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Optimum dose of chitin and chitosan for organization of non-woven fabric in the subcutaneous tissue

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Abstract

This study was performed to evaluate tissue reactions induced in rats by polyester non-woven fabric (NWF, 1×1 cm^2, 0.6 mm thick) impregnated with chitin or chitosan suspension ranging from 0.1 to 50 mg/ml (chitin-NWF, chitosan-NWF) and NWF impregnated with phosphated buffer solution (control). Evaluations were based on macroscopic and microscopic observations, and image analysis of histological samples stained with Alcian blue, Safranin O, and Masson trichrome for identifying glycosaminoglycan, proteoglycan, and collagen, respectively. Macroscopically, the NWFs impregnated with higher concentrations of chitin (50 mg/ml) and chitosan (10 mg/ml) suspensions evoked more strong reactions than those impregnated with weaker concentrations of chitin (1.0 and 10 mg/ml) and chitosan (0.1 and 1.0 mg/ml) suspensions and were characterized by thickening of the NWF at Day 7 of the implantation. There was swelling and accumulation of exudates in the NWF impregnated with 50 and 10 mg/ml of chitin and chitosan suspensions, respectively. However, in microscopic examination, organization of the NWF implants was found to be superior in the NWF impregnated with weaker suspensions of chitin and chitosan. The chitin groups at higher concentrations showed higher value than the controls in the sections prepared with Alcian blue staining, but lower values in the sections prepared by Safranin O staining. In the Masson's trichrome staining, the chitin groups at lower concentrations showed lower values than the control. The chitosan groups were similar to the control in all special stainings.

Keywords: Chitin; Chitosan; Tissue reaction; Rats

1. Introduction

Chitin and chitosan, a pair of polysaccharides have been widely studied in engineering and medicine. In the field of medicine, the effects of chitin and chitosan on wound healing have been the focus of great attention since Prudden et al. published a paper on the topic in 1970 (Prudden, Migel, Hanson, Friedlich & Ballassa, 1970). Numerous reports describe the advantage of chitin and chitosan on tissue reaction (Allan, Altman, Bensinger, Ghosh, Hirabayasi & Neogi, 1984; Ballasa & Prudden, 1978; Minami, Okamoto, Miyatake, Matsuhashi, Kitamura, Tanigawa et al., 1996b; Minami, Okamoto, Tanioka, Sashiwa, Saimoto, Matsuhashi et al., 1993; Okamoto, Minami, Matsuhashi, Sashiwa, Saimoto, Shigemasa et al., 1993a; Okamoto, Minami, Matsuhashi, Shigemasa, Saimoto, Tanigawa et al., 1993b; Okamoto, Shibazaki, Minami, Matsuhashi, Tanioka & Shigemasa, 1995; Okamoto, Southwood, Stashak, Norrdin, Nelson, Minami et al., 1997). Recently, Minami, Oh-oka, Miyatake, Matsuhashi, Shigemasa and Fukumoto (1996a) indicated that large amounts of chitosan cause pneumonia in dogs. We also clinically encountered the development of excessive granulation tissue and accumulation of exudate when large amounts of chitin and chitosan were administered in subcutaneous tissue. While these phenomena suggest that the tissue reaction to chitin and chitosan may be influenced by the volume or concentration of these materials, there have been no reports on this potential influence. Though some papers describe the difference between tissue reactions to chitin and chitosan under the same condition in vitro (Minami, Mura-e, Okamoto, Sanekata, Matsuhashi, Tanioka et al., 1997; Suzuki, Okamoto, Morimoto, Sashiwa, Saimoto, Shigemasa et al., 2000; Usami, Okamoto, Minami, Matsuhashi, Kumazawa, Tanioka et al., 1994a,b; Usami, Okamoto, Takayama, Shigemasa & Minami, 1998a,b), only two papers describe this difference in vivo (Kojima, Okamoto, Miyatake, Kitamura & Minami, 1998; Okamoto et al., 1995).

In this paper, we investigated the optimum dose of chitin and chitosan in the organization of non-woven fabric (NWF) in vivo.

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2. Materials and methods

2.1. Animals

Eight Wistar rats (adult female, weight 270–300 g) were used for this study. The animals were purchased from Hamaguti Laboratory Co., Ltd. (Japan).

2.2. Reagents

Chitin and chitosan were supplied by Sunfive Co., Ltd. (Tottori, Japan). Chitin (MW: 300 kD) and chitosan (MW: 80 kD) with a mean particle size of 3.5 μm were each sterilized using ethylene oxide and suspended in phosphate buffered solution (PBS, pH 7.2) at a concentration of 10 mg/ml. These agents showed <10 and $>80\%$ deacetylation, respectively. The molecular weight and deacetylation were determined by viscosity method (Tokura & Nishi, 1995) and IR method (Shigemasa, Matsuura, Sashiwa & Saimoto, 1996), respectively. Both chitin and chitosan suspensions were adjusted with PBS to final concentrations of 1.0, 10 and 50 mg/ml, and 0.1, 1.0 and 10 mg/ml, respectively, before use.

2.3. Preparation of implants

A polyester NWF (Sontara 8100, Dupont, USA, $1.0 \times 1.0\ cm^2$, 0.6 mm thick) was sterilized by ethylene oxide gas using automatic sterilizer (Semmel-706, Ikiken, Co. Ltd., Japan), and then was impregnated with 0.1 ml of chitin suspension (1.0, 10 and 50 mg/ml) or chitosan suspension (0.1, 1.0 and 10 mg/ml). For a control, NWF was impregnated with 0.1 ml of PBS alone. The NWF impregnated with the 0.1 mg/ml chitin suspension was named the 0.1 mg chitin-NWF, and the other NWFs impregnated with chitin and chitosan suspensions at different concentrations were named accordingly.

2.4. Experimental design

The rats were divided into chitin and chitosan groups ($n = 4$ each). After induction of anesthesia with intramuscular injection of atropine sulfate (0.05 mg/kg), ketamine-HCl (15 mg/kg), and proponyl promadine (0.05 mg/kg), the dorsal supracostal region was shaved and disinfected by chlorhexidine gluconate. Each rat received a straight full-thickness skin incision extending about 10 cm over the dorsal midline. In the first rat in the chitin group, the 1 mg chitin-NWF, 10 mg chitin-NWF, 50 mg chitin-NWF, and NWF impregnated with PBS were placed at cranial-right, caudal-right, caudal-left, and cranial-left sites, respectively. The implants on the right and left sides were placed at a distance of 2 cm from each other, and the implants at the cranial and caudal sides were placed at a distance of 3 cm (Fig. 1). In the second rat, the four different NWFs were placed at the caudal-right, caudal-left, cranial-left, and cranial-right sites, respectively. In the third rat, the materials were placed at the caudal-left, cranial-left, cranial-right, and caudal-right sites, respectively. In the fourth rat, the materials were placed at the cranial-left, cranial-right, caudal-right, and caudal-left sites, respectively. The same arrangement was performed in the chitosan group. The implants were fixed at each corner by interrupted sutures with 3-0 nylon, and then the skin was surgically closed by interrupted sutures with 0.15 mm diameter wire at intervals of 1 cm.

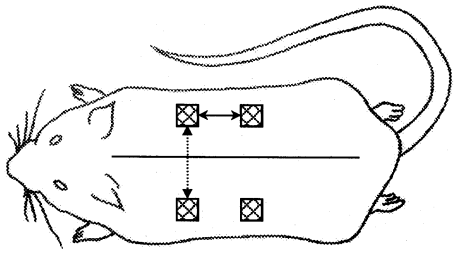

Fig. 1. Three implants ($1 \times 1\ cm^2$, 0.6 mm thick) were placed subcutaneously on each side of the dorsal midline at an interval of 2 cm (solid line arrow). At each site, there is at a distance of 3 cm between them (broken line arrow).

2.5. Macroscopic and microscopic observations

After euthanasia at 7 days post implantation, each implant was taken from the wound with surrounding tissues, observed macroscopically, and then fixed in 10% phosphate-buffered formalin. The samples were dehydrated, embedded in paraffin, sectioned into 3–4 μm thick specimens, and stained with hematoxylin and eosin.

2.6. Image analysis

Specimens stained with Alcian blue, Safranin O, and Masson's trichrome were used for quantitative evaluations of glycosaminoglycan (GAG), proteoglycan (PG), and collagen. At $20\times$ magnification, 5 fields in the implants were randomly selected for each group, the fields were photographed by a Fujix HC-300 Digital Camera (Fuji Photo Film Co, Ltd., Tokyo) and uploaded to a computer using Photograb™-300 SH-3 software (Version 1.0 for Windows and Macintosh, Fuji Photo Film Co. Ltd., Tokyo), and then the images were digitized by using Adobe Photoshop 5.0 (Macintosh software, Adobe System, Tokyo). Next, the proportion of pixels showing the desired hue within a total of 100,000 pixels (random sampling of 20,000 pixels at 5 fields) was calculated through an image processing technique. The value obtained were tested by a statistical processing method (Duncan's multiple range test) with p-values below 0.05 regarded as statistically significant.

Table 1
Summary of histological finding (±, slight; +, moderate; ++, severe; +++, more severe)

Agent	Concentration (mg/ml)	Granulation tissue in implant	Granulation tissue surrounding implant	Inflammatory cells	Giant cells
Control	–	+	±	±	±
Chitin	1	++	±	+	+
	10	+++	+	+	++
	50	+	+++	+++	+
Chitosan	0.1	++	±	+	+
	1	++	++	+	+
	10	+	+++	+++	±

3. Results

3.1. Macroscopic findings

In the 1.0 and 10 mg chitin groups, there were no abnormal changes in the skin above the implants. The surfaces of these two NWFs and the control NWF were covered with thin granulation tissue. In the 50 mg chitin-NWF, however, swelling and accumulation of exudates were observed. The surface of the 0.1 mg chitosan-NWF was covered with thin granulation tissues without inflammation, while slight exudate and swelling were observed in the tissue covering the 1.0 mg chitosan-NWF implant. In the 10 mg chitosan group, granulation tissues surrounding the implant were thick and rough with severe exudates as well as in the 50 mg chitinf-NWF.

3.2. Histological findings

Histological findings are summarized in Table 1. The tissue reaction was evaluated at the inside and outside of the implants. In the 1.0 and 10 mg chitin-NWFs, the organization of tissues (Fig. 2) was clearly superior to that in the control group (Fig. 3). In the 10 mg chitin group, the appearance of giant cells and organization were prominent all over the implant. The tissue surrounding 50 mg chitin-NWF was organized only slightly compared with that surrounding the 10 mg chitin-NWF, and the influx of many inflammatory cells was observed in the implant (Fig. 4). Histological findings in the 0.1 and 1.0 chitosan-NWFs were similar to those in the 1.0 and 10 mg chitin-NWFs. However, the influx of the inflammatory cells was prominent in the 10 mg chitosan-NWF, and the degree of tissue organization was slight in both the 10 mg chitosan-NWF and 50 mg chitin-NWF.

3.3. Image analysis

Table 2 shows an image analysis of the special staining. In the Alcian blue staining, the chitin groups at higher concentrations showed higher values than the control ($p < 0.05$). In the Safranin O staining, the chitin groups showed lower values than the control ($p < 0.05$). In the Masson's trichrome staining, the chitin groups at lower concentrations showed lower values than the control. The chitosan groups were similar to the control in all special stainings.

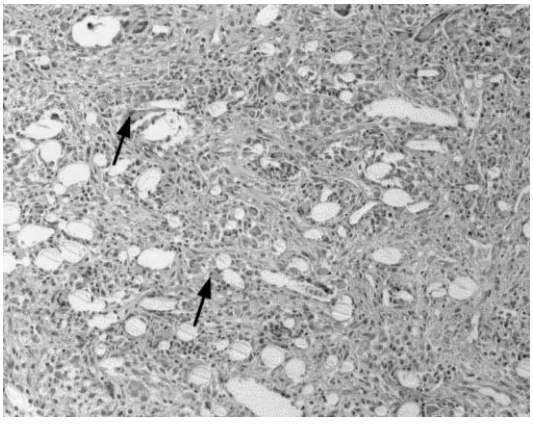

Fig. 2. 10 mg chitin-NWF. The implant was organized almost completely. Many giant cells also observed (arrows). Hematoxylin eosin staining (×200).

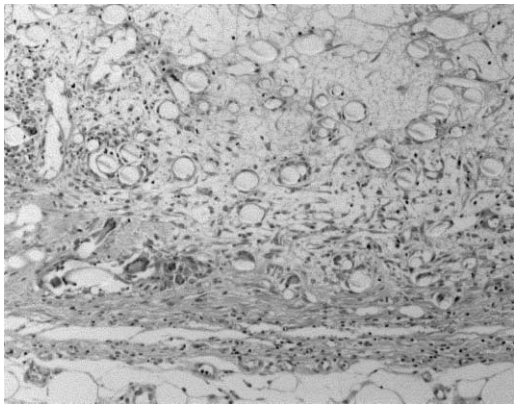

Fig. 3. Control NWF. The organization of the implant was observed at the margin area, but not at the central area. Hematoxylin eosin staining (200×).

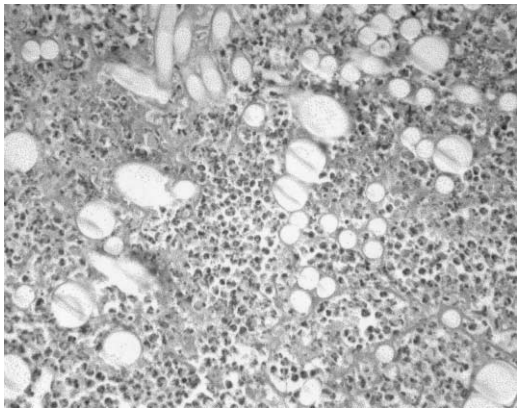

Fig. 4. 50 mg chitin-NWF. Numerous inflammatory cell influx was observed in the implants. There is no organization of the implants. Hematoxylin eosin staining (200×).

4. Discussion

In the present study, suitable levels of chitin and chitosan for organization of the implants in the rat were found to be 1.0–10 mg/ml and 0.1–1.0 mg/ml, respectively. Furthermore, excessive levels of chitin and chitosan were found to induce excessive inflammatory reaction and lead to reduce formation of granulation tissue. This phenomenon is consistent with our previous reports, which showed that large amounts of chitosan induce hemorrhagic pneumonia (Minami et al., 1996a) and small amounts of chitosan activate peripheral polymorphonuclear cells (Minami et al., 1997). We also have found clinically that excessive administration of chitin and chitosan induces excessive formation of granulation tissue and inflammatory reactions such as swelling and exudate. These tendencies are especially prominent when the chitin and chitosan agents are buried in subcutaneous tissue. These clinical events also are consistent with the present results.

The histological reactions of chitosan at the concentrations of 0.1 and 1.0 mg/ml were similar to those of chitin at the concentrations of 1.0 and 10 mg/ml, and the same similarity was seen between the 50 mg chitin and 10 mg chitosan groups. These relative values suggest that chitosan influences histological reaction about 5–10 times more strongly than chitin. To our knowledge, there is no report that evaluated the histological reaction of chitin and chitosan under the same condition in vivo. Minami, Suzuki, Okamoto, Fujinaga and Shigemasa (1998) reported similar events on induction of comlement component 3 activity in vitro. The present result indicates that chitosan influences the tissue reaction more than chitin in vivo as well as in vitro. In a further study, we investigated the effects of 0.1 mg of chitin and 0.01 mg of chitosan suspensions, i.e. suspensions of lower concentrations than those investigated in the study reported here. As a result, these materials had no affect on the organization of the implants.

In image analysis of specimens stained with Masson's trichrome, chitin was found to influence the synthesis of collagen. Our present results support our previous reports, which found no scar formation or excessive granulation tissues in the presence of chitin in vivo (Okamoto et al., 1993b, 1995). The present results also indicate that chitosan has no significant influence on collagen synthesis. Though no reports have described any effect of chitosan influence on collagen synthesis, chitosan is known to induce granulation tissue formation in vivo (Minami et al., 1993). Our data from the present study offers no exact explanation of this paradox. It is possible that another mechanism of granulation tissue formation may be at work in the presence of chitosan. Further study is necessary.

Regarding syntheses of GAG and PG, there is no report that chitin and chitosan have any influence on them. Recently, some investigators report that oral administration of glucosamine (GlcN), a monomer of chitosan, improves arthritis in humans (Kajimoto, Sakamoto, Takamori, Kajitani, Imanishi, Matsuo et al., 1998; Tapadinhas, Rivera & Bignamini, 1982; Theodosaikis, Adderly & Fox, 1997). They speculate that GlcN induces extracellular matrix in the cartilage. GAG and PG are major components of extracellular matrix together with collagen. GAG is also a major component of PG and plays an important role in tissue regeneration. The present study showed that synthesis of GAG was enhanced by chitin, but not chitosan. In the present study, we evaluated quantitatively GAG and PG in the granulation tissue using image anlaysis. For further

Table 2
Image analysis of the implants in the special staining sample (data are displayed as mean ± SD; the different superscripts in the figure indicate a significant difference between them: ab, a′b′, a″b″ ($p < 0.05$))

Agent	Concentration (mg/ml)	Alcian blue staining	Safranin O staining	Masson's trichrome staining
Control	–	95.6 ± 3.9^{a}	$218.7 \pm 25.6^{a'}$	$160.5 \pm 9.5^{a''}$
Chitin	1	99.6 ± 5.7^{a}	$189.5 \pm 2.9^{b'}$	$146.0 \pm 4.2^{a''}$
	10	103.4 ± 3.3^{b}	$185.8 \pm 3.0^{b'}$	$144.7 \pm 5.3^{b''}$
	50	101.8 ± 5.1^{b}	$186.9 \pm 5.5^{b'}$	$158.9 \pm 5.3^{a''}$
Chitosan	0.1	93.7 ± 4.5^{a}	$211.9 \pm 15.1^{a'}$	$155.6 \pm 7.8^{a''}$
	1	92.7 ± 3.4^{a}	$214.3 \pm 6.2^{a'}$	$168.8 \pm 6.2^{a''}$
	10	97.8 ± 3.0^{a}	$212.8 \pm 6.4^{a'}$	$161.3 \pm 7.0^{a''}$

interpretation, it is necessary to analyze biochemically as well as by using image analysis.

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