Chitosan promotes immune responses, ameliorates glutamic oxaloacetic transaminase and glutamic pyruvic transaminase, but enhances lactate dehydrogenase levels in normal mice in vivo

MING-YANG YEHW1, YUNG-LUEN SHIH2,2*, HSUEH-YU CHUNG3, JASON CHOU6, HSU-FENG LU7, CHIA-HUI LIU8, JIA-YOU LIU7, WEN-WEN HUANG9, SHU-FEN PENG9, LUNG-YUAN WU10 and JING-GUNG CHUNG9,11

1Office of Director, Cheng Hsin General Hospital; 2Department of School of Medicine, Fu-Jen Catholic University; 3Department of Pathology and Laboratory Medicine, Shin Kong Wu Ho-Su Memorial Hospital; 4School of Medical Laboratory Science and Biotechnology, Taipei Medical University, Taipei; 5Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli; Departments of 6Anatomical and 7Clinical Pathology, Cheng Hsin General Hospital, Taipei; 8The Center of General Education, Chia-Nan University of Pharmacy and Science, Tainan; 9Department of Biological Science and Technology, China Medical University, Taichung; 10The School of Chinese Medicine for Post Baccalaureate, I-Shou University, Kaohsiung; 11Department of Biotechnology, Asia University, Taichung, Taiwan, R.O.C.

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Abstract. Chitosan, a naturally derived polymer, has been shown to possess antimicrobial and anti-inflammatory properties; however, little is known about the effect of chitosan on the immune responses and glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and lactate dehydrogenase (LDH) activities in normal mice. The aim of the present study was to investigate whether chitosan has an effect on the immune responses and GOT, GPT and LDH activities in mice in vivo. BALB/c mice were divided into four groups. The negative control group was treated with a normal diet; the positive control group was treated with a normal diet plus orally administered acetic acid and two treatment groups were treated with chitosan at doses of 5 and 20 mg/kg, respectively, every other day for 24 days. Mice were weighed during the treatment, and following the treatment, blood was collected, and liver and spleen samples were isolated and weighted. The blood samples were used for measurement of white blood cell markers, and the spleen samples were used for analysis of phagocytosis, natural killer (NK) cell activity and cell proliferation using flow cytometry.

The results indicated that chitosan did not markedly affect the body, liver and spleen weights at either dose. Chitosan increased the percentages of CD3 (T-cell marker), CD19 (B-cell marker), CD11b (monocytes) and Mac-3 (macrophages) when compared with the control group. However, chitosan did not affect the phagocytic activity of macrophages in peripheral blood mononuclear cells, although it decreased it in the peritoneal cavity. Treatment with 20 mg/kg chitosan led to a reduction in the cytotoxic activity of NK cells at an effector to target ratio of 25:1. Chitosan did not significantly promote B-cell proliferation in lipopolysaccharide-pretreated cells, but significantly decreased T-cell proliferation in concanavalin A-pretreated cells, and decreased the activity of GOT and GPT compared with that in the acetic acid-treated group. In addition, it significantly increased LDH activity, to a level similar to that in normal mice, indicating that chitosan can protect against liver injury.

Introduction

The emergence and growth of tumors are known to be associated with tumor immunosurveillance and antitumor immune responses (1). However, one of the drawbacks of many therapeutic technologies for cancer patients is the inadvertent induction of host immune responses (2). Thus, previous studies have focused on immune-mediated protection against cancer in immunocompromised patients with cancer and mouse
models (3). Treatments for human cancers remain a therapeutic challenge, and the identification and development of novel agents to induce immune function is necessary.

Chitosan, a linear heteropolysaccharide composed of β-(1,4)-linked D-glucosamine (GlcN) and β-(1,4)-linked D-N-acetylg glucosamine (GlcNAc), can be derived from chitin (4), which is a naturally occurring polysaccharide composed of GlcNAc units (5). Chitosan can be used as a biomaterial for tissue regeneration, and has antibacterial, anti-inflammatory and drug delivery functions (6). Numerous studies have demonstrated that chitosan may inhibit the growth of microbial organisms, such as Porphyromonas gingivalis (7), Actinobacillus actinomycetemcomitans, Streptococcus mutans (8,9), Pseudomonas aeruginosa, Staphylococcus aureus (10) and Aggregatibacter actinomycetemcomitans (11).

In human astrocytoma cells, the secretion and expression of the pro-inflammatory cytokines tumor necrosis factor (TNF)–α and interleukin (IL)–6 has been shown to be markedly inhibited following pretreatment with water-soluble chitosan (9). It has also been reported that chitosan-induced macrophages exhibit markedly downregulated expression of pro-inflammatory markers, such as cluster of differentiation CD86 and major histocompatibility complex II (MHCII), and decrease the expression of pro-inflammatory cytokines, specifically TNF–α; however, the anti-inflammatory markers IL-10 and TGF-β were found to be increased (12,13).

Despite the reports of several studies that chitosan has an anti-inflammatory effect in vitro, knowledge concerning the effect of chitosan on the immune responses of normal mice is lacking. In the present study, the promoted immune responses in BALB/c mice were evaluated in vivo. Furthermore, the levels of certain enzymes, including glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and lactate dehydrogenase (LDH), were analyzed in BALB/c mice following oral treatment with chitosan. The expression levels of the white blood cell markers CD3, CD11b, CD19 and Mac-3 were also investigated.

Materials and methods

Materials and reagents. Acetic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum, L-glutamine and penicillin-streptomycin were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Tissue culture plastic wares and Tissue culture plastic wares and phycoerythrin (PE)-conjugated anti-mouse-CD3 (cat. no. 553062), PE-conjugated anti-mouse-CD19 (cat. no. 553786), FITC-conjugated anti-mouse-CD11b (cat. no. 553310) and FITC-conjugated anti-mouse-Mac-3 (cat. no. 553322) were purchased from BD Pharmigen (San Diego, CA, USA).

Preparation of chitosan. Chitosan powder with a molecular weight of ~86,000 kDa (Koyo Chemical Co., Ltd., Sakaaiminato, Japan) was obtained from the National Taiwan University College of Medicine Animal Medicine Center (Taipei, Taiwan). The doses of 5 and 20 mg/kg were separately suspended in 0.2 ml acetic acid for 1 h at room temperature prior to use (14).

Male BALB/c mice and chitosan treatment. Forty male BALB/c mice aged 4 weeks and weighing 22-25 g, were obtained from the National Laboratory Animal Center (Taipei, Taiwan). All mice were maintained at 25°C on 12 h light/dark cycles in the animal center of China Medical University (Taichung, Taiwan). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University (approval ID, 103-215-B). All animal care was in accordance with the institutional animal ethical guidelines of the China Medical University (15). The 40 mice were randomly divided into the following four groups (10 mice per group): Negative control group, comprising untreated mice; positive control group, treated with acetic acid; 5 mg/kg group, treated with 5 mg/kg chitosan in acetic acid, and 20 mg/kg group, treated with 20 mg/kg chitosan in acetic acid. Mice in all four groups were fed a normal diet. Chitosan in acetic acid was administered by gavage every 2 days for a total of 24 days (12 times), during which the body weight was recorded. Upon termination of the treatment, mice from each group were weighed and sacrificed with CO2 as previously described (15).

Immunofluorescence staining for surface markers. Upon termination of the treatment, all mice were individually weighed and blood samples, as well as the liver and spleen of the mice were individually collected. The collected spleens were used for the isolation of splenocytes and measurement of natural killer (NK) cell activity, as previously described (15). A blood sample of 1 ml from each mouse was lysed to destroy the red blood cells using 1X BD Pharm Lyse®*14 lysing buffer (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol, and leukocytes were collected as previously described (15). Phycocerythrin (PE)-labeled anti-mouse-CD3, PE-labeled anti-mouse-CD19, fluorescein isothiocyanate (FITC)-labeled anti-mouse-CD11b and FITC-labeled anti-mouse-Mac-3 antibodies (all dilution 1:40) were used to stain the isolated leukocytes for 30 min, and then all samples were washed with phosphate-buffered saline (PBS). After this, all samples were analyzed using flow cytometry (BD FACSCalibur; BD Biosciences) to measure the percentages of white blood cell markers, as previously described (15).

Measurements of the phagocytic activity of macrophages. Macrophages were isolated from the peripheral blood mononuclear cells (PBMCs) and peritoneum of each mouse as previously described (15) and were placed in plates containing 50 µl target E. coli-FITC according to PHAGOTEST® kit manufacturer's instructions (ORPÈGEN Pharma GmbH, Heidelberg, Germany). All samples were individually mixed, then examined for phagocytosis using flow cytometry. Quantification of phagocytosis was performed using CellQuest software (version 5.1; BD Biosciences) as previously described (15).

Measurements of NK cell cytotoxic activity. Splenocytes were isolated from each spleen as previously described (15) and were placed in 96-well plates (1x10⁴ cells/well) with 1 ml RPMI-1640 medium. Target YAC-1 cells (2.5x10⁵ cells; Food Industry Research and Development Institute, Hsinchu, Taiwan) and PKH-67/Diluent C buffer (Sigma-Aldrich) were individually added to the cell-containing wells, according
to the manufacturer's protocol. The samples were mixed thoroughly for 2 min at 25˚C and 2 ml PBS was added to each well for 1 min together with 4 ml medium. The mix was then incubated for 10 min. Following incubation, all samples were centrifuged for 2 min at 290 x g rpm (25˚C). NK cell cytotoxic activity was measured using flow cytometry as previously described (15).

Measurements of T- and B-cell proliferation. Isolated splenocytes (1x10^5 cells/well) from each mouse were placed in 96-well plates containing 100 µl RPMI-1640 medium. Following stimulation by incubation with concanavalin A (Con A; 0.5 µg/ml; Sigma-Aldrich) for 3 days, T-cell proliferation was measured. In addition, B-cell proliferation was measured following stimulation with lipopolysaccharide (LPS, 1 µg/ml; Sigma-Aldrich) for 5 days. Cell proliferation was measured using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, USA) as previously described (15).

Measurement of blood levels of GOT, GPT and LDH in BALB/c mice following exposure to chitosan. Following treatment, blood samples were collected from all mice in order to measure the levels of GOT, GPT and LDH using quantitative kits. The kits were liquiUV (aspartate aminotransferase) for GOT, liquiUV (alanine aminotransferase) for GPT and liquiUV (lactate dehydrogenase) for LDH, respectively, which were purchased from HUMAN Gesellschaft für Biochemica und Diagnostica mbH (Wiesbaden, Germany), and were used as previously described (16,17).

**Statistical analysis.** The data from three independent experiments were expressed as the mean ± standard error. Statistical comparison between the chitosan and control groups was performed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Chitosan affects the body, liver and spleen weights of normal BALB/c mice. Representative images of the mice in the four groups, and animal body, liver and spleen weights are presented in Fig. 1. These results indicate that chitosan did not significantly affect the appearance of the animals (Fig. 1A) or the body (Fig. 1B), liver (Fig. 1C) or spleen (Fig. 1D) weights when compared with those of the vehicle-treated group.

Chitosan affects cell markers of white blood cells in normal BALB/c mice. Blood samples were collected from each mouse and the levels of CD3, CD19, CD11b and Mac-3 cell markers were measured. The results presented in Fig. 2 indicate that chitosan promoted CD3 (Fig. 2A), CD19 (Fig. 2B), CD11b (Fig. 2C) and Mac-3 (Fig. 2D) expression at both doses, when compared with the acetic acid-treated group.

Chitosan affects the phagocytic activity of macrophages from the PBMCs and peritoneal cavity of normal BALB/c mice. Following treatment, cells were isolated from the PBMCs and peritoneal cavity of each animal, in order to measure the percentage of phagocytosis and results are shown in Fig. 3A and B, respectively. Neither of the two doses of chitosan (5 and
20 mg/kg) was found to significantly affect phagocytosis by macrophages from PMBCs (Fig. 3A); however, both doses were found to decrease phagocytosis by macrophages from the peritoneal cavity (Fig. 3B).

Chitosan affects the cytotoxic activity of NK cells from normal BALB/c mice. YAC-1 cells were used as targets for isolated splenocytes and were examined using flow cytometry. The results (Fig. 4) indicated that chitosan did not significantly affect the cytotoxic activity of NK cells at an effector to target ratio of 50:1; however, at the dose of 20 mg/kg chitosan and an
A effector to target ratio of 25:1 led to a significant reduction in the cytotoxic activity of the NK cells when compared with that in the acetic acid-treated group (P<0.05; Fig. 4).

**Chitosan affects T- and B-cell proliferation in normal BALB/c mice.** Isolated splenocytes were assayed for T- and B-cell proliferation using flow cytometry and the results are presented in Fig. 5. The two chitosan doses (5 and 20 mg/kg) notably decreased T cell proliferation when compared with the acetic acid-treated group (Fig. 5A), but did not significantly affect B-cell proliferation (Fig. 5B).

**Chitosan affects the activity of blood enzymes GOT, GPT and LDH in BALB/c mice.** Following treatment, the mice were sacrificed and blood samples were collected from each mouse in order to measure the activity of GOT, GPT and LDH (Fig. 6). Chitosan significantly decreased GOT and GPT activity when compared with that in the acetic acid-treated group (P<0.05; Fig 6A and B). However, GPT activity in the 20 mg/kg chitosan group was slightly higher than that in normal mice. Furthermore, chitosan significantly increased LDH activity when compared with that in the acetic acid-treated group (P<0.05).

**Discussion**

Although numerous studies have shown that chitosan is able to inhibit the growth of microbial organisms (7-11), it has also been shown to cause significant downregulation of the expression of pro-inflammatory markers CD86 and MHCII on macrophages, decrease the expression of the pro-inflammatory cytokine TNF-α and increase that of the anti-inflammatory cytokines IL-10 and TGF-β1 (12,13). In addition, our earlier study has shown that the hypolipidemic effect of chitosan is partly attributed to its suppression of intestinal lipid absorption and hepatic acyl-coenzyme A: cholesterol acyltransferase-2 expression (18). Furthermore, chitosan has also been found to slow down the rate of tumor growth without inhibiting tumor formation (14); however, no detailed analysis of the immune responses in chitosan-treated animals, including mice, has been reported.

In the present study, normal BALB/c mice were randomly divided into four groups. The negative control group received a normal diet, the positive control group received a normal diet and acetic acid, and two treatment groups received a normal diet and the oral administration of 5 or 20 mg/kg chitosan.
in acetic acid. During the treatment, chitosan and/or acetic acid was administered, and the animals were weighed, every 2 days. At the end of the treatment period, blood samples were collected from all mice for cell marker analysis and measurement of phagocytosis, and splenocytes were isolated in order to examining NK cell activities and T- and B-cell proliferation.

To the best of our knowledge, this is the first study evaluating the effect of chitosan on immune responses in normal mice in vivo. The present results showed the following: i) chitosan did not significantly affect the appearance (Fig. 1A) or body (Fig. 1B), liver (Fig. 1C) and spleen (Fig. 1D) weights of the mice when compared with the acetic acid group; ii) chitosan increased CD3 (T cell; Fig. 2A), CD19 (B cell; Fig. 2B), CD11b (monocyte; Fig. 2C) and Mac-3 (macrophage; Fig. 2D) markers when compared with the acetic acid group; iii) chitosan treatment did not significantly increase the phagocytic activity of macrophages in PBMCs (Fig. 3A) but significantly decreased it in the peritoneal cavity (Fig. 3B); iv) chitosan at 20 mg/kg significantly decreased the cytotoxic effect of NK cells compared with that in the acetic acid group (Fig. 4); v) 20 mg/kg chitosan treatment significantly decreased T cell proliferation (Fig. 5A) compared with that in the acetic acid group, but B cell proliferation was not significantly affected by treatment with 5 and 20 mg/kg doses (Fig. 5B), and vi) chitosan decreased GPT and GPT activity compared with that in the acetic acid group, with GPT activity in the 20 mg/kg group being slightly higher than the levels in normal mice (Fig. 6B). Chitosan significantly increased LDH levels when compared with those in the acetic acid-treated group (Fig. 6C).

Chitosan promoted the cell markers CD3 (T cell), CD19 (B cell), CD11b (monocytes) and Mac-3 (macrophages) when compared with the acetic acid-treated mice. A previous study has reported that these four cell types play an important role in immune responses, particularly against the invasion of foreign antigens (19). Other studies have shown that macrophages play an important role in innate immunity (20,21). Despite reports suggesting the involvement of chitosan in inflammatory responses, reliable data in the literature regarding the effects of chitosan on immune responses in normal mice in vivo are lacking. The aim of the present study, therefore, was to investigate the effects of chitosan on the immune responses of normal BALB/c mice in vivo.

A notable observation of the present study is that chitosan did not significantly affect the phagocytic activity of macrophages in PBMCs (Fig. 3A), but significantly decreased this activity in the peritoneal cavity (Fig. 3B); thus, the effects of chitosan on the Mac-3 marker and macrophage function require further study. It has been suggested that chitosan may exert an anti-inflammatory activity in astrocytoma cells (11) and macrophages (12,13). Furthermore, it has been reported that chitosan exerts anti-inflammatory activity by modulating prostaglandin E synthase 2 levels through the c-Jun N-terminal kinase pathway, which has been suggested to be useful in the prevention or treatment of periodontal inflammation (22). Treatment with 20 mg/kg chitosan significantly decreased the cytotoxic effect of NK cells from normal mice. Compared with the acetic acid-treated group, chitosan did not significantly affect B-cell proliferation following LPS stimulation (Fig. 5B) but both doses of chitosan decreased T-cell proliferation following Con A stimulation (Fig. 5A). Further investigations are necessary to investigate this. It has been suggested that the great variability observed in chitosan samples, such as degrees of deacetylation, molecular weight, viscosity, and pKa may affect its properties (4).

Chitosan decreased the levels of GOT and GPT compared with those in the acetic acid-treated group; although the GPT level in the 20 mg/kg group was slightly higher than the level in normal mice (Fig. 6B). High levels of GPT and GPT activity in the serum have been recognized to be a reflection of hepatic cell destruction (23). The results of the present study indicate that chitosan may have a protective effect against hepatic cell death following exposure to acetic acid. Chitosan significantly increased LDH levels when compared with those in the acetic acid-treated group. Abnormal hepatic transaminase and LDH levels have been suggested to be associated with liver injury in patients with abdominal trauma (24). Acetic acid treatment in mice may lead to liver injury; on the basis of the increased levels of LDH in the blood observed in the present study following treatment with acetic acid and chitosan, it appears that chitosan may have a protective effect.

In conclusion, these findings suggest that chitosan modulates immune responses by increasing T-cell, B-cell, monocyte and macrophage cell markers in normal mice in vivo. Furthermore, comparisons between mice treated with acetic acid and chitosan, or chitosan alone indicate that chitosan treatment may protect against liver injury in vivo.

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References

1. Jones LM, Broz ML, Ranger JJ, Ozcelik J, Ahn R, Zuo D, Ursini-Siegel J, Hallett M, Krummel M and Muller WF: Stat3 establishes an immunosuppressive microenvironment during the early stages of breast carcinogenesis to promote tumor growth and metastasis. Cancer Res: 30 Dec, 2015 (Epub ahead of print).
2. Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E, Lichitor T, Decker WK, Whelan RL, Kumarma HM, et al.: Immune evasion in cancer: Mechanistic basis and therapeutic strategies. Semin Cancer Biol (Suppl 35): S185 –S198, 2015.
3. Vesely MD, Kershew MH, Schreiber RD and Smyth MJ: Natural innate and adaptive immunity to cancer. Annu Rev Immunol 29: 235-271, 2011.
4. Domard A: A perspective on 30 years research on chitin and chitosan. Carbohydr Polym 84: 696-703, 2011.
5. Chung MJ, Park JK and Park YI: Anti-inflammatory effects of low-molecular weight chitosan oligosaccharides in IgE-antigen complex-stimulated RBL-2H3 cells and asthma model mice. Int Immunopharmacol 12: 453-459, 2012.
6. Francesko A and Tzanov T: Chitin, chitosan and derivatives for wound healing and tissue engineering. Adv Biochem Eng Biotechnol 125: 1-27, 2011.
7. Ikinci G, Senel S, Akincibay H, Kars S, Ercis S, Wilson CG and Hincal AA: Effect of chitosan on a periodontal pathogen Porphyromonas gingivalis. Int J Pharm 235: 121-127, 2002.
8. Choi BK, Kim KY, Yoo YJ, Oh SJ, Choi JH and Kim CY: In vitro antimicrobial activity of a chitoooligosaccharide mixture against Actinobacillus actinomycetemcomitans and Streptococcus mutans. Int J Antimicrob Agents 18: 553-557, 2001.
9. Sarasaam AR, Brown P, Khajotia SS, Dmytryk JJ and Madighally SV: Antibacterial activity of chitosan-based matrices on oral pathogens. J Mater Sci Mater Med 19: 1083-1090, 2008.
10. Chung YC, Wang HL, Chen YM and Li SL: Effect of abiotic factors on the antibacterial activity of chitosan against waterborne pathogens. Bioreour Technol 88: 179-184, 2003.

11. Kim MS, Sung MJ, Seo SB, Yoo SJ, Lim WK and Kim HM: Water-soluble chitosan inhibits the production of pro-inflammatory cytokine in human astrocytoma cells activated by amyloid beta peptide and interleukin-1beta. Neurosci Lett 321: 105-109, 2002.

12. Chou TC, Fu E and Shen EC: Chitosan inhibits prostaglandin E2 formation and cyclooxygenase-2 induction in lipopolysaccharide-treated RAW 264.7 macrophages. Biochem Biophys Res Commun 308: 403-407, 2003.

13. Yoon HJ, Moon ME, Park HS, Im SY and Kim YH: Chitosan oligosaccharide (COS) inhibits LPS-induced inflammatory effects in RAW 264.7 macrophage cells. Biochem Biophys Res Commun 358: 954-959, 2007.

14. Yeh MY, Wu MF, Shang HS, Chang JB, Shih YL, Chen YL, Hung HF, Lu HF, Yeh C, Wood WG, et al: Effects of chitosan on xenograft models of melanoma in C57BL/6 mice and hepatoma formation in SCID mice. Anticancer Res 33: 4867-4873, 2013.

15. Lu HF, Tung WL, Yang JS, Huang FM, Lee CS, Huang YP, Liao WY, Chen YL and Chung JG: In vitro suppression of growth of murine WEHI-3 leukemia cells and in vivo promotion of phagocytosis in a leukemia mice model by indole-3-carbinol. J Agric Food Chem 60: 7634-7643, 2012.

16. Nagamatsu Y, Yamamoto J, Fukuda A, Ohta M, Tsuda Y and Okada Y: Determination of leukocyte elastase concentration in plasma and serum by a simple method using a specific synthetic substrate. Haemostasis 21: 338-345, 1991.

17. No authors listed: Recommendations of the German Society for Clinical Chemistry. Standardization of methods for the determination of enzyme activities in biological fluids. Z Klin Chem Klin Biochem 8: 658-660, 1970.

18. Wu CC, Lin SY, Chen CT, Chang YP, Huang YS, Li CK, Yu CC, Hsieh SL and Chung JG: Differential blood lipid-lowering effects of alkylsulfonated chitosan of different molecular weights in Syrian hamsters in vivo. Mol Med Rep 5: 688-694, 2012.

19. Arpinati M and Curti A: Immunotherapy in acute myeloid leukemia. Immunotherapy 6: 95-106, 2014.

20. Gordon S, Plüddemann A and Mukhopadhyay S: Sinusoidal immunity: Macrophages at the lymphohematopoietic interface. Cold Spring Harb Perspect Biol 7: a016378, 2015.

21. Kim KH, Kim TS, Lee JG, Park JK, Yang M, Kim JM, Jo EK and Yuk JM: Characterization of proinflammatory responses and innate signaling activation in macrophages infected with Mycobacterium scrofulaceum. Immune Netw 14: 307-320, 2014.

22. Arancibia R, Maturana C, Silva D, Tobar N, Tapia C, Salazar JC, Martinez J and Smith PC: Effects of chitosan particles in periodontal pathogens and gingival fibroblasts. J Dent Res 92: 740-745, 2013.

23. Yamashita T, Ohshima H, Asanuma T, Inukai N, Miyoshi I, Kasai N, Kon Y, Watanabe T, Sato F and Kuwabara M: The effects of alpha-phenyl-tert-butyl nitrone (PBN) on copper-induced rat fulminant hepatitis with jaundice. Free Radic Biol Med 21: 755-761, 1996.

24. Bilgic I, Gelecek S, Akgun AE and Ozmen MM: Predictive value of liver transaminases levels in abdominal trauma. Am J Emerg Med 32: 705-708, 2014.