrobial, and cytotoxic characteristics of a chitosan film cross-linked by naturally occurring cross-linking agent, aglycone genipocidic acid. *J. Agric food chem.* 2006;54 (9):3290-96.
10. Sebti I, Chollet E, Degraeve P, Noel C, Peyrol E. Water sensitivity, antimicrobial, and physicochemical analysis Of edible films based on HPMC and/or chitosan. *J Agric food chem.* 2007;55:693-99.
11. 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 *Mar* 15;76(4):711-20.
12. Akıncıbay, H., Şenel, S. and Yetkin Ay, Z. Application of chitosan gel in the treatment of chronic periodontitis. *J. Biomed. Mater. Res.*, (2007), 80B: 290–296. doi: 10.1002/jbm.b.30596.
13. Jaroslav A. Kralovec and Colin J. Barrow. *Glucosamine Production and Health Benefits in Colin Barrow, Fereidoon Shahidi .Marine Nutraceuticals and Functional Foods. CRC Press Taylor & Francis Group. FL2008:197.*
14. Gartant PR. *Oral cells and tissues. Quintessence Publishing Co, Inc. 2003; 195-238.*
15. Si-Feng Shi, Jing-Fu Jia, Xiao-Kui Guo, Ya-Ping Zhao, De-Sheng Chen, Yong-Yuan Guo, Tao Cheng and Xian-Long Zhang. Biocompatibility of chitosan-coated iron oxide nanoparticles with osteoblast cells. *Int J Nanomedicine.* 2012; 7: 5593–5602.
16. Melcher AH. On the repair potential of periodontal tissue. *J Periodontol* 1976;47:256-60.
17. Lin G LG, Banie L , Wang G , Ning H , Lue TF , Lin CS . Soft Tissue Distribution of Mesenchymal Stem Cell Marker Stro-1. *Stem Sel Dev.* Feb 24, 2011.
18. Gronthos S MK, Shi S, Bartold PM. Ovine periodontal ligament stem cells: Isolation, characterization, and differentiation potential. *Calcif Tissue Int* 2006;79(5):3120-317.
19. Von Kossa J. Ueber die im Organismus kunstlich erzeugbaren Verkalkungen. *Beit Path Anat*, 1901:163.
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal Of Immunological Methods* December 1983;65(1–2):55–63.

21. Patil A S, Merchant Y, Nagarajan P. Tissue Engineering of Craniofacial Tissues – A Review. Journal of Regenerative Medicine & Tissue Engineering. 2013:1-19.
22. Nassif L, Jurjus A, Nassar J, Ghafari J, Sabban Mel. Enhanced in-Vivo Bone Formation by Bone Marrow Differentiated Mesenchymal Stem Cells Grown in Chitosan Scaffold. J Bioengineer & Biomedical Sci. 2012:106:1-6
23. Nighojkar UR, Lele AP, Agrawal M. Scope of Stem Cells in Periodontal Regeneration. International Journal of Scientific Research. 2013(2):5:1-4
24. Tatullo M, Marrelli M, Paduano F. The Regenerative Medicine in Oral and Maxillofacial Surgery: The Most Important Innovations in the Clinical Application of Mesenchymal Stem Cells. Int. J Med Sci. 2015; 12(1):72- 77.
25. Alberto Busilacchi, Antonio Gigante, Monica Mattioli-Belmonte, Sandra Manzotti, Riccardo A.A. Muzzarelli. Chitosan stabilizes platelet growth factors and modulates stem cell differentiation toward tissue regeneration. Carbohydrate Polymers 98. 2013:665–676.

	Periodontitis group														
	Control			Chitosan 0.15 %			p (mean per time with control)	Chitosan 0.20 %			p (mean per time with control)	Chitosan 0.25 %			p (mean per time with control)
	Mean	±	SD	Mean	±	SD		Mean	±	SD		Mean	±	SD	
Osteocalcin D3	0.09	±	0.16	0.52	±	0.04	0.01	0.33	±	0.43	0.43	5.45	±	8.70	0.35
Osteocalcin D7	0.04	±	0.07	0.20	±	0.17	0.22	0.31	±	0.28	0.18	3.54	±	5.69	0.35
Osteocalcin D14	0.21	±	0.37	0.92	±	0.12	0.04	0.46	±	0.05	0.32	8.02	±	7.06	0.13
Osteocalcin D21	0.07	±	0.12	0.13	±	0.13	0.61	0.00	±	0.00	0.37	0.27	±	0.24	0.27
Collagen D3	467.36	±	320.91	897.55	±	882.15	0.47	2000.79	±	637.85	0.02	1066.39	±	865.68	0.24
Collagen D7	536.98	±	505.49	799.88	±	225.63	0.46	536.71	±	397.86	1.00	1205.35	±	1718.93	0.55
Collagen D14	1308.64	±	2281.17	832.19	±	804.15	0.75	885.31	±	1346.71	0.79	1864.86	±	1410.21	0.74
Collagen D21	1673.58	±	2173.71	981.82	±	626.41	0.62	1513.58	±	1085.20	0.92	1658.24	±	486.43	0.99
Alcaline phosph D3	0.49	±	0.12	0.60	±	0.02	0.06	0.51	±	0.07	0.74	0.53	±	0.07	0.50
Alcaline phosph D7*	0.70	±	0.03	0.70	±	0.04	0.98	0.56	±	0.01	0.00	0.60	±	0.06	0.01
Alcaline phosph D14*	0.67	±	0.06	3.87	±	5.05	0.15	0.57	±	0.01	0.00	0.46	±	0.03	0.00
Alcaline phosph D21*	0.82	±	0.06	0.72	±	0.06	0.02	0.68	±	0.11	0.02	0.72	±	0.10	0.05
Calcium depo D7	1244000.00	±	201690.69	1336666.67	±	282908.70	0.67	1910666.67	±	481698.38	0.09	2589000.00	±	163514.53	0.00
Calcium depo D14	580333.33	±	31069.81	1600666.67	±	329439.42	0.01	1312666.67	±	67211.11	0.00	2741666.67	±	287828.81	0.00
Calcium depo D21	1714666.67	±	300380.35	3797666.67	±	322295.41	0.00	1409666.67	±	135396.95	0.18	2986000.00	±	331018.13	0.01

Table 1. Differences in biomarkers among periodontitis patients.

Note : * p of alkaline phosphatase at chitosan 0.15 % between day 3 and 7 = 0.03; p of alkaline phosphatase at chitosan 0.25 % between day 3 and 7 = 0.03; p of alkaline phosphatase at chitosan 0.25 % between day 7 and 14 = 0.03; p of alkaline phosphatase at chitosan 0.25 % between day 14 and 21 = 0.03.

	Healthy group														
	Kontrol			Chitosan 0.15 %			p (comparison with control)	Chitosan 0.20 %			p (comparison with control)	Chitosan 0.25 %			p (comparison with control)
	Mean	±	SD	Mean	±	SD		Mean	±	SD		Mean	±	SD	
Osteocalcin D3	0.29	±	0.21	0.12	±	0.13	0.11	0.32	±	0.17	0.82	5.39	±	8.52	0.17
Osteocalcin D7	0.18	±	0.21	0.06	±	0.05	0.23	1.94	±	4.56	0.37	2.56	±	6.14	0.36
Osteocalcin D14*	2.31	±	4.56	0.28	±	0.18	0.30	0.37	±	0.29	0.32	2.30	±	5.19	1.00
Osteocalcin D21*	0.18	±	0.22	0.14	±	0.14	0.70	0.18	±	0.31	0.98	2.05	±	4.89	0.37
Collagen D3	1182.06	±	1156.52	616.77	±	735.44	0.51	413.18	±	369.58	0.33	1441.06	±	895.19	0.77
Collagen D7	1634.66	±	2401.56	880.52	±	811.26	0.63	474.22	±	81.01	0.45	858.62	±	339.13	0.61
Collagen D14	1043.92	±	137.61	1072.33	±	446.24	0.92	582.78	±	729.85	0.34	3425.85	±	1431.27	0.05
Collagen D21	679.89	±	582.71	1666.43	±	1671.77	0.39	1014.34	±	1402.59	0.72	2970.55	±	1841.14	0.11
Alcaline phosph D3	0.40	±	0.04	0.40	±	0.02	0.72	0.39	±	0.06	0.72	0.40	±	0.03	0.93
Alcaline phosph D7*	0.43	±	0.03	0.47	±	0.02	0.02	0.47	±	0.04	0.09	0.41	±	0.09	0.66
Alcaline phosph D14*	0.45	±	0.01	0.44	±	0.01	0.18	0.38	±	0.03	0.00	0.40	±	0.03	0.00
Alcaline phosph D21*	0.59	±	0.01	0.57	±	0.03	0.14	0.43	±	0.04	0.00	0.54	±	0.12	0.33
Calcium depo D7	1578333.33	±	375895.38	1995333.33	±	111383.72	0.14	2256666.67	±	87933.69	0.04	3204333.33	±	1060130.34	0.07
Calcium depo D14	2661000.00	±	307413.40	1215000.00	±	93214.81	0.00	1186333.33	±	110273.90	0.00	3491333.33	±	266049.53	0.03
Calcium depo D21	2766666.67	±	126183.73	4945333.33	±	108098.72	0.00	5791000.00	±	404834.53	0.00	4125333.33	±	71164.13	0.00

Table 2. Differences in biomarkers among healthy subjects.

Note : * p of alkaline phosphatase at chitosan 0.15 % and 0.20 % between day 3 and 7 = 0.03; p of alkaline phosphatase chitosan 0.15 % and 0.20 % between day 7 and 14 = 0.03; p of alkaline phosphatase at 0.15 %, 0.20 % and 0.25 % between day 14 and 21 = 0.03.
 * p of Calcium deposition at chitosan 0.15 % between day 14 and 21 = 0.00 , 0.20 % between day 7 and day 14 = 0,04 and between day 14 and 21 = 0.00 and 0,25% between day 14 and 21=0.00.