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Anti-inflammatory effect of chemically modified chitin

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Abstract

Anti-inflammatory effects of the three types of chitin derivatives namely phosphated chitin (P-chitin), phosphated–sulfated chitin (PS-chitin), and sulfated chitin (S-chitin) were investigated using a canine model of chitosan-induced pneumonia. After simultaneous administration of chitosan with or without each chitin derivative (chitosan alone: $n = 6$, chitosan and P-chitin: $n = 6$, chitosan and PS-chitin: $n = 1$, and chitosan and S-chitin: $n = 3$), hematological examination and X-ray image processing were performed for up to 24 h. Then the lungs were recovered and were evaluated by softex imaging after inflation and fixation. The hematological findings showed that PS-chitin and S-chitin did not prevent the decrease in white blood cell (WBC) count as seen in dogs administered chitosan, while P-chitin prevented such decrease in WBC count. The surface of the inflated and fixed lung specimens was hemorrhagic in the PS- and S-chitin groups as well as in the chitosan group, while the lung looked like normal in the P-chitin group. The pulmonary blood vessels of the chitosan group showed severe change while the P-chitin group showed no changes with softex findings. Furthermore, the pattern of histogram density obtained with image processing of thoracic X-ray in P-chitin group did not change among pre and post administration while chitosan group showed rightward movement and significant changes on parameters. The cause of which is attributed to an attenuation of X-ray permeability by angiectasis of the lung.

Keywords: Phosphated chitin; Phosphated–sulfated chitin; Sulfated chitin; Chitosan; Pneumonia; Adult respiratory distress syndrome

1. Introduction

In the recent years, chitosan and chitin derivatives have been reported to have dramatic beneficial effects on wound healing (Minami et al., 1997a,b, 1998; Okamoto et al., 1993a,b, 1997). However, extremely high doses of chitosan (200 mg/kg body weight, 2000 times of routine clinical dosage) injected subcutaneously have been reported to induce pneumonia in dogs (Minami et al., 1996). Histopathological findings in chitosan-induced pneumonia closely resemble those of acute respiratory distress syndrome (ARDS), where a severe hemorrhage results from of the interstitial spaces of the lung (Minami et al., 1996).

Chitin and its derivatives are studied worldwide, however, studies on application to medical fields started recently. Some investigators have reported that certain chemically modified chitin derivatives, including sulfated and carboxymethylated chitin, cannot degrade certain enzymes including collagenase, and this mechanism was thought to be responsible for the inhibition of tumor cell metastasis (Murata et al., 1991; Saiki, Murata, Nakajima, Tokura, & Azuma, 1990). Neutrophil emigration, as occurs in ARDS, is comparable to the mechanism of tumor cell metastasis.

In this study, the anti-inflammatory effect of chitin derivatives was investigated using a model of chitosan-induced pneumonia in dogs.

2. Materials and methods

Sixteen female mongrel dogs (approximately 2 to 3 years old) weighing 7.0–16.0 kg were used for this study. All dogs were found to be normal on routine physical, hematological and biochemical screenings.

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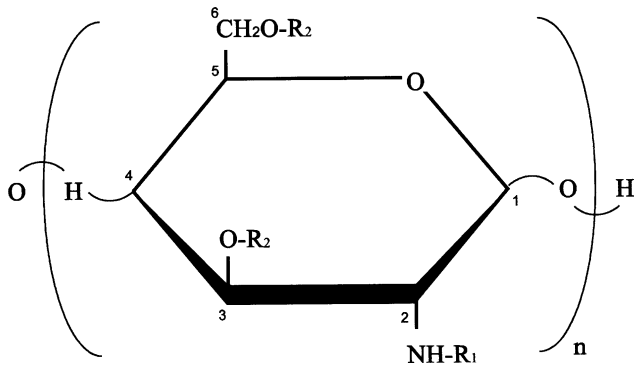

Fig. 1. Chemical structures of chitin and chitin derivatives. $R_1 = COCH_3$ (over 90%), H (under 10%) $R_2 = H$(Chitin), H/PO_3H_2 (P-Chitin), H/SO_3H (S-Chitin), PO_3H_2/SO_3H (PS-Chitin) Molecular weight (MW) of P-chitin was 111,000 (Pullulan conversion: PC), degree of substitution (DS) was 1.18 at C-3 and C-6. MW of PS-chitin was 111,000 (PC), DS was 0.89 and S was 0.16 at C-3 and 6, S-chitin was 111,000 (PC), DS was 1.87 at C-3 and C-6.

Chitosan powder made from snow crab shells (Sunfive Inc., Japan) with $>82\%$ deacetylation and particles <10 μm (mean particle size was 5 μm) was used. The powder was sterilized with ethylene oxide gas, and suspended in 50 mg/ml of sterile physiological saline before use.

The chitin derivatives used were phosphated chitin, phosphated–sulfated chitin and sulfated chitin (Fig. 1). These samples were synthesized by phosphorylation or sulfation of chitin as in the method described by Tokura and Tamura (1998). P-chitin was synthesized by phosphorylation of chitin. In brief, 150 g of urea was suspended in 150 ml of DMF (*N*, *N*-Dimethyl formamide) and prepared a homegeneous solution by stirring at 100 °C. A 10 g of dried chitin fine powder from squid pen was added to above solvent at 100 °C until chitin was swollen. After 26 ml of *ortho*-phosphoric acid was added and the reaction was proceeded for 3 h at 150 °C. The reaction mixture was rinsed by methanol extensively to remove phosphoric acid and DMF. The residue was dissolved in water and water soluble fraction of pH 10–11 was dialyzed against deionized water repeatedly to remove urea and phosphoric acid. The residue was then lyophilized and insoluble part separated was treated by acetone followed by methanol rinse to dry in air.

P-chitin is modified by substitution with a phosphoric acid base, with a molecular weight of 111,000 (Pullulan conversion, PC), and the degree of substitution by phosphate (DS-P) is 1.18 at C-3. The molecular weight of PS-chitin is 111,000 (PC), with a DS-P of 0.89 and substitution by sulfate (DS-S) of 0.16 at C-3 and C-6, respectively. MW of S-chitin was 111,000 (PC), and DS-S of 1.87 at C-3 and C-6.

2.1. Experiment 1

Ten dogs were randomly assigned to four groups. Chitosan alone (C) ($n = 3$), chitosan and P-chitin (C/P-c) ($n = 3$), chitosan and PS-chitin (C/PS-c) ($n = 1$), and chitosan and S-chitin (C/S-c) ($n = 3$), respectively. In the chitosan group, chitosan suspension (200 mg/kg) was injected subcutaneously. In other groups, each modified chitin material (10 mg/kg) was injected intravenously within 10 min of chitosan (200 mg/kg) administration.

Blood examination was performed to evaluate the blocking effect of each chitin derivative on chitosan-induced pneumonia. Blood samples were collected from the jugular vein of each dog using a syringe with 18 G needle before administration of chitosan (pre-administration) and at 1, 3, 5, 7, 12, and 24 h after administration. The measured parameters were red blood cell count (RBC), white blood cell count (WBC, Table 1), total plasma protein (TP), hemoglobin concentration (Hb) and packed cell volume (PCV).

At 24 h after administration, all animals were euthanized by an overdose of anesthetic agent (90 mg/kg i.v. pentobarbital sodium injection, Nembutal, Dainippon-Seiyaku, Japan) and a thoracotomy performed to evaluate the surface of the lungs. The lung was inflated and fixed as described previously and softex survey was performed to observe the changes in the lung blood vessels (Markarian & Dailey, 1984; Miyatake, Okamoto, & Minami, 1999).

Table 1
Variation in the WBC count after chitosan and chitin derivatives administration

Hour	White blood cell (WBC) count			
	Chitosan	Chitosan + P-chitin	Chitosan + PS-chitin	Chitosan + S-chitin
Pre	110.3 ± 34.9	156.0 ± 27.8	101	136.3 ± 26.5
1	82.0 ± 13.9	96.7 ± 19.7*	33	112.7 ± 32.7
3	30.7 ± 15.9*	94.7 ± 19.1*	28	26.7 ± 14.8*
5	27.0 ± 3.6*	128.3 ± 51.9	32	25.3 ± 6.5*
7	64.7 ± 30.9	103.0 ± 44.2	78	50.7 ± 25.8*
12	79.0 ± 38.0	111.7 ± 30.6	117	72.7 ± 15.0*
24	93.0 ± 38.0	150.0 ± 44.2	142	111.3 ± 21.5

Values are expressed in mean ± standard deviation; Hour means pre and post administration of chitosan; WBC: White blood cell count ($\times 100\ \mu l^{-1}$); Values are significantly different between pre and post administration designated by asterisk (*); *$P < 0.05$ compared with the pre administration value by Student's *t*-test.

2.2. Experiment 2

Six dogs were divided into two groups: C and C/P-c groups ($n = 3$ each). In the C group, chitosan was administered subcutaneously (200 mg/kg) as in Experiment 1. In the C/P-c group, P-chitin was administered intravenously (10 mg/kg) within 10 min of chitosan administration. For the evaluation of chitosan-induced pneumonia, thoracic radiography was performed at pre-, 1, 3, 5, 7, 12, and 24 h after administration. The thoracic radiograph was taken with ventoradorsal position at the time of maximum inspiration using X-ray devices (KXO-80F and TF-6, Toshiba, Japan). The Medical X-ray film SR-G, Konica, Japan and intensifying screen (KM-250, Konica, Japan) were used. Focal distance to film is set at 100 cm.

The radiographs taken were analyzed by image processing. Image analysis of grey level on thoracic X-ray films was performed with an image analyzer (MV4000, Nippon Data General Co. Ltd., Japan), and a multi-color data system (4200F, NAC Co. Ltd., Japan). The analysis procedures were done as described in our previous paper (Miyatake et al., 1999). In brief, X-ray image was input into MV4000 via 4200F, and redisplayed on the screen of 4200F using the ID300's image file support programs, Define image file status (DIFS) and Define image status program (DISP). Image analysis on the lung field was performed by MV4000 image processor. The pulmonary field was divided into right and left fields by the spine, and into cranial, middle and caudal parts at the level of the fourth intercostal space and at the eighth intercostal space, respectively. Density histograms and parameters in 5% of the lung field area were obtained by ID300's analysis software program HGRAM.

Histogram analysis was performed in the right caudal lobe, because it is the easiest to evaluate because of its wide anatomical lung field. The parameters used were minimum grey level value (Min), maximum grey level value (Max), mean grey level value (Mean), median grey level value (Med), maximum frequency grey level value (Mode), and maximum frequency value (MaF) as shown in Fig. 2.

3. Results and discussion

Chitosan induces pneumonia by subcutaneous administration of 200 mg/kg in dog. However, chitins do not induce such fatal disease even in dogs. We were agreeable for chitin in quite safe materials, but 200 mg/kg of chitosan of which case induced pneumonia in dog, is 2000 times of the clinical dose. We used 0.01–1 mg/kg of chitosan for wound treatment in clinical care. For 1 cm^2 wound, 0.01 mg/cm^2 of chitosan is adequate dose for treatment. We do not have any side effects when we used chitosan within 1 mg/kg or 1 mg/cm^2 of wound from the data of 300,00 heads of dogs on clinical wound treatment. Chitosan also is quite safe material and is quite effective remedy. Chitosan is ordinary wound remedy in Japan.

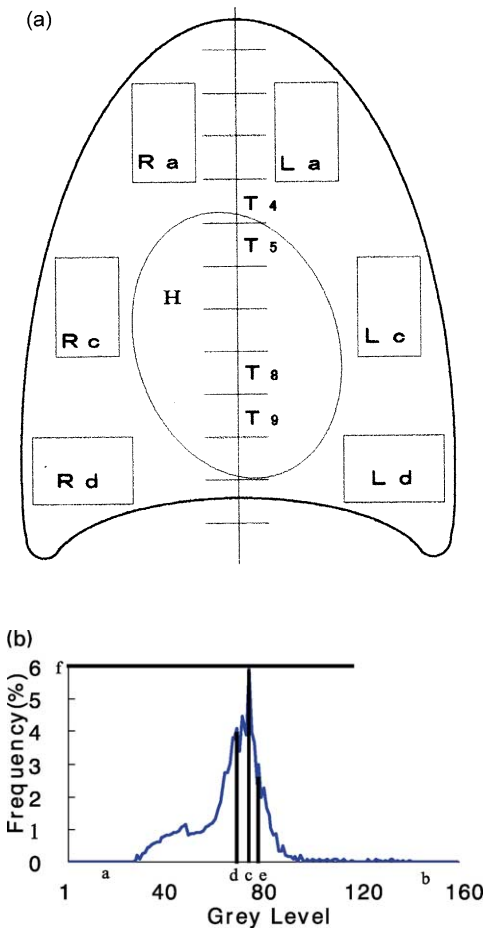

Fig. 2. (a) Showing analysis of the pulmonary field The pulmonary field was divided into right and left fields from the impression left by the thoracic vertebrae into cranial, middle and caudal parts at the level of the fourth and eighth intercostal space, respectively. Density histogram and parameters were obtained by 5% of the lung field area. R is right side lung, L is left side lung, a, c and d are cranial, middle and caudal, respectively. (b) Histogram pattern of the normal lung field a: Minimum grey level (Min); b: Maximum grey level (Max); c: Median; d: Maximum frequency grey level (Mode); e: Mean; f: Maximum frequency value (MaF).

In C/P-c group showed leucopenia within 3 h post administration, and the decrease of WBC was significant compare to pre-value (Fig. 3), however, variation was limited within normal range of Canine WBC (6000–17,000, Jain, 1986). In C group, Leucopenia continued to 12 h post-administration, and most severe leucopenia was observed at 3 and 5 h. The variation of WBC was closely resembled to C/P-s, and C/S-c groups.

In the gross finding of the lungs, the best anti-inflammatory effect was observed with C/P-c group. The lung surface of the chitosan group was hemorrhagic, edematous and showed red hepatization (Fig. 4(a)). In the C/S-c and C/PS-c groups, the lung surfaces were also hemorrhagic but less than that of the chitosan group (Fig. 4(b) and (c)). Hemorrhagic changes in the C/S-c group were observed over approximately 50% of the lung surface. In the C/P-c group, hemorrhage observed was less than half of the lung surface, with more healthy surface. The lung surface

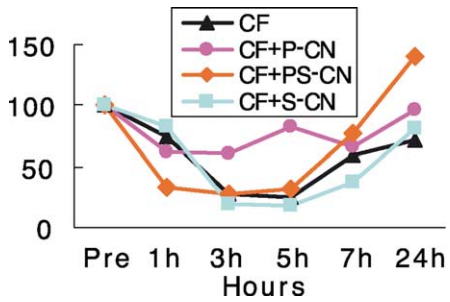

Fig. 3. Changes in WBC count the WBC in each group. Values were lower than Pre-injection count (Pre). Pre-administration values were expressed in 100 to compare with post administration. Need to write Labels, CF: Chitosan, CF + P-CN: Chitosan + P-Chitin, CF + PS-CN: Chitosan + PS-Chitin, CF + S-CN: Chitosan + S-Chitin.

of the C/P-c group was normal and light pink in color and there was no evidence of hemorrhage (Fig. 4(d)).

In the softex findings, pulmonary arterial lesions were clearly demonstrated in the lung of the C group. The main morphological change of C group was angiectasis (Fig. 5). The peripheral arteries were indistinct due to hemorrhage. Normal peripheral arteries of C/P-c group are very clear, but those of the C group were blurred.

Variation in the parameters after administration of chitosan and P-chitin was shown in Table 2. In the C group parameters, MaF showed a significant decrease and Max showed a significant increase with respect to time. Other parameters of each group did not show any changes. In the analysis of the radiographic images, the histogram

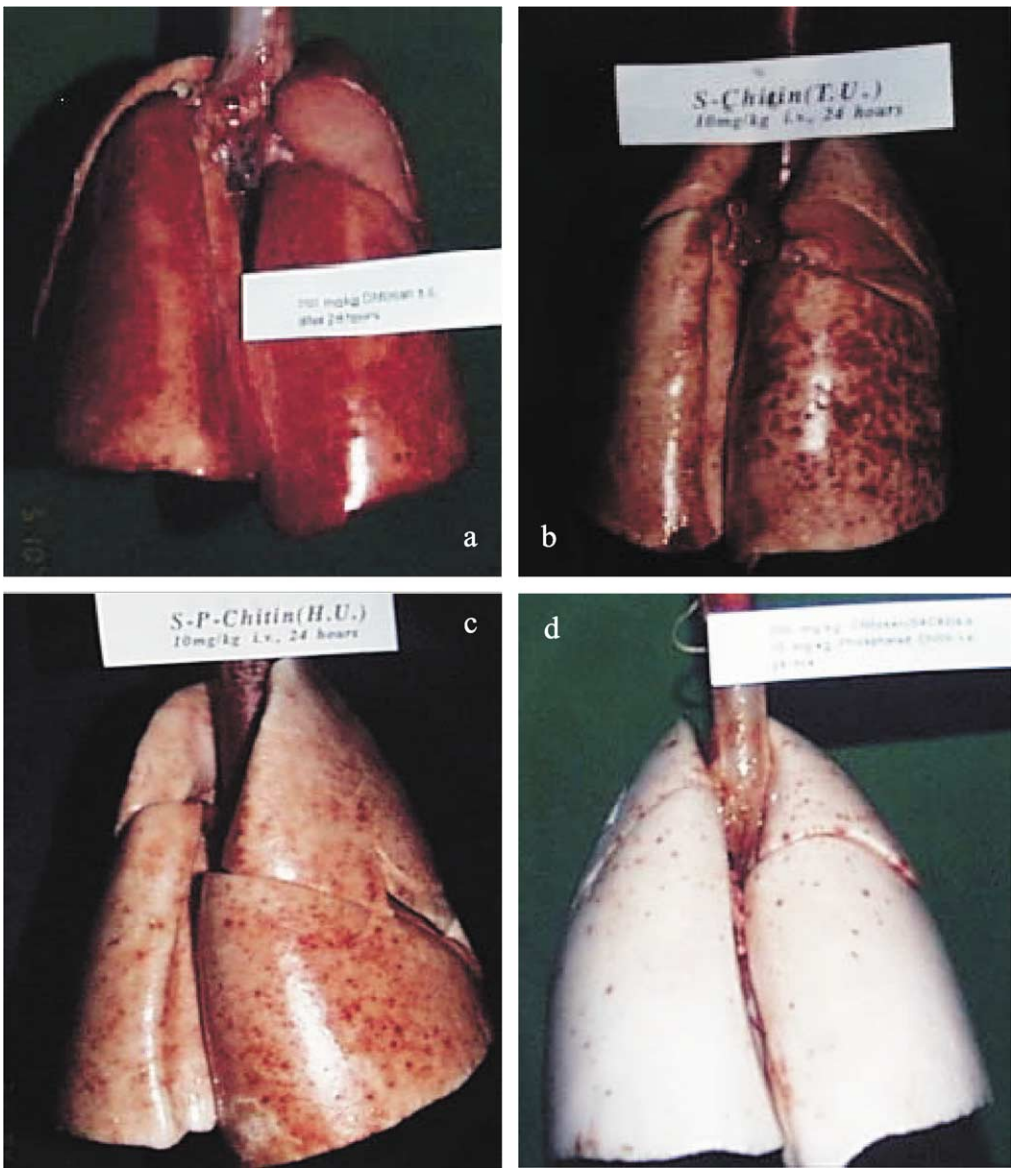

Fig. 4. Photographs showing inflated lung specimens 24 h after the administration of test materials (a: only chitosan, b: chitosan and S-chitin, c: chitosan and SP-chitin, d: chitosan and P-chitin). The lung lobes from dog receiving P-chitin were pink in color. P-chitin inhibited hemorrhage in the lung and appeared almost normal.

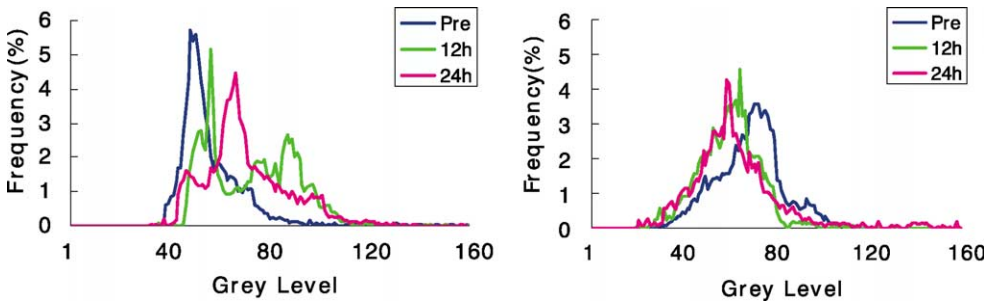

Fig. 5. Showing histograms of the lung field from dog receiving chitosan (a) and chitosan + P-chitin (b). Histogram pattern was dramatically changed in the chitosan case at 12 and 24 h post administration, but in P-chitin and chitosan case showed slight change in the histogram patterns.

pattern of the C group showed a decreasing MaF pattern with rightward movement. In contrast C/P-c group showed no significant changes. The histogram pattern showed minor leftward moving changes (Table 2, Fig. 6).

Pneumonia, especially adult respiratory distress syndrome is a form of acute respiratory failure occurring as a complication of a wide variety of factors including trauma, shock, oxygen therapy, and fat embolism. For that reason, this syndrome can be very difficult to treat and it is estimated that about 150,000 cases occur each year and approximately one half of these terminate fatally (Heizman, 1984). Furthermore, the study of this disease is difficult, as there are no suitable models exclusively for pneumonia.

Overdoses of chitosan (200 mg/kg body weight) injected subcutaneously have been reported to induce pneumonia especially in dogs (Minami et al., 1996), and it is the only material to induce a pneumonia similar to ARDS. Khanal et al. (2001) report that phosphated chitin is effective to chitosan-induced pneumonia which is characterized a severe hemorrhage and inflammation in the lung. This event suggests that P-chitin has potency for being an anti-inflammatory agent. Therefore, the model of chitosan-induced pneumonia was used in this study to investigate possible treatments. Hematological and radiographic findings by the chitosan-induced pneumonia in dogs have been reported in veterinary studies (Minami et al., 1996).

We reported earlier that P-chitin inhibit chitosan-induced pneumonia in mice (Khanal et al., 2001). However, we could not get real time data because of smaller experimental animal so, we used dogs as a experimental animal for more detail experiment. In this study, it was found that chitin derivatives exhibited anti-inflammatory effects against chitosan-induced pneumonia in dogs. Of the chitin derivatives, the most effective anti-inflammatory agent was P-chitin, followed by PS-chitin and S-chitin.

Pre- and post-administration histograms showed differences in concentration and arterial changes. Histogram patterns in the chitosan group moved rightward and MaF was decreased. By comparison, MaF of the C/P-c group did

Table 2
Variation in the parameters after administration of chitosan and P-chitin

Hour	Min	Max	Mean	Med	Mode	MaF
Chitosan group (C group)						
Pre	27.7 ± 9.5	157.3 ± 11.6[a]	50.2 ± 7.9	56.0 ± 8.7	53.7 ± 7.4	5.1 ± 0.7[b]
1	26.7 ± 11.5	154.0 ± 14.4	43.1 ± 7.2	49.0 ± 10.5	47.3 ± 10.4	5.5 ± 1.1
3	27.3 ± 10.7	149.7 ± 15.5	48.0 ± 11.2	54.7 ± 8.5	49.7 ± 11.2	4.8 ± 0.4
5	26.7 ± 6.5	160.3 ± 5.1	52.2 ± 13.8	61.7 ± 13.7	56.3 ± 17.6	4.3 ± 0.8
7	25.0 ± 12.5	164.7 ± 8.7	54.6 ± 18.4	62.7 ± 16.9	63.3 ± 16.4	3.7 ± 0.6
12	27.0 ± 10.8	178.3 ± 22.3	58.9 ± 1.9	64.7 ± 8.1	58.3 ± 4.2	3.4 ± 0.7[b]
24	29.7 ± 7.1	184.7 ± 6.7[a]	58.4 ± 1.4	57.3 ± 4.7	61.3 ± 10.7	2.8 ± 0.5
Chitosan + P-chitin group (C/P-c group)						
Pre	23.7 ± 4.9	140.3 ± 6.6	64.5 ± 5.7	64.0 ± 4.6	64.0 ± 7.0	5.1 ± 1.3
1	24.7 ± 4.9	124.7 ± 8.7	58.2 ± 6.0	58.7 ± 5.1	57.0 ± 5.3	5.1 ± 0.9
3	24.0 ± 4.6	122.0 ± 12.8	57.7 ± 6.8	58.3 ± 5.9	57.0 ± 5.6	5.0 ± 0.4
5	26.0 ± 7.2	130.3 ± 20.0	61.5 ± 11.4	62.3 ± 9.3	60.0 ± 8.5	5.4 ± 0.2
7	23.3 ± 4.0	126.3 ± 12.9	56.6 ± 5.6	56.3 ± 6.0	55.7 ± 4.5	5.5 ± 0.4
12	22.3 ± 5.1	138.0 ± 26.5	57.7 ± 8.6	60.7 ± 6.1	60.0 ± 5.3	5.0 ± 0.4
24	23.0 ± 5.2	142.7 ± 29.5	57.0 ± 5.8	58.7 ± 5.1	58.0 ± 5.0	4.9 ± 0.5

Values are expressed in mean ± standard deviation; Hour means pre and post administration of chitosan; Min: minimum grey level value, Max: maximum grey level value; Mean: mean grey level value, Med: median grey level value; Mode: maximum frequency grey level value and MaF: maximum frequency value; Values significantly different between same alphabetical superscript (a-a, b-b).

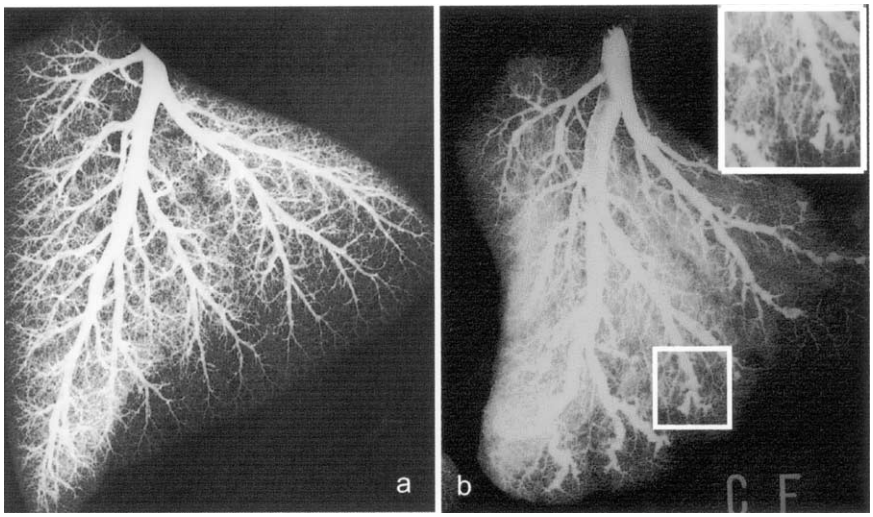

Fig. 6. Photographs showing plumonary artery changes with inflated specimens of the lungs 24 h after Chitosan and P-chitin (a), Chitosan (b) administration. Artery became angiectactic and the peripheral arteies were indistinct by hemorrhage in the chitosan case (b), but in the P-chitin and chitosan case, arterial changes were minimum.

not show any significant changes. A rightward shift means that opacity increased on the radiographic image, probably due to hemorrhage. From the previous study, we know that histogram patterns of lung arteries with angiectasis show decreased MaF and rightward movement (Miyatake et al., 1999).

Minami et al. (1997) reported that WBC decreased in the dog with chitosan-induced pneumonia. This phenomenon could be explained by adhesion of neutrophils that are main component in WBC to the blood vessel wall in the lung, and pneumonia caused multiple Neutrophil migration in the interstitial structure of lung. Neutrophil migration bears counter resemblance to tumor cells' metastasis. There have been some reports about the inhibition of metastasis of tumor cells by chitin derivatives (Murata et al., 1991; Saiki et al., 1990). Khanal et al. (2002) had reported P-chitin potentially recovered neutrophils deformability which was lost by incubation with chitosan activated serum. Minami et al. (1997a) already reported that chitosan activated complement system, and C5a was contained in the activated serum. C5a is a well known strong chemoattractant of neutrophils and is also attributed for the loss of deformability of neutrophils.

These results show P-chitin to be the most effective of the chitin derivatives against chitosan-induced hemorrhagic pneumonia. The phosphated derivatives proved to exhibit the greatest anti-inflammatory property. In conclusion, chitin derivatives, especially P-chitin, may become suitable drug for the treatment of inflammatory disease. In the acute toxicity test of chitin, the LD_{50} was reported as over 10 g/kg subcutaneously in mice (Mita, 1987). Using doses are mg order in medical use. Thus, chitin and chitin derivatives are safe agents for medical treatment administration. Further investigation will be necessary to fully understand the mechanism of the inhibitory effect of chitin derivatives against chitosan-induced pneumonia.

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