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Chitosan-inducing hemorrhagic pneumonia in dogs

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Various amounts of chitosan (10–200 mg/kg) were administered subcutaneously to dogs. Anorexia and mortality were observed in dogs given doses above 50 mg/kg, and above 150 mg/kg, respectively. In hematologic findings leukocytosis and increasing of serum LDH_2 and LDH_3 isoenzymes were characteristic. From the findings of autopsy, severe hemorrhagic pneumonia was observed in all dead dogs. Chitosan causes lethal pneumonia to dogs. Copyright © 1996 Elsevier Science Limited.

INTRODUCTION

Dramatic effects of chitosan (polymeric D-glucosamine) and chitin (polymeric *N*-acetyl-D-glucosamine) on wound healing have been reported in veterinary studies (Minami *et al.*, 1991, 1992, 1993; Okamoto *et al.*, 1992, 1993). Alternatively, the safety of chitosan has been demonstrated in mice and rats when used as food additives and base materials for cosmetics (Mita, 1987; Seo, 1990). In the acute toxicity test, the LD_{50} was reported as over 1.5 g/kg orally in rats, over 10 g/kg subcutaneously in mice, 5.2 g/kg intraperitoneally in mice and 3.0 g/kg in rats (Mita, 1987). Thus chitosan does not seem to be in the category of toxic substances; however, the toxicity of chitosan for larger animals such as cats, dogs, cows, and other domestic animals has not been demonstrated.

In our preliminary examination, 200 mg/kg chitosan administered subcutaneously caused no physiological or hematological responses in cats, mice, and cows; however, a characteristic effect was found in dogs. Therefore, in order to use chitosan as biomaterial, it is very important to clarify how administered chitosan affects animals, especially dogs. In this paper we would like to report the relationship between the amount of chitosan administered subcutaneously and the effects on dog.

EXPERIMENTAL

Preparation of chitosan

Chitosan powder from snow crab shells (Sunfive Inc., Japan) with > 82% deacetylation and particles < 10 μm (mean particle size: 5 μm) were used. The distribution of granule size was measured with an SK Laser Micron Sizer 7000S (Seisin K.K., Japan). The endotoxin content in a hot water extract (70°C, 6 h) of this powder was undetectable by the specific calorimetric determination (Endospecy, Seikagaku-kogyo, Japan). The chitosan powder was sterilized with ethylene oxide gas, and was suspended in 40 ml of aseptic physical saline before use.

ANIMALS

Twenty-four adult male mongrel dogs weighing 7–11 kg were used in this study. They were divided into seven groups by the dose of chitosan administered: 0 (control, physical saline), 10, 30, 50, 100 and 150 mg/kg were given to three dogs each and another six dogs were given 200 mg/kg chitosan. Each dose of chitosan was administered as several subcutaneous injections around the neck.

CLINICAL EXAMINATION

Up to 1 month of administration, the surviving dogs were observed everyday; the rectal temperature, pulse rate and respiration rate were measured. The determination of pain at the injection site was performed by direct palpation. Thoracic radiographic examination was performed when symptoms of respiratory disturbance appeared.

Blood was collected from the jugular vein of each dog using a syringe with 18 G needle before administration of chitosan (pre-administration) and at first, 3rd, 7th, 14th, 21st and 28th days post-administration. An aliquot

(1 ml) of collecting blood was treated by EDTA 2Na for anticoagulation and the serum was separated from the residue by centrifugation at 2500 rpm for 10 min after clotting. Red blood cell count (RBC) and white blood cell count (WBC) were measured by a hemocytometer (Thoma) method. Packed cell volume (PCV) was measured by micro-hematocrit method. Differential leukocyte count (DLC) was measured by light microscopic observation of Giemsa-stained blood smear. Plasma total protein was measured by a refractometric method. Serum transaminase (GOT, GPT), lactated dehydrogenase (LDH), creatine kinase (CK), alkaline phosphatase, blood glucose, total cholesterol, blood urea nitrogen, and calcium were measured by dry chemistry system (Cobas ready, Japan Rosh, Japan). LDH (iso-LDH, Iatron, Japan) and CK isoenzymes (Titan III-iso Flur) were measured by the method of cellulose acetate electrophoresis. LDH and CPK isoenzymes were examined in the serum of four cases of the 200 mg/kg group at Day 3, which showed higher activity in both enzymes. The endotoxin concentration in all collected serums was measured by the specific calorimetric determination (Endospecy, Seikagaku-kogyo, Japan).

AUTOPSY AND HISTOLOGICAL EXAMINATION

Autopsies for all dead dogs were performed. Tissues were fixed in 20% neutral buffered formalin. Specimens were embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (HE). Selected sections of lung were stained by the phosphotungstic acid–hematoxylin (PTAH) method.

RESULTS

Clinical findings

In the dogs given doses above 50 mg/kg chitosan, vigor loss and anorexia were observed in over 60% on the next day (Day 1) or within 3 days after injection. In the dogs given a 30 mg/kg dose, these symptoms were only transient. Control dogs and the dogs given 10 mg/kg chitosan demonstrated no clinical abnormality. At 200 mg/kg chitosan, all animals developed these signs on Day 1. Two dogs (66.7%) given 150 mg/kg chitosan developed continuous severe dyspnea with an increasing respiration rate and one dog died on Day 6. Eight dogs (88.9%) given 200 mg/kg chitosan also developed the same dyspnea and seven of them died between Days 4–9. In the dog that died on Day 4, collapse and hemoptysis were observed. One dog with severe dyspnea after the administration of 200 mg/kg chitosan survived on Day 14 with apparent lung dysfunction. No significant increase of body temperature was observed.

Radiographic findings

Chest radiographs from a dog with severe dyspnea (200 mg/kg) revealed a slight enlargement of heart with dilated pulmonary arteries and showed 'cotton wool' appearance with both an air bronchogram and air alveologram in a lung field.

Hematological findings

No significant changes were observed in RBC and PCV values. WBC, however, were significantly increased in the 150 mg/kg group during the experimental period after chitosan injection and temporarily in 50, 100, and 200 mg/kg chitosan groups (Table 1). From the results of DLC, the increase of WBC was caused by neutrophilia. On the other hand, two dogs survived over 21 days in the 200 mg/kg group, which demonstrated a significant decrease of WBC at Days 21 and 28. In the serum biochemical analysis, no significant changes were observed except for LDH and CK concentrations (Tables 1 and 3). As shown in Table 2, LDH concentration in the 200 mg/kg group increased continuously during 7 days after the administration of chitosan and temporarily in the 100 and 10 mg/kg groups. The results of LDH isoenzymes in the 200 mg/kg group at Day 3 is summarized in Table 4. The mean LDH concentration was 883.5 IU/L and mean percent of each isoenzyme of

Table 1. Variation in the white blood cell count (WBC) after subcutaneous administration of chitosan

Day*	Dose (mg/kg)						
	200	150	100	50	30	10	0
1	1.04±0.19**	1.87±0.37[a]	0.89±0.01[b]	1.58±0.51[a]	0.92±0.31	1.23±0.26	1.03±0.11
3	0.97±0.32	1.46±0.18[a]	1.17±0.25	1.25±0.78	0.96±0.11	1.02±0.15	1.02±0.09
7	1.56±0.05[a](5)[c]	2.92±1.73[a] (5)	1.67±0.13[a]	1.12±0.23	1.11±0.08	0.75±0.22	0.96±0.25
14	0.83±0.17 (2)	1.85±0.09[a] (5)	1.58±0.46[a]	0.96±0.04	1.35±0.30	0.94±0.14	1.03±0.15
21	0.59±0.01[b] (2)	2.13±0.01[a] (5)	1.49±0.20[a]	0.97±0.05	0.97±0.06	1.83±0.81	0.93±0.26
28	0.84±0.01[b] (2)	1.55±0.16[b] (5)	1.29±0.47	1.08±0.08	1.00±0.05	0.82±0.21	0.92±0.22

*Day 1 means first day after administration of chitosan.
**WBC of post-administration/WBC of pre-administration (mean±S.D., $n=6$).
[a]Values were significantly increased ($p<0.05$); [b]values were significantly decreased ($p<0.05$).
[c]In 200 and 150 mg/kg groups, the number of experimental dogs was decreased by the deaths of dog with chitosan-inducing pneumonia.

Table 2. Variation in the serum LDH concentration after subcutaneous administration of chitosan

Day*	Dose (mg/kg)						
	200	150	100	50	30	10	0
1	1.52±0.32[a]**	1.81±0.99	1.64±0.25[a]	1.16±0.46	0.82±0.04	0.89±0.69	1.03±0.32
3	2.66±1.16[a]	2.00±1.26	2.68±0.5[a]	1.36±0.55	1.47±0.44	2.34±0.87[a]	1.33±0.55
7	2.55±0.89[a]	1.05±0.41	1.05±0.75	2.06±1.29	1.25±0.32	1.05±0.35	1.06±0.30

*Day 1 means first day after administration of chitosan.
**LDH of post-administration/LDH of pre-administration (mean±S.D., $n=6$ except for 200 and 150 mg/kg groups at Day 7, $n=5$), [a]Values are significantly increased ($p<0.05$).

Table 3. Variation in the serum CK concentration after subcutaneous administration of chitosan

Day*	Dose (mg/kg)						
	200	150	100	50	30	10	0
1	1.06±0.16**	1.54±0.58	1.66±1.01	0.96±0.35	1.10±0.16	1.37±0.30	1.23±0.20
3	2.71±0.45[a]	3.00±0.59[a]	3.00±0.73[a]	3.20±2.36	2.20±0.26[a]	1.74±0.80	1.55±0.35
7	3.34±2.19[a]	1.17±0.01	1.83±0.01[a]	1.44±0.66	1.48±0.45	1.84±1.24	1.42±0.15

*Day 1 means first day after administration of chitosan,
**CK of post-administration/CK of pre-administration (mean±S.D., $n=6$ except for 200 and 150 mg/kg groups at Day 7, $n=5$), [a]Values are significantly increased ($p<0.05$).

Table 4. LDH and CK isoenzymes of 200 mg/kg group at Day 3

No.	Serum LDH concentration (IU/L)	Isoenzymes (%)					Serum CK concentration (IU/L)	Isoenzymes(%)		
		1	2	3	4	5		BB	MB	MM
1	799	3	20	23	15	39	693	34	11	49
2	706	3	14	14	21	48	790	29	4	64
3	1360	3	14	19	15	49	381	43	5	45
4	669	6	14	16	17	47	606	57	4	32
mean	883.5	3.8	15.5	18	17	45.8	617.5	40.8	6	47.5

1–5 was 3.8, 15.5, 18, 17, and 45.8, respectively. The mean CK concentration was 617.5 IU/L and mean percent of each isoenzyme of BB, MB, and MM was 40.8, 6, and 47.5, respectively. The endotoxin level in serum was below 9 pg/ml in all tested samples.

Autopsy and histological findings

Macroscopic observation of a control lung at 7 days after the subcutaneous administration of 40 ml physical saline was shown in Fig. 1. The lung lobes were pink in color and well extended with oxygen flash via trachea. On the other hand, all dead dogs had the same macroscopic findings of the red hepatization in the majority of the lung lobes. Numerous hemorrhagic lesions of various sizes were obvious on the surface of the lung (Fig. 2). In the bronchi, moderate amounts of bloody catarrhal exudate were pooled. There were no abnormal findings in the other organs. Characteristic histological findings are shown in Figs 3 and 4. Inflammatory cells and hemorrhage were observed in the alveoli, bronchioles, and interstitial tissue (Fig. 3). The interstitial tissue was hypertrophied and edematous. Fibrin deposition was obviously observed in the interstitial tissue on PTAH-stained sections (Fig. 4). Chitosan particles were not observed in any of the lung specimens. The lung changes were diagnosed as chronic hemorrhagic fibrinous pneumonia.

DISCUSSION

It was demonstrated clearly that subcutaneous administration of large doses of chitosan caused a severe pneumonia in dogs. This response was completely unknown in the toxicity testing of chitosan (Arai *et al.*, 1968). In the case of wild animals, we have experienced the overdosing of two raccoon dogs (*Nyctereutes procyonoides*) which were injured severely by automobiles. Approximately 200 mg/kg chitosan was administered to the wound. On the second day, those raccoon dogs showed weakness, anorexia and dyspnea. Chest X-ray findings were quite similar to those of the dead dogs that were administered chitosan. The raccoon dog is a member of the dog family. Furthermore, in our preliminary experi-

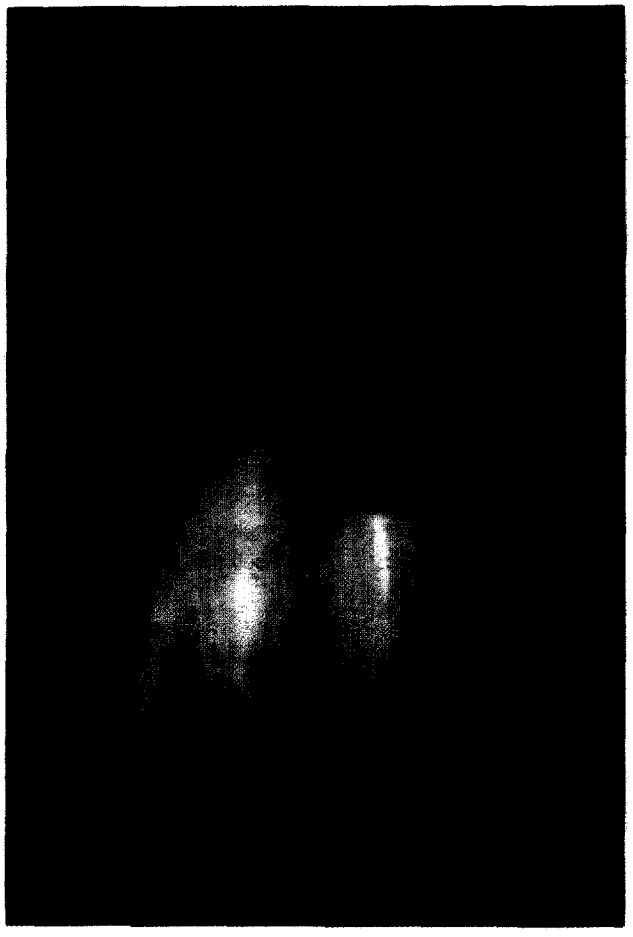

Fig. 1. Normal lung appearance in a control 7 days after administration of 40 ml physical saline.

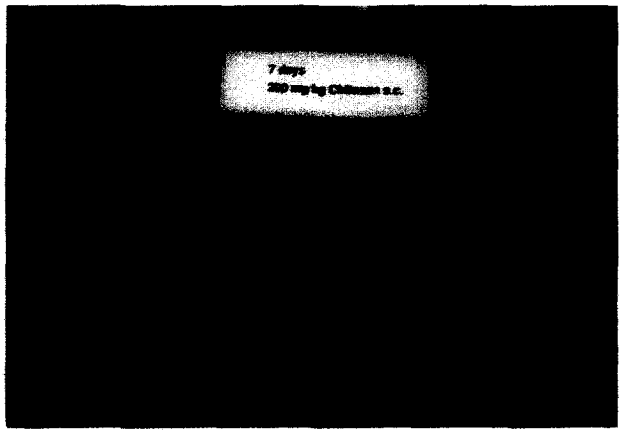

Fig. 2. Autopsy findings of lung in the dead dog. The majority of the lung lobes showed severe hemorrhage and focal red hepatizations.

ment of acute toxity in cows, cats, and mice, chitosan caused dogs and raccoon dogs serious pneumonia, but not in the former groups of animal; hence, the dog family are subjected to serious pneumonia by the subcutaneous administration of chitosan.

In 150 and 100 mg/kg groups, neutrophilia had been

Fig. 3. Histological findings of lung in the dead dog (HE-stain). Hemorrhage and severe inflammatory cell invasion (arrows) to interstitial tissue of lung are visible.

Fig. 4. Histological findings of lung in the dead dog (PTAH-stain). Fibrin deposition was obviously observed (arrows).

observed, this might be an inflammatory reaction to subcutaneous chitosan injection and pneumonia. In the 200 mg/kg group, however, neutrophilia had not been observed except for Day 7, and at Days 21 and 28, leukopenia was observed (Table 1). At 5 h after subcutaneous administration of 5 μm chitosan particles (200 mg/kg), some leukopenia also occurred (data not shown). On the other hand, such leukopenia was never observed in dogs given the same subcutaneous administration of 5 μm chitin particles or of 5 μm latex beads (200 mg/kg). The leukopenia in the chitosan-administrated dog may not be caused by a physical effect of 5 μm chitosan particles, but by a biochemical effect of chitosan which induced an adherent of neutrophils to capillary endothelium. During the long-term, the reason that leukopenia occurred intermittently, as described above, may be because of the slow degradation of chitosan in the subcutaneous tissue (Minami *et al.*, 1993). In the chemical examination of serum, activations of LDH and CK were observed. In comparison with the normal values (Jordan, 1977), the concentrations of LDH and CK of 200 mg/kg group at Day 3 were 17 and four times, respectively. LDH isoenzymes were increased in LDH_2 and LDH_3 in comparison

with the normal values (Heavner *et al.*, 1986). It is known that LDH_2 and LDH_3 are the main constituents of the organ LDH isoenzymes of lung (Kitamura *et al.*, 1985). Therefore, chitosan-induced LDH activation may be caused by the pneumonia. However, the origin of the temporary increase in LDH concentration in 100 and 10 mg/kg groups was not able to be clarified. On the other hand, there was no characteristic increase of CK isoenzymes (Kikuta & Onishi, 1987). In pigs, it had been reported that serum CK activity increased significantly in various stress situations (Moss, 1979), but LDH activity did not. The chitosan-induced pneumonia was a severe stress to animals, the increasing of CK activity, therefore, may be a reaction to these biological invasions.

From the histopathological findings, the chitosan-related pneumonia featured the infiltration of polymorphonuclear cells and severe hemorrhage into the interstitial spaces and alveoli in the lung. These findings closely resembled those of adult respiratory distress syndrome (ARDS) (Murray, 1977) and those of murine lung affected by cobra venom factor as a model of ARDS (Mulligan *et al.*, 1993). The mechanism of this syndrome involves complement activation which upregulates endothelial P-selectin (Mulligan *et al.*, 1993). There are no reports concerning the complement activation by chitosan, however, we already reported that chitosan particles were phagocytized by PMN in the presence of normal serum, but not in the presence of the heat-treated serum (Minami *et al.*, 1993). On the other hand, chitosan was a chemotactic substance for canine PMN (Usami *et al.*, 1994) and induced more active chemotactic migration of PMN by the addition of serum (Minami *et al.*, 1993). Furthermore, the incubated serum separated from chitosan particles by centrifugation also induced the migration of PMN and this migration was not inhibited by the heat treatment of the incubated serum at 56°C for 30 min (data were not shown). The heat-stable chemotactic complement for PMN was known as C5a (Honda & Hayashi, 1982). C5a also induced Mac-1 upregulation of neutrophils (Kishimoto *et al.*, 1989). Mac 1(CDb/CD18 molecule) participates in neutrophil adhesion to endothelium and is critical for effective neutrophil localization into inflamed tissues (Jutila *et al.*, 1989). On the other hand, the inflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor (TNF), and bacterial endotoxin act directory on cultured human vascular endothelium to increase the adhesion of leukocytes (Bevilacqua *et al.*, 1989), and also induce Mac-1 upregulation of neutrophils (Kishimoto *et al.*, 1989). It was reported that chitosan showed a biological aptitude for activating macrophages for stimulating the production of IL-1 (Nishimura *et al.*, 1989). The serum endotoxin was not increased in the chitosan-administered group (below 9 pg/ml in all tested samples). The characteristic features of the endotoxin shock in dogs were an increase in liver weight by severe portal hypertension (MacLean *et al.*, 1956) and intestinal mucosal hemorrhage (Schweinburg *et al.*, 1957) and edema by extreme increasing of intestinal lymph flow (Ballin & Meyer, 1960). There are many differences in pathological findings between the endotoxin shock and the chitosan subcutaneous administration in dog.

The pneumonia caused by subcutaneous injection of chitosan may be induced by immunological reactions and various cytokine activations. Further investigation must be performed, especially in an estimation of complement components and various cytokines including interleukin 8 in a bronchi-alveolar lavage fluid (Miller, 1992).

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