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Effects of chitin and chitosan on blood coagulation

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Abstract

The effects of chitin and chitosan on blood coagulation and platelet aggregation using canine blood were evaluated. Whole blood was mixed with chitin and chitosan suspensions (0.0001–1.0 mg/ml), and then the blood coagulation time (BCT) was measured using the modified Ree-White method. Chitin and chitosan reduced BCT in a dose-dependent manner. Platelet rich plasma (PRP) was mixed with chitin and chitosan suspensions, and then platelet aggregation (PA) was measured using a Dual aggregometer. The PA level induced by chitin was the strongest in all samples including chitosan, cellulose, and latex. When the washed platelet was used, the PA level induced by chitin was similar to that of chitosan, while the rate of coagulation was lower than that of PRP. Chitin and chitosan enhanced the release of the platelet derived growth factor-AB (PDGF-AB) and the transforming growth factor-β1(TGF-β1) from the platelets, particularly, more with chitosan

Keywords: Chitin/chitosan; Blood coagulation; Platelet; Platelet derived growth factor; Transforming growth factor

1. Introduction

The first step in the early wound healing process is hemostasis due to blood coagulation. Platelets are the most important component in blood coagulation. Furthermore, platelets release some cytokines that enhance the healing process during blood coagulation (Beatriz, Porras-Reyes, & Mustoe, 1992). We have investigated the mechanism of wound healing acceleration by chitin and chitosan (Kojima, Okamoto, Miyatake, Kitamura, & Minami, 1998; Kojima et al., 2001; Minami et al., 1996; Minami et al., 1997; Mori et al., 1997; Okamoto et al., 1993a,b; Okamoto et al., 1995a, b; Okamoto et al., 2002; Suzuki et al., 1997; Usami et al., 1994a,b; Usami, Minami, Okamoto, Matsuhashi, & Shigemasa, 1997). However, we could not elucidate all evidence of wound healing acceleration by chitin and chitosan including the effects of blood coagulation. Some reports indicate that chitosan accelerate blood coagulation in vivo (Brandenberg, Leibrock, Shuman, Mallete, & Quigley, 1984; Klokkevold, Lew, Ellis, & Bertolami, 1991; Klokkevold, Subar. Fukuyama, & Bertolami, 1992; Klokkevold, Fukuyama, Sung, & Bertolami, 1999; Malette, Quigley, Gaines, Johnson, & Rainer, 1983). These reports suggest that chitosan influence platelets. To our knowledge, there are no reports regarding the direct effects of chitin and chitosan on platelets.

In this paper, the effects of chitin and chitosan on blood coagulation were evaluated, particularly in the function of platelets.

2. Experimental

2.1. Reagents

Chitin and chitosan: Chitin (MW, 300 kD) and chitosan (MW, 80 kD) were supplied by Sunfive Co., Ltd (Japan). The mean particles of chitin and chitosan were 2.8 and 6.9 μm, 2.8 and 6.2 μm, respectively. They were each sterilized using ethylene oxide gas, and suspended in phosphate-buffered solution (PBS, pH 7.2) at a concentration of 30 mg/ml. The chitin and chitosan had degrees of deacetylation of $<10\%$ and $>80\%$, respectively.

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The molecular weight and degree of deacetylation were determined using the viscosity method (Tokura & Nishi, 1995) and IR method (Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996), respectively.

Other reagents: Latex (Polybead R 2.5%; Solid Latex Polyscienec, Inc., USA; particle size, 2.8 μm) and cellulose (Funakoshi, Co. Ltd, Tokyo; mean particle size, 8.6 μm) were prepared for physical and chemical control, respectively. Cellulose was sterilized using ethylene oxide gas. They were suspended in PBS at a concentration of 20 mg/ml.

Each sample was adjusted to 0.0001–20 mg/ml with PBS before use.

2.2. Blood coagulation time (BCT)

BCT was measured using the modified Ree-White method (Lee & White, 1913). In this study, we used chitin and chitosan with the mean particle of 2.8 μm. In brief, we prepared 5-ml disposable syringes containing 0.2 ml of each sample. Blood (1.8 ml) was collected using the prepared syringes with 21-gauge needles from the cephalic vein. The blood was transferred to 2 glass tubes (1 cm in diameter, 10 cm in length) at a volume of 1 ml each other, which were previously incubated in a water bath at 37 °C. The tubes were kept in the water bath for 5 min, then one tube was picked up and the blood coagulation was observed by inclining the tube at 30 s intervals. We justified the blood coagulation when blood flow was not observed even if the tube was inclined at a 90° angle. We checked the remaining tube in a similar manner. BCT was justified as the time from the start of the blood collection until blood coagulation in the second tube.

2.3. Platelet aggregation (PA)

Preparation of platelet rich plasma (PRP): We preloaded a 20-ml disposable syringe with 2 ml of 3.8% sodium citrate. Eighteen ml of blood was collected using the prepared syringe with a 18-gauge needle from the jugular vein. The blood was transferred to a 50-ml plastic tube, and then centrifuged at 200 × *g* for 15 min. The supernatant was harvested. The supernatant was named PRP. The tube was centrifuged at 1600 × *g* for 15 min again, then the supernatant was harvested. This supernatant was named platelet poor plasma (PPP). The number of platelets in the PRP was adjusted to 30×10^4 cells/ml with the PPP.

Preparation of the washed platelet: A 20-ml disposable syringe containing 2 ml of acid-citrated dextrose solution was prepared. Eighteen ml of blood was collected using the prepared syringe with 18-gauge needle from the jugular vein. The blood was transferred to a 50-ml plastic tube, and then centrifuged at 200 × *g* for 15 min. The supernatant was harvested and then centrifuged at 500 × *g* for 15 min again. The supernatant was removed, and then the same volume of calcium-free modified Tyrode's buffer was added and suspended. The tube was centrifuged again under the same condition. This procedure was performed 2 times. Finally, the number of platelets was adjusted to 15×10^4 cells/ml with a modified Tyrode's buffer.

Measurement of PA: Four hundred and fifty μl of the adjusted PRP and washed platelets suspensions were transferred into tubes (4 mm in diameter, 5 cm in length) which were previously incubated in a water bath at 37 °C. Fifty μl of each sample was added to the tubes and the light penetration was measured the using dual aggrerometer (AHS Japan, Co. Ltd, Tokyo) for 15 min. The degree of light penetration was used as a platelet aggregation index (PA ratio).

2.4. Scanning electron microscopy

After measurement of PA with PRP, 10 μl of each of the aggregated platelets suspensions were dropped on filter paper (No.1, Advantec, Co. Ltd, Tokyo, Japan). The filter paper was fixed with 0.1 M phosphate-buffered solution contained 2.5% glutaraldehade and 2.0% paraformaldehayde (pH 7.4), and postfixed with 1% osmium tetroxide in the same buffer. Samples were dehydrated in a graded series of ethanol, immersed in *t*-butyl alcohol and freeze-dried. Dried samples were coated with plantinum using an ion-sputtering coater, and observed with a field emission scanning electron microscope X-650 (Hitachi, Tokyo, Japan).

2.5. Release of cytokine from platelet

After the PRP was mixed with each sample for 15 min, the tubes were centrifuged at 1000 × *g* for 10 min. The supernatants were harvested and then centrifuged again at

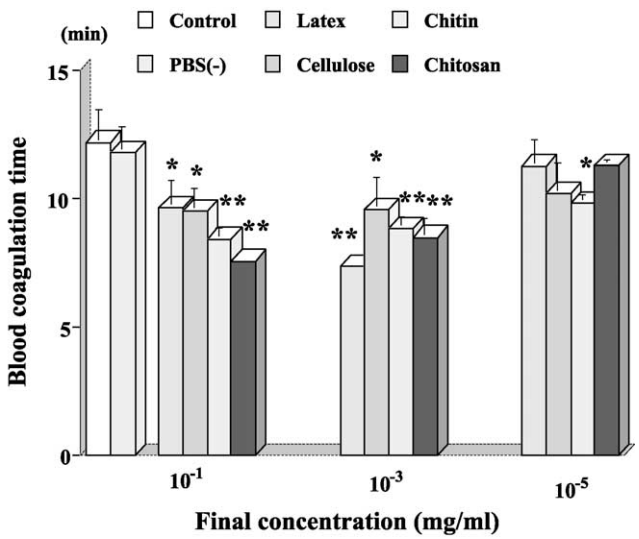

Fig. 1. Effect of chitin and chitosan on blood coagulation time. Chitin and chitosan with particle size of 2.8 μm were used. *: $p < 0.05$, **: $p < 0.01$ vs. Control.

Table 1
Platelets aggregation (PA) ratio in the presence of plasma

Final concentration of samples (mg/ml)	Platelet aggregation (PA) ratio (%)					
	Cellulose	Latex	Chitin (2.8 μm)	Chitin (6.9 μm)	Chitosan (2.8 μm)	Chitosan (6.2 μm)
0.01	1.0[a]	0.3[a]	4.8[ae]	3.7[ab]	1.0[a]	0.9[a]
0.03	2.6[ab]	0.7[a]	9.8[abe]	7.0[b]	4.0[b]	1.3[a]
0.1	6.5[b]	19.7[b]	19.8[be]	18.5[c]	9.9[c]	6.8[bc]
0.3	19.7[c]	26.3[b]	46.2[c]	32.3[de]	15.3[d]	16.5[d]
1	41.6[d]	23.7[b]	57.3[d]	61.2[f]	20.3[e]	27.9[e]
2	50.0[e]	7.0[a]	14.3[e]	29.0[e]	1.0[a]	5.8[b]
PBS(−)	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]

*Different superscript (a, b, c, d, e) in each column shows significant difference ($p < 0.05$). **There was no significant difference between particle size and PA ratio in the chitin and chitosan groups.

10,000 × *g* for 10 min. The platelet derived growth factor AB (PDGF-AB) and the transforming growth factor β1 (TGF-β1) in the supernatants were measured using EIA kit (R D Systems, Inc., USA).

2.6. Statistical analysis

A statistical analysis was performed using Student's *t*-test for 2 groups and Fisher's PLSD for multiple groups.

3. Results and discussion

3.1. Effect of chitin and chitosan on blood coagulation time (BCT)

The effect of the samples on BCT is shown in Fig. 1. Chitin and chitosan reduced BCT significantly in a dose-dependent manner. In the final concentration of 0.1 mg/ml, BCTs in the chitin and chitosan groups shortened by 3.7 and 4.7 min, respectively (control BCT was 12 min). Latex, which was used as a physical control, also reduced BCT significantly but not in a dose-dependent manner. Cellulose, which was used as a chemical control, also reduced BCT. However, the effect of cellulose was weak compared to those of chitin and chitosan.

Regarding the effects of chitosan on hemostasis, there are some reports of an in vivo experiment (Brandenberg et al., 1984; Klokkevold et al., 1991; Klokkevold et al., 1992; Klokkevold et al., 1999; Malette et al., 1983). They reported that chitosan enhanced hemostasis. The present in vitro result of chitosan was consistent with previous in vivo results. To our knowledge, there is no report about the effects of chitin on hemostasis. The present study indicated that chitin also has a potency of hemostasis as that of chitosan. Cellulose and latex at higher concentrations also reduced BCT, however, chitin and chitosan were found to be stronger than those materials. This suggests that the effects of chitin and chitosan on hemostasis are not only due to a physical effect, but also are related to their chemical structure, particularly amino residue.

In blood coagulation, chitosan was found to be more effective than chitin, while chitin was found to aggregate platelets more than chitosan. This means that the platelets aggregation does not directly reflect blood coagulation. Klokkevold et al. (1999) report indicates that chitosan shows high-compatibility against erythrocytes under the morphological observation. Rao and Sharma (1997) suggests that chitosan influences erythrocytes directly and induces their aggregation. Therefore, the shortening of BCT by chitosan may be related to not only platelets aggregation, but also erythrocyte aggregation.

3.2. Effect of chitin and chitosan on platelet aggregation (PA)

Table 1 shows the PA ratio in the presence of plasma. The PA ratios increased until at the concentration of 1 mg/ml, but decreased at 2 mg/ml in all groups except for the cellulose group. The PA ratio in the chitin group was the highest in all groups. Also, compared to that of the control (PBS), the PA ratios in all samples were significantly

Table 2
Platelets aggregation (PA) ratio in the absence of plasma

Samples (0.3 mg/ml)	Platelets aggregation (PA) ratio (%)
Chitin (2.8 μm)	14.0[b]
Chitin (6.9 μm)	9.6[b]
Chitosan (2.8 μm)	14.2[b]
Chitosan (6.2 μm)	14.2[b]
Cellulose	1.0[c]
Latex	40.8[a]
PBS(−)	0[c]

*Different superscript (a, b, c) shows significant difference ($p < 0.05$). **There was no significant difference between particle size and PA ratio in the chitin and chitosan groups.

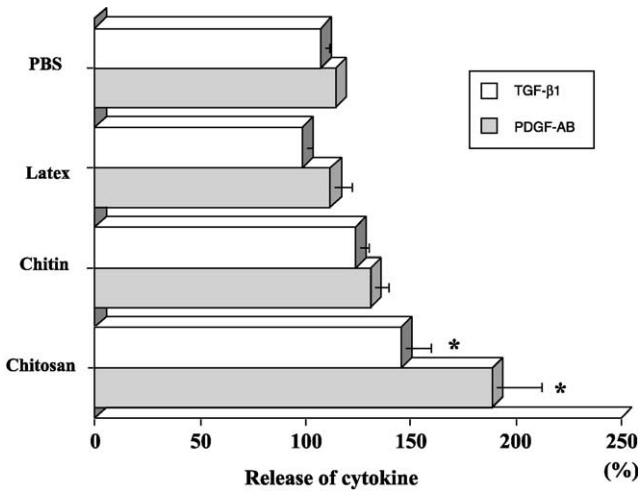

Fig. 2. Release of cytokine from platelet. *: $p < 0.05$ vs. PBS.

increased. There was no significant difference between the particle size and the PA ratio in the chitin and chitosan groups.

Table 2 shows the PA ratio in the absence of plasma. The PA ratios in the chitin and chitosan groups were weak compared to those in the presence of plasma. Also, the PA ratio in the latex group was the highest in all groups. Furthermore, the PA ratios in the chitin and chitosan groups were the same, while PA was not observed in the cellulose group.

Chitin and chitosan were found to aggregate platelets. Chitin induced PA strongly in the presence of plasma compared to chitosan, while the PA of both chitin and chitosan were similar when the washed platelets were used. In addition, PA of the washed platelets was weaker than that in the presence of plasma. This evidence suggests that chitin and chitosan directly influence platelets themselves and this effect was enhanced in the presence of plasma. This effect was found to be not due to a physical effect because cellulose did not induced PA when the washed platelets were used.

3.3. Morphology of aggregated platelets

Fig. 2 shows the morphology of the aggregated platelets in each sample in the presence of plasma. In the chitin and chitosan groups, platelets adhered strongly on the surface of chitin and chitosan particles with an elongated process. Furthermore, platelets were bound to each other and formed the aggregated mass. These phenomena were prominent in the chitosan group. In the latex group, platelets adhered on the surface of the latex beads spread out. It was observed that the latex beads were bound to each other by elongated process of platelets. In the cellulose group, platelets adhered to the cellulose particles with the elongated process. In the absence of plasma, it was observed that platelets adhered to the surface of chitin and chitosan particles and the latex beads with their elongated process, but they did not adhere to the surface of cellulose particles.

From these results, the platelets were found to adhere strongly to chitin and chitosan particles. Furthermore, this effect enhanced in the presence of plasma. This result is

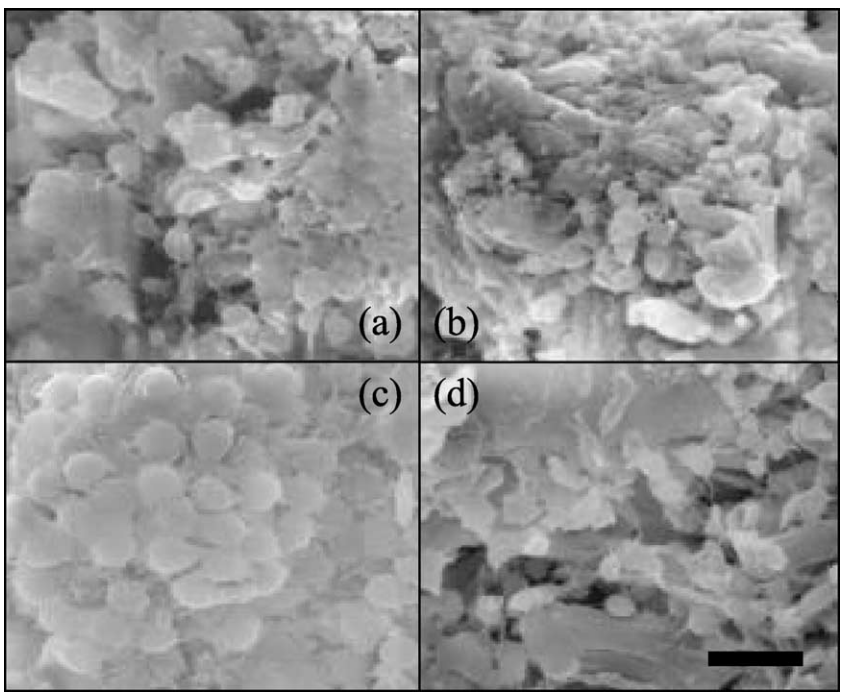

Fig. 3. Morphology of platelet aggregation. (a) Chitin (6.9 μm), (b) chitosan (6.2 μm), (c) latex, (d) cellulose. After measurement of platelet aggregation (PA) with PRP, each sample was made. bar = 5 μm.

consistent with the data of PA. Chitin and chitosan have amino residue in their chemical structure, but cellulose did not although another chemical structure is the same. This suggests that amino residue is important in platelets aggregation.

3.4. Release of cytokine from platelets

Fig. 3 shows the release of PDGF-AB and TGF-β1 from platelets, respectively. The levels of PDGF-AB in the chitin and chitosan groups were increased to 130 and 190%, respectively, while the level in the latex group was 112% (the level before addition of the samples was 100%). The level of PDGF-AB in the chitosan group was significantly higher than that of the control (PBS alone addition). The levels of TGF-β1 in the chitin and chitosan groups were increased to 123 and 146%, respectively, while the level in the latex group did not change. The level of TGF-β1 in the chitosan group was significantly higher than that of the control (PBS alone addition).

The present results indicate that chitin and chitosan enhance the release of PDGF-AB and TGF-β1 from platelets. Some cytokines including PDGF-AB and TGF-β1 play important roles on the wound healing process (Clark & Denver, 1985). PDGF-AB and TGF-β1 retract inflammatory cells (Beatriz et al., 1992; Deuel, Senior, Huang, & Griffin, 1982), which suggests that they enhance the early phase of the wound healing process. In the present study, chitosan was found to induce PDGF-AB and TGF-β1 strongly compared to chitin. This evidence cannot be explained exactly. One possible answer is the following: Chitosan makes platelets aggregate strongly compared to chitin, which suggests that chitosan influences the membranes of platelets and subsequently damage their membranes. Therefore, it may be that degree of cytokine release depends on the degree of the membrane injury in platelets. At this point, further study is necessary.

4. Conclusion

Chitin and chitosan was found to enhance blood coagulation. This phenomenon was be more effective in chitosan than chitin. On the other hand, chitin was found to aggregate platelets more than chitosan. This means that the platelets aggregation does not directly reflect blood coagulation. Therefore, the shortening of BCT by chitosan may be related to not only platelets aggregation, but also erythrocyte aggregation. The present results also indicate that chitin and chitosan enhance the release of PDGF-AB and TGF-β1 which play important roles on the wound healing process. These results support combined use of chitin and chitosan for biomedical application, especially wound treatment including hemostasis.

References

Brandenberg, G., Leibrock, L. G., Shuman, R., Mallete, W. G., & Quigley, H. (1984). Chitosan: A new topical hemostatic agent for diffuse capillary bleeding in brain tissue. *Neurosurgery*, *15*, 9–13.

Clark, R. A. F., & Denver, M. D. (1985). Cutaneous tissue repair: Basic biologic considerations. *Journal of American Academic Dermatology*, *13*, 701–725.

Deuel, T. F., Senior, R. M., Huang, J. S., & Griffin, G. L. (1982). Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. *Journal of Clinical Investigation*, *69*, 1046–1049.

Klokkevold, P. R., Fukuyama, H., Sung, E. C., & Bertolami, C. N. (1999). The effects of chitosan (poly-*N*-acetyl glucosamine) on lingual hemostasis in heparinized rabbits. *Journal of Oral Maxillofacial Surgery*, *57*, 49–52.

Klokkevold, P. R., Lew, D. S., Ellis, D. G., & Bertolami, C. N. (1991). Effects of chitosan on lingual hemostasis in rabbits. *Journal of Oral Maxillofacial Surgery*, *49*, 858–863.

Klokkevold, P. R., Subar, P., Fukuyama, H., & Bertolami, C. N. (1992). Effect of chitosan on lingual hemostasis in rabbits with platelet dysfunction induced by epoprostenol. *Journal of Oral Maxillofacial Surgery*, *50*, 41–45.

Kojima, K., Okamoto, Y., Miyatake, K., Kitamura, Y., & Minami, S. (1998). Collagen typing of granulation tissue induced by chitin and chitosan. *Carbohydrate Polymers*, *37*, 109–113.

Kojima, K., Okamoto, Y., Miyatake, K., Tamai, Y., Shigemasa, Y., & Minami, S. (2001). Optimum dose of chitin and chitosan for organization of non-woven fabric in the subcutaneous tissue. *Carbohydrate Polymers*, *46*, 235–239.

Lee, R. I., & White, P. D. (1913). A clinical study of the coagulation time of blood. *American Journal of Medical Science*, *145*, 495–503.

Malette, W. G., Quigley, H. J., Gaines, R. D., Johnson, N. D., & Rainer, W. G. (1983). Chitosan: A new hemostatic. *Annual Thoracic Surgery*, *36*, 55–58.

Minami, S., Masuda, M., Suzuki, H., Okamoto, Y., Matsuhashi, A., Kato, K., & Shigemasa, Y. (1997). Subcutaneous injected chitosan induces systemic activation in dogs. *Carbohydrate Polymers*, *33*, 285–294.

Minami, S., Okamoto, Y., Miyatake, K., Matsuhashi, A., Kitamura, Y., Tanigawa, T., Tanaka, Y., & Shigemasa, Y. (1996). Chitin induces type IV collagen and elastic fiber in implanted non-woven fabric of polyester. *Carbohydrate Polymers*, *29*, 295–299.

Mori, T., Okumura, M., Matsuura, M., Ueno, K., Tokura, S., Okamoto, Y., Minami, S., & Fujinaga, T. (1997). Effect of chitin and its derivatives on the proliferation and cytokine production of fibroblasts in vitro. *Biomaterials*, *18*, 947–951.

Okamoto, Y., Minami, S., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., Tanigawa, T., Tanaka, Y., & Tokura, S. (1993a). Polymeric *N*-acetyl-D-glucosamine (chitin) induces histionic activation in dogs. *Journal of Veterinary Medical Science*, *55*, 739–742.

Okamoto, Y., Minami, S., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., Tanigawa, T., Tanaka, Y., & Tokura, S. (1993b). Application of polymeric *N*-acetyl-D-glucosamine (chitin) to veterinary practice. *Journal of Veterinary Medical Science*, *55*, 743–747.

Okamoto, Y., Tomita, T., Minami, S., Matsuhashi, A., Kumazawa, N. H., Tanioka, S., & Shigemasa, Y. (1995a). Effects of chitosan on experimental abscess with *Staphylococcus aureus* in dogs. *Journal of Veterinary Medical Science*, *57*, 765–767.

Okamoto, Y., Shibazaki, K., Minami, S., Matsuhashi, A., Kumazawa, N. H., Tanioka, S., & Shigemasa, Y. (1995b). Evaluation of chitin and chitosan on open wound healing in dogs. *Journal of Veterinary Medical Science*, *57*, 851–854.

Okamoto, Y., Watanabe, M., Miyatake, K., Morimoto, M., Shigemasa, Y., & Minami, S. (2002). Effects of chitin/chitosan and their oligomers/

monomers on migrations of fibroblasts and vascular endothelium. *Biomaterials*, *23*, 1975–1979.

Porres-Reyes, B. H., & Mustoe, T. A. (1992). Platelet-activating factor: Improvement in wound healing by chemotactic factor. *Surgery*, *111*, 416–423.

Rao, S. B., & Sharma, C. P. (1997). Use of chitosan as biomaterial: Studies on its safety and hemostatic potential. *Journal of Biomedical Materials Research*, *34*, 21–28.

Shigemasa, Y., Matsuura, H., Sashiwa, H., & Saimoto, H. (1996). Evaluation of different absorbance ratios from infrared spectroscopy for analyzing the degree of deacetylation in chitin. *International Journal of Biological Macromolecules*, *18*, 237–242.

Suzuki, Y., Okamoto, Y., Morimoto, M., Sashiwa, H., Saimoto, H., Tanioka, S., Shigemasa, Y., & Minami, S. (1997). Influence of physiochemical properties of chitin and chitosan on complement activation. *Carbohydrate Polymers*, *42*, 307–310.

Tokura, S., & Nishi, N. (1995). Specification and characterization of chitin and chitosan. In M. B. Zaria, W. M. Wan Muda, & Md. P. Abdullah (Eds.), *Chitin and Chitosan* (pp. 67–86). Malysia: Penerbit Universiti Kebangsaan.

Usami, Y., Minami, S., Okamoto, Y., Matsuhashi, A., & Shigemasa, Y. (1997). Influence of chain length of *N*-acetyl-D-glucosamine and D-glucosamine residues on direct and complement-mediated chemotactic activities for canine polymorphonuclear cells. *Carbohydrate Polymers*, *32*, 115–122.

Usami, Y., Okamoto, Y., Minami, S., Matsuhashi, A., Kumazawa, N. H., Tanioka, S., & Shigemasa, Y. (1994a). Chitin and chitosan induce migration of bovine polymorphonuclear cells. *Journal of Veterinary Medical Science*, *56*, 761–762.

Usami, Y., Okamoto, Y., Minami, S., Matsuhashi, A., Kumazawa, N. H., Tanioka, S., & Shigemasa, Y. (1994b). Migration of canine neutrophils to chitin and chitosan. *Journal of Veterinary Medical Science*, *56*, 1215–1216.