Chitin and chitosan stimulate canine polymorphonuclear cells to release leukotriene B_4 and prostaglandin E_2

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Abstract: The effects of chitin and chitosan on the release of arachidonic acid products were investigated in this study. Supernatants of canine polymorphonuclear cell (PMN) suspensions incubated with chitin and chitosan contained a leukotriene B_4 (LTB_4) concentration high enough to induce canine PMN migration *in vitro.* The supernatants also contained the same concentration of prostaglandin E_2 (PGE_2) as that normally found in the peripheral blood of dogs. Intraperitoneal administration of chitosan to dogs induced peritoneal exudative fluid (PEF), but chitin did not. The PEF contained numerous PMNs and macrophages. The supernatant of PEF contained both heat-stable and heat-labile chemotactic factors for canine PMNs. It also contained enough LTB_4 to attract the canine PMNs *in vitro*. © 1998 John Wiley & Sons, Inc. J Biomed Mater Res, 42, 517–522, 1998.

Key words: chitin; chitosan; canine polymorphonuclear cells; leukotriene B_4; prostaglandin E_2

INTRODUCTION

Chitin and chitosan [poly(*N*-acetyl-D-glucosamine) GlcNAc) and poly(D-glucosamine) (GlcN)] have been demonstrated to act as wound healing accelerators[1–4] and are now used in human and veterinary medicine.[5,6] Wounds to which chitin and chitosan are applied show active and rapid accumulation of polymorphonuclear cells (PMNs), angiogenesis, and healing with little scarring.[7,8] Some investigators have reported on the mechanism by which chitin and chitosan act at a wounded site, which might involve activation of PMNs and macrophages,[9–12] a protective effect against microorganisms,[13] or promotion of granulation tissue formation with angiogenesis.[2,14] PMN migration is one of the important steps in wound healing,[15] and defects of PMN migration not only induce delayed wound healing but also severe infection.[16,17] Many kinds of chemotactic factors for PMN have been reported[18–21]; these are divided into three categories: microorganism-derived factors such as formyl peptides and lipids, enzyme-linked factors such as C5a and leukotriene B_4 (LTB_4), and gene expression factors such as IL-8. We found that C5a was produced when chitin and chitosan were mixed with canine serum and that chitin and chitosan induced complement-mediated migration of the canine PMNs. Also, an increased concentration of prostaglandin E_2 (PGE_2) was detected at the site of chitin implantation,[22] suggesting that chitin induces the production of arachidonic acid products (AAPs). Furthermore, PMNs not only have receptors for AAP but also have the ability to produce AAPs.[19] However, it has not been clear whether chitin and chitosan induce AAPs such as LTB_4 that promote PMN migration and upregulation of endothelial leukocyte adhesion molecule expression.[23,24]

In this study we investigated the effects of chitin and chitosan on the release of AAPs *in vitro* and *in vivo* in dogs.

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MATERIALS AND METHODS

Chemoattractants

A chitin powder (Sunfive, Japan) purified from squid pen and a chitosan powder chemically deacetylated from snow crab shell chitin (Sunfive) were suspended in Hank's balanced salt solution (HBSS, Nissui Seiyaku, Japan) for use in this study. The chitin and chitosan powders showed <10% and >80% deacetylation, respectively. The mean particle size of each powder was 3.5 μm as measured with an SK Laser Micron Sizer 7000S (Seisin K. K., Japan). Preparation of each powder was based on the method of Tanioka et al.[6] Latex

beads (Polybead, Polysciences) with an average diameter 2.8 μm were suspended in HBSS.

Animals

Fifteen mongrel dogs aged 1–4 years (seven males and eight females) were used in this study. Animal treatments were performed on the basis of the Guidelines for Animal Experimentation of the Faculty of Agriculture of Tottori University, the Japanese Government Animal Protection and Management Law (no. 105), and the Japanese Government Notification on Feeding and Safekeeping of Animals (no. 6) with the permission of the Animal Committee of the Faculty of Agriculture of Tottori University (approval no. 1996-1).

Preparation of canine PMN suspension

Ten milliliters of peripheral blood was collected from the jugular vein using a syringe containing 10 U of heparin solution and an 18-gauge needle. Separation of canine PMNs from the collected blood was done by density gradient centrifugation [specific gravity (spgr) = 1.082] as reported previously.[11]

Incubation of PMNs with chitin and chitosan

Two milliliters of canine PMN suspension (2×10^6 to 1×10^7 cells/mL) was placed in a 24-well tissue culture plate (Greiner, Germany) and incubated with 2 mg of chitin, chitosan, and latex beads for 15 min to 3 h at 37°C in humidified air with 5% CO_2. After incubation, each canine PMN suspension was centrifuged at 700 *g* for 10 min to remove the cells and the particles of chitin, chitosan, and latex beads. The supernatants of PMNs incubated with chitin, chitosan, and latex were designated as NGIS, GIS, and LIS, respectively. Supernatant of canine PMNs (1×10^7/mL) incubated without any stimulants was used as the control (HBIS).

Measurement of PMN migration

The migration of canine PMNs was measured using the blind-well chamber method (Nucle Pore) as reported previously.[10] In brief, 200 μL of sample was placed into the lower chamber and a 5-μm pore polycarbonate filter (Neuro Probe) was positioned between the lower and upper chambers. Then 200 μL of cell suspension was added to the upper chamber and incubation was done at 37°C for 60 min. Each filter was then removed, air dried, fixed in methanol, stained with Wright–Giemsa solution, and mounted on a glass slide. The migrating cells that had completely passed through the pores and became attached to the lower surface of the filter were counted in 30 randomly selected oil immersion fields under a microscope at a magnification of 1000. The results were expressed as the number of PMNs/mm^2 of filter surface.

Collection of peritoneal exudative fluid (PEF)

Chitin or chitosan suspension (10 mg/kg body weight) was administered intraperitoneally with an 18-gauge intravenous catheter. After 24 h the PEF was collected through the catheter and the supernatant (SPEF) was obtained by centrifugation at 700 *g* for 10 min at room temperature. Some SPEF samples were heated at 56°C for 30 min (HSPEF).

Analysis of PEF

PEF was analyzed as follows: the quantity was measured in a Messchlinder with macroscopic observation of color and clearness. Specific gravity (SpGr) and total protein (TP) were measured by a protein refractometer. The concentration of LTB_4 in SPEF was also analyzed. The migration of canine PMNs to SPEF and HSPEF was also examined by the blind-well chamber method. PEF sediment was investigated by microscopy. In brief, the sediment smeared on a glass slide was fixed in methanol and stained with Wright–Giemsa solution. The average number of each kind of cell (neutrophil, monocyte, lymphocyte, eosinophil, platelet, erythrocyte, and mesothelial) per three fields on the slide was counted at a magnification of 400 and was expressed as follows: grade I, >500; grade II, 50–500; grade III, <50.

Measurement of LTB_4, LTC_4, and PGE_2

The concentrations of LTB_4, LTC_4, and PGE_2 in NGIS, GIS, LIS, HBIS, SPEF, and HSPEF were measured with enzyme immunoassay kits (Cayman). In brief, after acetylcholinesterase linked to LTB_4 (LTC_4) or PGE_2 was placed into a 96-well plate precoated with a mouse monoclonal antibody or with a goat anti-mouse polyclonal antibody, respectively, specific antiserum to LTB_4 (LTC_4) or PGE_2 was added to each well. After incubation for 18 h, Ellman's reagent was added to each well to develop the plate. The absorbance was measured at 412 nm with a microplate reader (model EAR 340 AT, SLT-Lab-Instruments, Austria).

Standard LTB_4

Standard LTB_4 (Cayman) was diluted with HBSS and adjusted to 0.5–5 ng/mL. Some standard samples were heated at 56°C for 30 min.

Statistical analysis

Statistical analysis was performed by Student's *t* test. Every assay was carried out in duplicate and repeated 3 times.

RESULTS

The effect of NGIS and GIS on canine PMN migration is shown in Figure 1. The migratory activity depended on the number of cells. NGIS and GIS incu-

bated with 1×10^7 cells/mL attracted twofold more canine PMNs compared to incubation with 2×10^6 cells/mL. The migratory activity was similar between NGIS and GIS incubated with 1×10^7 cells/mL, reached a peak at 30 min, and subsequently decreased gradually. The migratory activity of NGIS incubated with 2×10^6 cells/mL reached a peak at 30 min and subsequently decreased gradually; however, that of GIS reached a peak at 30 min and decreased rapidly at 60 min.

Table I shows the concentrations of LTB_4, LTC_4, and PGE_2 in NGIS and GIS incubated with canine PMNs (1×10^7/mL) for 60 min. The LTB_4 level in NGIS and GIS was higher than in LIS and HBIS ($p < 0.01$). The LTC_4 level in GIS was higher than in both NGIS and LIS ($p < 0.01$), which had higher levels than HBIS ($p < 0.05$). The PGE_2 level in NGIS and GIS was higher than in LIS and HBIS ($p < 0.01$).

The time course of changes of the concentrations of LTB_4 and PGE_2 in NGIS and GIS is shown in Figure 2. LTB_4 levels in NGIS and GIS reached a peak at 30 min and subsequently showed a plateau. The LTB_4 level in GIS was significantly greater than in NGIS at 120 min ($p < 0.05$). The changes of PGE_2 in NGIS and GIS were similar to those of LTB_4.

Figure 3 shows the effect of LTB_4 on canine PMN migration. Both 0.5 and 5 ng/mL of LTB_4 attracted significantly more canine PMNs than HBSS. The migratory activity induced by 5 ng/mL LTB_4 was higher than at 0.5 ng/mL. Heated LTB_4 also induced the migration of canine PMNs similar to unheated LTB_4.

TABLE I
Concentrations of LTB_4, LTC_4, and PGE_2 in PMN-Incubated Fluids with Chitin (NGIS), Chitosan (GIS), Latex (LIS), or HBSS (HBIS)

Agents*	LTB_4 (pg/mL)	LTC_4 (pg/mL)	PGE_2 (pg/mL)
NGIS	8300 ± 1500^{a}	$64 \pm 24^{a'}$	$3700 \pm 1000^{a''}$
GIS	12000 ± 2500^{a}	$200 \pm 32^{b'}$	$5200 \pm 3000^{a''}$
LIS	150 ± 20^{b}	$73 \pm 16^{a'}$	$60 \pm 15^{b''}$
HBIS	16 ± 5^{c}	$19 \pm 7^{c'}$	$21 \pm 11^{c''}$

*Chitin, chitosan, or latex beads were suspended in canine PMN suspension at a volume/weight of 1 mg/mL.

Supernatants of canine PMN suspension were incubated with chitin, chitosan, latex, or HBSS. Data are shown as mean ± SD for three experiments. The different superscripts in the figure indicate a significant difference between them: ab, ac, bc, a′b′, a′c′, a″b″, b″c″ ($p < 0.01$).

Chitosan induced 12 ± 8 mL of PEF, but chitin did not induce PEF (Table II). The PEF induced by chitosan was clear and light red in color and had a SpGr and TP content of 1.020 ± 0.002 and 2.5 ± 0.3 g/dL, respectively. The LTB_4 concentration in SPEF was 10 ± 8 ng/mL. The PEF sediment contained many kinds of blood cells and mesothelial cells (Table III). Neutrophils and erythrocytes were observed throughout. Monocytes were observed more frequently than lym-

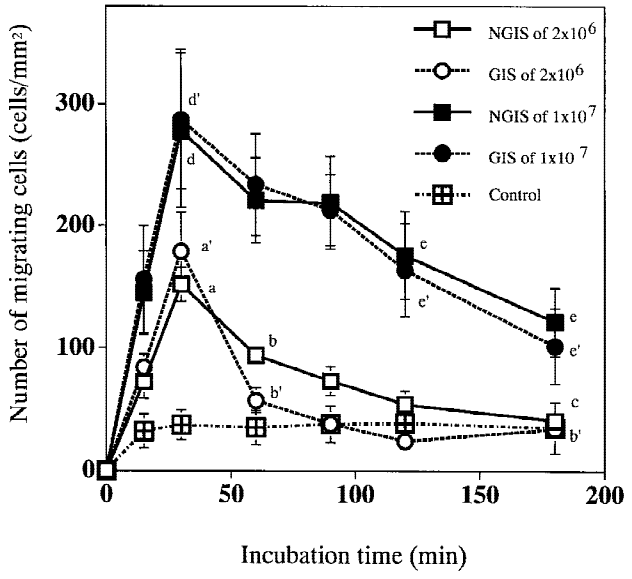

Figure 1. The effects of the supernatant of canine PMNs incubated with chitin and chitosan suspension on their migration. NGIS (GIS) of 2×10^6 (1×10^7) cells/mL; the supernatant of the 2×10^6 (1×10^7) cells/mL of the canine PMN suspension incubated with chitin (chitosan). Control: the supernatant of 1×10^7 cells/mL of canine PMN suspension incubated without chitin (chitosan). Data are shown as mean ± SD for three experiments. The different superscripts in the figure indicate a significant difference between them: ab, a′b′, ac, de, d′e′ ($p < 0.05$).

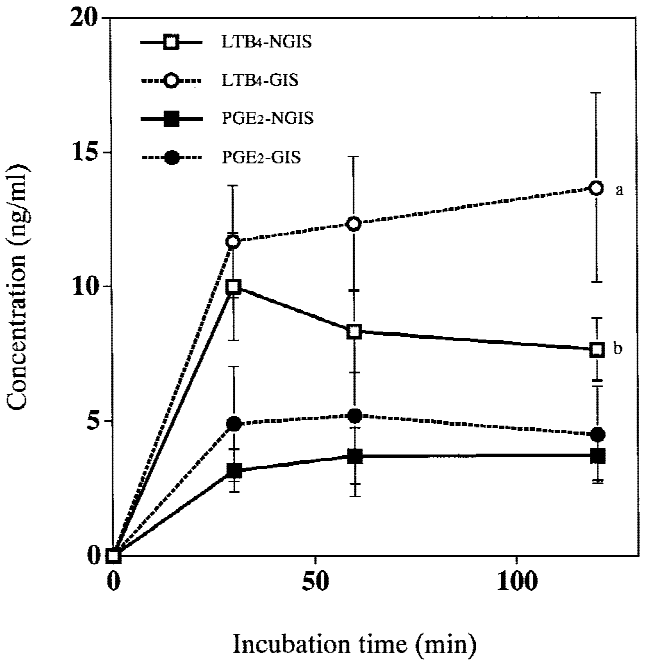

Figure 2. The concentration of LTB_4 and PGE_2 in the supernatant of canine PMNs incubated with chitin and chitosan suspension for 60 min. LTB_4 (PGE_2)-NGIS (GIS): the concentration of LTB_4 (PGE_2) in the NGIS (GIS). The different superscripts in the figure indicate a significant difference between them: ab ($p < 0.05$).

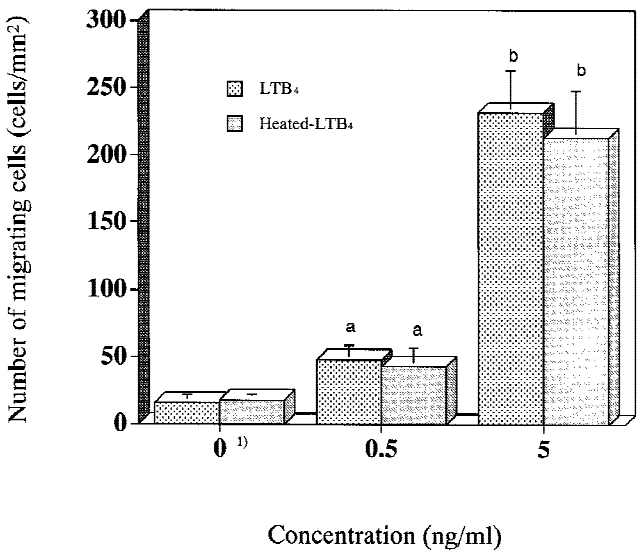

Figure 3. The effect of LTB_4 and heated LTB_4 on canine PMN migration. Data are shown as mean ± SD for three experiments. 1) The control was HBSS. The different superscripts in the figure indicate a significant difference between the control and them: a, $p < 0.05$; b, $p < 0.01$.

phocytes and eosinophils. Figure 4 shows the effect of SPEF on canine PMN migration. From 1 to 100% SPEF induced more migration of canine PMNs than HBSS ($p < 0.01$). HSPEF induced less migration of canine PMNs than SPEF but still had a higher migration than HBSS ($p < 0.01$).

DISCUSSION

The present study showed that the supernatant of canine PMNs incubated with chitin and chitosan (NGIS and GIS) attracted canine PMNs and contained enough LTB_4 to enhance migration *in vitro.* Additionally, the migratory activities induced by NGIS and GIS depended on the number of canine PMNs incubated with chitin and chitosan. PMNs play an important role in host defenses, not only by phagocytosis and bactericidal activity,[25] but also by producing chemical mediators and cytokines (such as LTB_4 and IL-8) when stimulated by invading microorganisms and other chemical mediators.[19,20] Many kinds of stimulants such as opsonized zymosan, lipopolysaccharide (LPS), and tumor necrosis factor cause neutrophils to release LTB_4 *in vitro,*[19,21,26,27] which enhances migration of PMNs. Our results suggest that chitin and chitosan were recognized as stimulants by canine PMNs and stimulated the PMNs to release LTB_4.

NGIS and GIS at 30 min induced the highest PMN migration in both the 2×10^6 and 1×10^7 cells/mL groups, and subsequently the migratory activity decreased. On the other hand, the LTB_4 concentrations in NGIS and GIS were the same as those at 120 min and the concentration of PGE_2, which inhibits the biosynthesis of LTB_4,[28] was not enough to actually inhibit LTB_4 synthesis. These results suggested two reasons for reduced migratory activity: one was that another chemoattractant had been synthesized and metabolized for 120 min; the other was that some inhibitors of canine PMN migration to LTB_4 had appeared in the NGIS and GIS. Other chemoattractants induced by the PMNs may have been phospholipid products such as platelet-activating factor and hydroxyeicosatetraenoic acid (HETE), and the induced cytokine IL-8. However, the effect of phospholipid products on canine PMN migration is regarded as far lower than that of LTB_4 and IL-8. Furthermore, NGIS and GIS obtained at 30 min contained more than 5 ng/mL of LTB_4 and induced the same level of migration as the 5 ng/mL LTB_4 standard. These results suggest that the migrations toward NGIS and GIS were mainly due to LTB_4 and that there were little effects of any other component in the NGIS and GIS on the canine PMNs migration. On the other hand, canine PMNs release some AAPs such as LTD_4, 15-HETE, and PGI_2 that inhibit

TABLE III
Cell Proportion of Sediment in Peritoneal Exudative Fluid Induced by Chitosan Suspension

Cells	Grade
Neutrophil	
Stab cells	III
Segmented cells	I
Monocyte	II
Lymphocyte	III
Eosinophil	III
Platelet	III
Erythrocyte	I
Mesothelial	III

The grades (cells/×400) are I, >500; II, 50–500; III, <50.

TABLE II
Character of Peritoneal Exudative Fluids Induced by Chitin and Chitosan

Sample	Quantity (mL)	Color	Clearness	Sp Gr	TP (g/dL)	LTB_4* (ng/mL)
Chitin	0	ND	ND	ND	ND	ND
Chitosan	12 ± 8	Light red	(+)	1.020 ± 0.002	2.5 ± 0.3	10 ± 8
Saline	0	ND	ND	ND	ND	ND

Sp Gr, specific gravity; TP, total protein; ND, no data.
*The concentration of LTB_4 in SPEF.

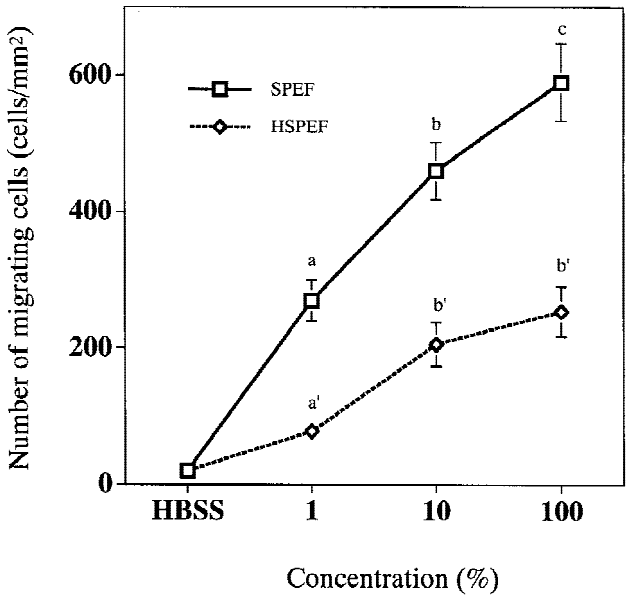

Figure 4. The effect of SPEF induced by chitosan suspension on canine PMN migration. SPEF, supernatant of peritoneal exudative fluid induced by chitosan suspension; HSPEF, heated SPEF (56°C, 30 min). Data are shown as mean ± SD for five experiments. The different superscripts in the figure indicate a significant difference between them: ab, ac, a'b' ($p < 0.01$).

the PMN chemotactic activity and adhesion to endothelial cells.[28–30] Furthermore, the migratory activity to NGIS in the 2×10^6 cells/mL decreased gradually; however, that to GIS decreased rapidly after 30 min of incubation, but that to NGIS and GIS in the 1×10^7 cells/mL groups decreased gradually. These findings imply that there were some inhibitors for the canine PMNs in the NGIS and GIS after 30 min of incubation and chitosan induced more inhibitors than chitin when the rate of the amount of chitin and chitosan mixed with canine PMNs against canine PMNs increased.

Chitosan induced PEF containing a large number of blood cells (PMNs, erythrocytes, and monocytes) while chitin did not. The findings in PEF looked like the early stage of inflammation.[31] Some investigators have reported that chitosan induced severe infiltration of peritoneal macrophages and weight loss with long-term intraperitoneal administration while chitin did not.[32] Components of microorganisms such as LPS and zymosan induce PEF with a large number of inflammatory cells as a result of enhancing complement activities, synthesis of AAPs, and production of cytokines.[21,25,29,33] Our results showed that chitin and chitosan stimulated canine PMNs to release LTB_4 *in vitro,* and the SPEF contained enough LTB_4 to induce canine PMN migration. When NGIS and GIS were incubated for 3 h, however, the GIS contained more LTB_4 than the NGIS. More LTB_4 was produced by human PMNs incubated with blood components than by PMNs alone.[29] On the other hand, GIS contained more LTC_4 than NGIS and LIS. LTC_4 shows many biological activities such as smooth muscle contraction and promoting the secretion of mucus.[29] Furthermore, because HSPEF induced less canine PMN migration than SPEF, SPEF appears to contain some kinds of heat-labile PMN chemoattractants. Recent studies have shown that chitin and chitosan induce fibroblasts to produce IL-8 *in vitro.*[34] These results suggest that one of the reasons chitosan induced PEF and not chitin is due to not only its higher *in vivo* activation of both the peptide–leukotriene pathway and the release of LTB_4 but also the production of heat-labile chemoattractants such as IL-1 and IL-8.

It is unclear why chitosan induced a stronger inflammatory response than chitin when they were administrated to dogs. Canine hemorrhagic pneumonia with numerous PMNs infiltrating the interstitial tissues of the lungs develops after subcutaneous administration of chitosan but not chitin.[35] On the other hand, a few hours after injection of chitin or chitosan, an increase in capillary permeability was observed as leakage of Evans blue.[36] Subcutaneous implantation of chitin induced a considerable amount of fluid at the site.[7] The structural difference between chitin and chitosan is the extent of deacetylation.[10] These results suggest that the stronger inflammatory response to chitosan depends on its amino residues, but more investigations about the effects of these residues in chitin and chitosan is needed.

Our study showed that chitin and chitosan stimulated canine PMNs to release LTB_4 *in vitro* and that chitosan induced PEF that contained enough LTB_4 to enhance canine PMN migration *in vitro.* Recent studies showed that chitin and chitosan induce fibroblasts to produce IL-8 and that oligomers of *N*-acetyl-D-glucosamine and D-glucosamine, which are components of chitin and chitosan, directly enhance canine PMN migration.[11,35] We also reported that chitin and chitosan enhance complement activity.[8] These results indicate that chitin and chitosan attract canine PMNs by at least four pathways *in vivo:* direct attraction, complement activation, arachidonic acid production, and cytokine production. Chitin and chitosan accelerate wound healing in humans and animals.[5,6] Various effects of chitin and chitosan on PMN migration, which is an important step in wound healing, were demonstrated.[8,10,11,34] Although it is clear that chitin and chitosan accelerate wound healing,[2–4] the mechanisms involved were not determined. More investigation of the relationship between PMN migration induced by chitin or chitosan and repair of normal tissue at wound sites is needed.

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