Various gelatinizers, which facilitate oral ingestion, are employed in patients with dysphagia. The purpose of this study was to histologically clarify the influence of various gelatinizers on the lung, using rats. We administered 0.2 ml/kg of 0.1% xanthangam, a 0.25% commercially available xanthangam gelatinizer, 0.35% ι-carrageenan, 0.5% κ-carrageenan, 1% gelatin, 0.15% agar, physiological saline, tap water, and isopropanol-purified 0.1% xanthangam/0.35% ι-carrageenan into the trachea of 8- to 9-week-old male SD rats. The lungs were extirpated after 24 and 72 hours. Neutrophil infiltration in the alveolar space was expressed as the mean number of neutrophils in 30 randomly selected high-power fields. In the xanthangam (451.0±204.0 cells)-, and the ι-carrageenan (424.4±257.2)-treated groups, the neutrophil counts after 24 hours was significantly greater than in the physiological saline (33.0±22.6)-treated group (p<0.05). In the available xanthangam gelatinizer (290.0±86.8)-treated group, no significant difference in the physiological saline-treated group. In the isopropanol-purified xanthangam (90.2±42.3)-treated group, the neutrophil counts after 24 hours were significantly smaller than in the non-purified xanthangam-treated group. These results suggest that lung tissue inflammatory response-inducing features depend on the type of gelatinizer. On the other hand, purification reduces the lung-damaging features of xanthangam.

Key words: dysphagia, lung-damaging features, gelatinizer, xanthangam, carrageenan

Introduction

In humans, the incidence of pneumonia increases with age. As an etiological factor, food aspiration is involved in pneumonia in most cases. Many commercially available foods contain gelatinizers to facilitate oral ingestion. These gelatinizers are useful for preventing aspiration. Furthermore, they are added to liquid or foods due to their aspiration-preventing characteristics, and utilized for deglutition function tests and eating training for patients with dysphagia. However, in patients with severe dysphagia, gelatinizer-containing liquid or foods are sometimes aspirated during deglutition function tests or eating training. As few studies have investigated the extent of pulmonary tissue disorder related to gelatinizers or influence on the onset of pneumonia, it is necessary to evaluate the effects of various gelatinizers.

The purpose of this study is to histologically clarify acute inflammatory responses, as the influence of various gelatinizers on the lung, using rats.

Materials and Methods

Animals and Experimental diets. We used 8- to 9-week-old male Sprague-Dawley (SD) rats (Japan SLC, Shizuoka, Japan) weighing 280 to 347 g. After arrival, these rats were acclimated for 5 to 9 days; commercially available solid food (CE-2, CLEA, Tokyo, Japan) and water were given ad libitum under the following conditions: temperature, 23±3°C; humidity, 55±15%; and lighting cycle, 12 hours (7:00 a.m. to 7:00 p.m.).

Experimental design. Samples including gelatinizers...
Among gelatinizers that are commonly used for foods, we selected gelatin, xanthan gum/its commercially available product, carrageenan, and agar. As control groups, physiological saline or tap water was administered. Usually, the manufacturers have shipped gelatinizers after purification, but in this study, we were provided xanthan gum and carrageenan before purification by the manufacturers.

Physiological saline and tap water were sterilized with a 0.22-μm filter prior to this study. The other samples were dissolved in physiological saline to prepare a specific concentration, and autoclaved (121 °C, 15 min). Because the gelatinizers to give to a rat might make influence to damage the lungs physically, we dragged and adjusted their viscosity in speed 10sec⁻¹ (ARES, Rheometric Scientific, Inc., New Jersey, United States). The specific concentrations of samples other than physiological saline, tap water, and gelatin were adjusted so that the viscosity was approximately 20 mPa·sec.

The rats were anesthetized with 5% sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) (inhalation anesthesia), and fixed in a dorsal position on a 45-degree tilting table. Under an otoscope, a 16G catheter (Terumo Co., Ltd., Tokyo, Japan) was inserted into the subglottic area. A 3Fr catheter (Atom Medical Co., Ltd., Tokyo, Japan) was inserted into the 16G catheter. Simultaneously, the 3Fr catheter's end was protruded by approximately 5 mm from the 16G catheter's end to accurately insert it into the trachea without deep insertion into the unilateral lung. Using a syringe pump (Harvard Apparatus, Massachusetts, United States), each sample at 0.2 ml/kg was administered into the trachea at a constant flow velocity (10 μl/sec). Subsequently, the rats were routinely raised.

The lungs were extirpated 24 or 72 hours after sample administration; the rats were exsanguinated by cutting the abdominal aorta under 5% sevoflurane anesthesia. Median incision of the cervix was performed to expose the trachea. The tracheal cartilage was incised at the inferior thyroid gland. A catheter was inserted through the section, and 10% neutral buffered formalin solution (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan) (corresponding to 5 to 7 ml) was infused at a pressure of 20 cmH₂O (1.96kPa) to fix the lung. Subsequently, the trachea was ligated so that the formalin solution did not leak, and the thorax was incised to expose the lung. The left and right lungs involving the trachea were extirpated, and immersed in formalin solution in a container. Hematoxylin and eosin (H&E) staining was performed to prepare tissue specimens.

The number of specimens was 4 in the physiological saline-, tap water-, 1% gelatin- and 0.15% agar-treated groups, and 5 in the 0.1% xanthan gum-, 0.25% commercially available xanthan gum gelatinizer-, 0.35% ι-carrageenan- and 0.5% κ-carrageenan-treated groups.

Subsequently, xanthan gum or ι-carrageenan was purified with isopropanol, and administered to rats to prepare tissue specimens. The number of specimens was 5 in the purified 0.1% xanthan gum- and 0.25% ι-carrageenan-treated groups. Purification was performed using the following procedures: after each gelatinizer was dissolved to prepare a specific concentration, 6 ml (3-fold volume) of isopropanol was added to 2 ml of each solution, placed at 4°C for 10

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>physiological saline</td>
<td>Otsuka Pharmaceutical Factory Co., Ltd.</td>
</tr>
<tr>
<td>tap water</td>
<td>Ooigawa Kouiki Suidou Kigyodos</td>
</tr>
<tr>
<td>gelatin (Super gelatin SSB)</td>
<td>Nippi Co., Ltd.</td>
</tr>
<tr>
<td>agar</td>
<td>Wako Pure Chemical Industries Co., Ltd.</td>
</tr>
<tr>
<td>xanthan gum (non-purified)</td>
<td>SIGMA Co., Ltd.</td>
</tr>
<tr>
<td>commercially available xanthan gum</td>
<td>Kissei Pharmaceutical Co., Ltd.</td>
</tr>
<tr>
<td>ι-carrageenan (non-purified)</td>
<td>MRC Polysaccharide Co., Ltd.</td>
</tr>
<tr>
<td>κ-carrageenan (non-purified)</td>
<td>MRC Polysaccharide Co., Ltd.</td>
</tr>
</tbody>
</table>
minutes, and centrifuged at 3,000 rpm for 10 minutes to precipitate the gelatinizer. After the supernatant was removed, 4 ml (2-fold volume) of isopropanol was added to each sample, and centrifuged at 3,000 rpm for 10 minutes. The supernatant was removed, and each sample was dried at 80°C for 30 minutes to remove isopropanol. Subsequently, it was dissolved in 2 ml of physiological saline.

**Analysis.** For histological assessment, neutrophil infiltration in the alveolar space was evaluated. The mean number of neutrophils in 30 randomly selected high-power fields (at a magnification of 400), consisting of 18 right and 12 left lung areas, was calculated. Among the gelatinizers used as samples, endotoxin tests (Endospecy, Seikagaku Biobusiness Corporation, Tokyo, Japan) with 0.15% agar, 0.1% xanthangam, the 0.25% commercially available xanthangam gelatinizer, 0.35% ι-carrageenan, 0.5% κ-carrageenan, and isopropanol-purified 0.1% xanthangam/0.35% ι-carrageenan were conducted to measure the endotoxin content.

**Statistical analysis.** All values were expressed as the mean ± SD. For statistical analysis, the cells were divided into groups 24 and 72 hours after administration, and the results were compared among the groups using the Tukey-Kramer method. P<0.05 was regarded as significant.

All analyses were performed with a commercially available statistical software package (Microsoft Excel 2010, Microsoft).

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Industrial Research Institute of Shizuoka Prefecture (H23-001).

### Table 2. The neutrophil count 24 or 72 hours after administration among the physiological saline-, tap water-, gelatin-, agar-, xanthangam-, commercially available xanthangam gelatinizer-, ι-carrageenan and κ-carrageenan-treated groups

<table>
<thead>
<tr>
<th>Sample</th>
<th>After 24 hours (cells)</th>
<th>After 72 hours (cells)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>physiological saline</td>
<td>33.0±22.6</td>
<td>19.5±12.5</td>
<td>4</td>
</tr>
<tr>
<td>tap water</td>
<td>27.8±10.6</td>
<td>20.8±1.6</td>
<td>4</td>
</tr>
<tr>
<td>1% gelatin</td>
<td>17.0±1.7</td>
<td>20.0±4.7</td>
<td>4</td>
</tr>
<tr>
<td>0.15% agar</td>
<td>14.5±3.0</td>
<td>9.8±6.0</td>
<td>4</td>
</tr>
<tr>
<td>0.1% xanthangam</td>
<td>450.8±204.0</td>
<td>63.2±44.6</td>
<td>5</td>
</tr>
<tr>
<td>0.25% commercially available xanthangam gelatinizer</td>
<td>290.0±86.8</td>
<td>24.0±4.3</td>
<td>5</td>
</tr>
<tr>
<td>0.35% ι-carrageenan</td>
<td>424.4±257.2</td>
<td>153.0±62.1</td>
<td>5</td>
</tr>
<tr>
<td>0.5% κ-carrageenan</td>
<td>80.2±54.4</td>
<td>24.6±9.3</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SE.

### Results

The neutrophil counts 24 hours after administration were 33.0±22.6 in the physiological saline-treated group, 27.8±10.6 in the tap water-treated group, 17.0±1.7 in the 1% gelatin-treated group, 14.5±3.0 in the 0.15% agar-treated group, 450.8±204.0 in the 0.1% xanthangam-treated group, 290.0±86.8 in the 0.25% commercially available xanthangam gelatinizer-treated group, 424.4±257.2 in the 0.35% ι-carrageenan-treated group, and 80.2±54.4 in the 0.5% κ-carrageenan-treated group.

The neutrophil counts 72 hours after administration were 19.5±12.5 in the physiological saline-treated group, 20.8±1.6 in the tap water-treated group, 20.0±4.7 in the 1% gelatin-treated group, 9.8±6.0 in the 0.15% agar-treated group, 63.2±44.6 in the 0.1% xanthangam-treated group, 24.0±4.3 in the 0.25% commercially available xanthangam gelatinizer-treated group, 153.0±62.1 in the 0.35% ι-carrageenan-treated group, and 24.6±9.3 in the 0.5% κ-carrageenan-treated group (Figure 1, Table 2).

There were no significant differences in the neutrophil count 24 hours after administration between the tap water-/gelatin-/agar-/commercially available xanthangam gelatinizer-/κ-carrageenan-treated and physiological saline-treated groups. However, the neutrophil counts in the xanthangam- and ι-carrageenan-treated groups were significantly greater than in the physiological saline-treated group (Figure 2a, b, c). The neutrophil count 72 hours after administration in the ι-carrageenan-treated group was significantly larger than in the physiological saline-treated group. However, neither the xanthangam- nor
commercially available xanthangam gelatinizer-treated groups showed any significant differences.

The neutrophil counts 24 and 72 hours after administration were compared between the ι- and κ-carrageenan-treated groups. At the two points, the neutrophil counts were significantly greater in the former.

The neutrophil counts 24 hours after the administration of isopropanol-purified gelatinizers were 90.2±42.3 in the isopropanol-purified 0.1% xanthangam-treated group and 86.2±44.5 in the isopropanol-purified 0.35% ι-carrageenan-treated group. In these groups, the neutrophil counts after 24 hours were smaller than in the non-purified xanthangam- and ι-carrageenan-treated groups (Figure 3a, b, Table 3).

The endotoxin contents of 0.15% agar, 0.1% xanthangam, the commercially available xanthangam gelatinizer, 0.35% ι-carrageenan, purified 0.1% xanthangam, and purified 0.35% ι-carrageenan were 58, 15,048 (3,000-fold that of tap water), 802, 26, 172 and 20 EU/ml, respectively.

**Discussion**

The results of this study suggest that acute pulmonary inflammatory response induction on aspiration differs among gelatinizers. In particular, pulmonary disorder marks in the xanthangam- and ι-carrageenan-treated groups.

Xanthangam is prepared by fermenting starch with *Xanthomonas campestris*. When it is mixed with water, it becomes viscous. Therefore, xanthangam is employed as a gelatinizer for dysphagia diets. Furthermore, xanthangam was approved as a less toxic substance that does not require the establishment of a daily allowance on safety assessment by the Joint FAO/WHO Expert Committee on Food Additives. However, *Xanthomonas campestris* is a gram-negative bacteria. The outer membrane of its cellular wall contains an endotoxin, lipopolysaccharide (LPS), as a component. A study indicated that xanthangam contained LPS as...
The pulmonary tissue damage with gelatinizers

a contaminant that cannot be completely removed in the purification process. LPS is known to induce various biological actions in vivo, represented by acute pulmonary injury. An experiment using an animal model demonstrated that the endotracheal administration of LPS promoted cytokine production and neutrophil inflow, causing pulmonary injury. LPS induces the expression of inflammatory cytokines such as TNF-α and IL-1β by binding to/activating macrophages, and promotes the lung tissue infiltration of activated neutrophils, causing pulmonary injury.

In this study, the neutrophil counts in the xanthangam-treated group were significantly greater than in the physiological saline-treated group 24 and 72 hours after administration to the rat lung. However, in the commercially available xanthangam gelatinizer-treated group, there was no significant difference in the neutrophil count. The commercially available xanthangam gelatinizer may have been purified. Indeed, the endotoxin content was lower than that of xanthangam. Therefore, the possibility of endotoxin-related pulmonary disorder was suggested. In this study, xanthangam was purified with isopropanol, and compared with an untreated sample. After purification, the endotoxin content and lung tissue neutrophil count decreased, suggesting that xanthangam induces inflammation.

Table 3. The neutrophil counts after 24 hours in the isopropanol-purified xanthangam- and ι-carrageenan-treated groups

<table>
<thead>
<tr>
<th>Sample</th>
<th>no purification (cells)</th>
<th>after purification (cells)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% xanthangam</td>
<td>450.8 ± 204.0</td>
<td>90.2 ± 42.3</td>
<td>5</td>
</tr>
<tr>
<td>0.35% ι-carrageenan</td>
<td>424.4 ± 257.2</td>
<td>86.2 ± 44.5</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SE.
endotoxin-related pulmonary disorder.

Isopropanol is an organic solvent, which is used to precipitate protein and to dissolve other materials. There is in particular the property to dissolve a cell wall in of the gram-negative bacillus, inactivating endotoxin.

Carrageenan is a negative-ion macromolecular compound consisting of D-galactose and sulfate. It is obtained from red seaweeds by alkali extraction.

As it forms a gel at room temperature, carrageenan is used for the manufacturing of various foods. An animal experiment with a rodent showed that the decomposition product of carrageenan caused ulcers and cancer of the digestive tract, and that the subcutaneous or intra-articular injection of carrageenan induced inflammation. However, the adverse effects of carrageenan is specific to rodents, and it was approved as a less toxic substance that does not require the establishment of a daily allowance on safety assessment by the Joint FAO/WHO Expert Committee on Food Additives. Furthermore, no study has reported additional serious toxic reactions other than watery stools after the oral ingestion of carrageenan in humans.

In this study, we purified ι-carrageenan with isopropanol, and compared the sample with an untreated sample. Purification decreased the pulmonary neutrophil count. However, the endotoxin content of ι-carrageenan before purification was low, suggesting that a factor other than endotoxin is involved in an increase in the neutrophil count. Concerning this, Ogata et al. reported that carrageenan increased the sensitivity of leukocytes to LPS.
promoting the production of TNF-α, an inflammatory cytokine. Furthermore, Goto et al. prepared an aspiration pneumonia model by infusing carrageenan into the mouse trachea, and reported that the antigen-presenting capacity of alveolar macrophages increased in comparison with mice in which the trachea was exposed, leading to the lung tissue accumulation of neutrophils. Based on these findings, ι-carrageenan may induce inflammatory responses in the lung tissue despite a low endotoxin level. Furthermore, purification with isopropanol may have altered ι-carrageenan-specific characteristics, different from endotoxin, reducing lung tissue-damaging features. When comparing specimens between the ι- and κ-carrageenan-treated groups, there is a significant difference in the pulmonary neutrophil count, suggesting that lung tissue damage depends on the type of carrageenan. The sulfate radical of the ι-carrageenan is double than that of the κ-carrageenan (Figure 4). From this, we thought the sulfate radical might affect the pulmonary toxicity, and the possibility that changes by isopropanol could decrease the pulmonary toxicity. There is no document which I examined about this, and examination will be necessary in future.

On the other hand, Miki et al. investigated the physical properties of gelatinizers, and reported the carrageenan- or agar-related enhancement of inflammatory responses in the lung tissue. They indicated that the physical properties of gelatinizers were involved in inflammatory responses based on differences in the physical properties from gelatin jelly, which did not induce any inflammatory response. In this study, we standardized the viscosity of xanthangam, carrageenan, and agar, and compared their lung tissue-damaging features. However, there were differences in the incidence of inflammatory responses among the substances administered. These results suggest that chemical rather than physical features are involved in the induction of inflammatory responses. Thus, to reduce the lung injury, gelatinizers, particularly xanthangam, should be purified.

Acknowledgments

We appreciated the advice by Dr. Hirohisa Takano, Kyoto University and Dr. Kikuo Ohno, Tokyo Medical and Dental University.

We declare that we have no conflicts of interest. All experimental protocols were approved by the Conflicts of Interest Committee of Hamamatsu City Rehabilitation Hospital.

References

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