Title

Ingredients of jelly products affect aspiration-related pulmonary inflammation; in an animal study.

Abstract

Diet modification is an important intervention in the management of patients with dysphagia. Food entering the airway, same as oral bacterium, causes pulmonary inflammation; therefore, the elucidation of inflammatory responses to different foods is important. This study aimed to investigate the differences in the severity of inflammatory response induced by intratrachial injection of foods with different nutritional components.

Two jelly products, the one containing only carbohydrates (KURIN jelly: Isocal Jelly KURIN®) and the other containing carbohydrates, proteins, and lipids (HC jelly: Isocal Jelly HC®), were prepared. These jelly products (dilution with saline, 50% volume/volume) and saline, as control, were intratracheally administered to Sprague-Dawley rats at a dose of 1 ml/kg (KURIN group (n=15), HC group (n=15), Saline group (n=15)). At 1, 2 and 7 days after administration, lungs were harvested and histological analysis was performed. The severity of induced inflammation was evaluated using the Acute Lung Injury (ALI) score with hematoxylin-eosin staining, and the expression of IL-1β, IL-6 and TNF-α, markers of airway inflammation, were observed with immunostaining.

The ALI score in the HC jelly group was significantly higher than the KURIN jelly group and the Saline group ($p<0.01$) at 1 and 2 days after administration, while the ALI score in the KURIN jelly group was higher than Saline group only at 2day after administration. Numerous positive cells for IL-1β, IL-6 and TNF-α were

observed only in the HC jelly group at 1 and 2 days after administration. There were no significant histological differences between the three groups at 7 days after administration.

Our data suggests that the severity of inflammation caused by aspiration differs depending on the ingredients of the foods, and the nutrients contained in foods might be considered in dietary management for the patients with dysphagia.

Keywords: dysphagia, diet modification, acute lung injury, jelly, three major nutrients

35 *Introduction*

36 Impaired swallowing function, dysphagia, was caused by various reasons and generally divided into
37 oropharyngeal dysphagia or esophageal dysphagia [1]. Impaired swallowing function permit the aspiration of
38 food, oral bacteria, and/or gastric contents that cause pulmonary inflammation, fatal complications such as acute
39 lung injury and acute respiratory distress syndrome [2]. In the management of the patient with dysphagia,
40 effective interventions to prevent the onset of pulmonary inflammation are essential, and the diet modification is
41 especially sufficient to avoid foods enter the respiratory tract against oropharyngeal dysphagia [1, 3, 4]. Various
42 classifications for the diet modification have been established, almost of these focuses only on viscosity and
43 syneresis of foods [1, 5]. However, the Japanese classification for diet modification covers not only viscosity and
44 syneresis but also nutritional content in the diets. Furthermore, the Japanese specification is unique in that jelly
45 products has been included in the classification [5, 6]. Jelly products are classified into two types depending on
46 the ingredients namely jelly with protein and jelly without protein in the Japanese classification and used to
47 evaluate patients' swallowing ability and for nutritional supplementation. [5, 7-9]. Depending on the severity of
48 patients' dysphagia, jelly, which is considered to be relatively safe, can also enter the respiratory tract and become
49 a material that induce pulmonary inflammation [10].

50 The main outcome of the intervention for the patients with dysphagia is to improve swallowing function,
51 provide safe and appropriate nutrition, and reduce the risk of developing severe pulmonary inflammation and

death [3]. Therefore, investigation of the pulmonary inflammatory response caused by the aspiration of jelly products with different nutritional content is beneficial to establish the developed management strategy for the patients with dysphagia. The severity of the inflammatory response in the lungs relate to substances that enter the respiratory tract and can vary by characteristics of aspirated materials such as pH and nutritional contents [11, 12]. However, severity of the pulmonary inflammatory response to aspirated different jelly products were unknown, and our hypothesis was that severity of pulmonary inflammation varied depending on the ingredients of jelly products.

This animal study was aimed to investigate the differences in the severity of pulmonary inflammation induced by intratrachial administration of two jelly products with different nutritional contents by histological and immunohistochemical analysis.

Materials and Methods

The experimental protocols, as described below, were approved by the Animal Care and Use Committee of Dokkyo Medical University, and the experiments were carried out according to the Guidelines for Animal Experimentation of Dokkyo Medical University (No.1196 and No.1301).

Preparation of jelly products and solutions for intratracheal administration

The two jelly products with different nutritional contents were prepared for intratracheal administration (Isocal jelly KURIN® and Isocal jelly HC®, Nestle Japan Ltd., Tokyo, Japan). The ratio of three major nutrients in the two jelly products are shown in Table 1 [13]. In the three major nutrients, Isocal jelly KURIN® contained only carbohydrates, while Isocal jelly HC® contained proteins, lipids and carbohydrates. Isocal jelly KURIN® is composed of dextrin and sucrose as carbohydrates. Soy protein as protein and, as lipids, vegetable oil, such as soybean oil and rapeseed oil, is added in Isocal jelly HC® [14].

These jelly products diluted to a concentration of 50% volume/volume with saline and saline, as the control, were prepared for intratracheal administration.

Measurement of pH

The pH of each solution, diluted jelly products and saline, was measured five times using the S2K712 pH meter (ISFETCOM Co. Ltd., Hidaka, Japan) to investigate the acidity that affect pulmonary inflammation after aspiration.

Intratracheal administration with prepared solutions and tissue collection

Total forty-five 8-week-old male Sprague Dawley rats were enrolled in this study. They were anesthetized by intraperitoneal administration of medetomidine (0.4mg/kg), midazolam (2mg/kg), and butorphanol tartrate (2.5mg/kg) before intratracheal administration. Prepared solutions were intratracheally administered through the vocal cord at a dose of 1 ml/kg using a 20G blunt needle. To ensure the solutions infiltrated the lungs, the rats were held in an upright position for 1 minute after the administration, then placed in a prone position and observed until recovered from the anesthesia.

(KURIN group (n=15): injected with diluted Isocal jelly KURIN®, HC group (n=15): injected with diluted Isocal jelly HC®, Saline group (n=15): injected with saline alone)

At 1, 2 and 7 days after intratracheal administration, the rats were anesthetized with intraperitoneal administration of medetomidine (0.4 mg/kg), midazolam (2 mg/kg), and butorphanol tartrate (2.5mg/kg), sacrificed by exsanguination, and finally the lungs were collected and fixed in 10% neutral buffered formalin over 24hours. The experimental flow chart of this study was shown in Figure 1.

Incisions were made in two horizontal sections on the left lobe of the lung and the sections were then embedded in paraffin. For histological and immunohistological analysis, sections prepared with 3 μm slices for each incision planes.

Histological assessment of lung inflammatory response in the lung

HE-stained specimens were observed using light microscopy (BZ-X800 Viewer, Keyence, Japan) at 4× and 400× objective magnifications. To assess the severity of the pulmonary inflammatory response to the aspirated solutions, the specimens were evaluated using the acute lung injury (ALI) score [15] with observation at 400× objective. The ALI score, consisted in the 5 items following, is expressed as 0 (normal) to 1 (severe) to evaluate the inflammatory response in the lungs: the neutrophils in the alveolar space, the neutrophils in the interstitial space, formation of a hyaline membrane, proteinaceous debris filling the airspaces, and alveolar septal thickening. Ten visual fields were randomly extracted from one slice, a totally of 20 visual fields were evaluated from two slices, and the mean value was determined as the ALI score for one individual [16-18].

Immunohistochemical analysis

The expression of TNF-α and IL-6 was confirmed using the prepared sections. The sections were stained with Rabbit polyclonal Anti-IL-1β antibody (Abcam, Cambridge, UK, CatNo.ab9722) and Anti-TNF-alpha antibody (Abcam, Cambridge, UK, CatNo.ab66579), and Mouse monoclonal Anti-IL-6 antibody (Abcam, Cambridge, UK, CatNo.ab9324); Histofine Simple Stain MAX PO(R) and Histofine Simple Stain MAX PO(M) (Nichirei, Tokyo, Japan) were used as secondary antibodies. Immunoreactivity against the antibody was examined using light microscopy at 400× objective magnification.

Statistical analysis

The data are expressed as the mean ± SD of the pH values, and the ALI scores at 1, 2 and 7 days after intratracheal administration. Normal distribution was assessed by Shapiro Wilk's test and homogeneity of variance by Levene's Test. Tukey's two-tailed test was performed to locate significant differences between three groups, using the SPSS version 26 (SPSS Inc., IBM, Chicago, IL, USA). A p-value of less than 0.05 was considered statistically significant.

136 *Results*

137 *pH measurement of prepared solutions*

138 Table 2 shows the pH of prepared solutions, demonstrating that acidity was strong in the order of the 50%

139 diluted Isocal jelly KURIN®, saline, and the 50% diluted Isocal jelly HC® ($p<0.05$).

140 *Histological assessment and Acute Lung Injury score*

141 When the whole lung section was observed at 4× objective magnification, significant inflammatory change was

142 not observed in the KURIN group and the Saline group; however, significant inflammatory change was observed

143 in the HC group at 1 and 2 days after intratracheal administration (Figure 2).

144 When sections were observed at 400× objective magnification, neutrophil infiltration was identified in the

145 alveoli and interstitial space at 1 and 2 days after tracheal administration in the HC group, while only mild

146 neutrophil infiltration was observed in the KURIN group and the Saline group. At 7 days after intratracheal

147 administration, no neutrophil infiltration was observed in the lungs of all groups (Figure 3).

148 When comparing the ALI score between the three groups, the ALI score of the HC group were significantly

149 higher than that of the KURIN group and Saline group at 1 day and 2 days after intratracheal administration

150 ($p<0.05$). In the KURIN group, only at 2 days after intratracheal administration, significant high scores were

151 observed compared with the Saline group. There was no significant difference between the three groups at 7

152 days after intratracheal administration ($p>0.05$) (Table 3).

153 *Immunohistochemical analysis*

154 IL-1β-positive mononuclear cells and polynuclear cells were observed in the alveoli in the HC group at 1 and

155 2 days after intratracheal administration compared to the other groups (Figure 4).

156 This trend was also observed in immunostaining for TNF-α and IL-6; TNF-α-positive mononuclear cells

157 were observed widely in the alveoli and alveolar septum in the HC group compared to the other groups at 1 and

158 2 days after intratracheal jelly administration (Figure 5). Increase in IL-6 -positive cells were observed in the HC

159 group only at 1 and 2 days after intratracheal administration (Figure 6). At 7 days after intratrachial

160 administration, there were no significant differences between the three groups.

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Discussion

In this study, two jelly products with different nutritional contents were administrated intratracheally and the histological and immunohistochemical analysis were performed to clarify the different pulmonary inflammation to the aspirated jelly products. Severe pulmonary inflammation was observed after intratracheal administration with diluted Isocal jelly HC® (HC jelly) compared to that of Isocal Jelly KURIN® (KURIN jelly) and saline.

Various interventions for the patients with dysphagia has been developed to improve the swallowing ability and to prevent foods entry into the airway while swallowing [3]. In diet modification, different classifications have been developed, and to avoid dehydration and malnutrition, an appropriate viscous meal and liquid were selected, taking into consideration the severity of the patient's dysphagia, risk of aspiration, and level of consciousness, sometimes with tube feeding; however, few classifications incorporated the dietary nutrient content and calorific value while most classifications focused on the viscosity and syneresis [5].

Jelly products are recommended applying for dysphagia patients, because it has more viscous than water and reduces the risk of entering the airways by slowing the descending speed during swallowing [5, 8]. The purposes of jelly products are different depending on their characteristic; when jelly products consisted of only carbohydrates and the energy is low, these jelly products are applied to evaluate swallowing function [10]. Jelly products with high energy, containing proteins, lipids and carbohydrates, are applied for, in addition to being used for swallowing rehabilitation, energy supplementation in patients with dysphagia, if they can be ingested

187 safely by mouth [9]. Jelly products with similar textures are used for different purposes depending on the different
188 nutrients that they contain. Even jelly products, which are considered to be relatively safe for dysphagia patients,
189 occasionally cause lung inflammation due to aspiration [10], among jelly foods, it is necessary to make an
190 appropriate selection in consideration of pulmonary response when aspirated. However, the difference in the
191 severity of pulmonary response, when jelly products with different nutrients were aspirated, has been unclear.

192 The mechanism of pulmonary response induced by foreign substances entering the respiratory tract has been
193 elucidated in previous studies. Firstly, aspirated foreign substances are recognized as antigens on specific
194 receptors such as toll-like receptors, and then inflammatory cytokines are produced by activated alveolar
195 macrophages. The cytokines activate neutrophils and increase vascular permeability, causing leakage of the
196 exudate and the migration of neutrophils into the alveoli, this resulting tissue injury and subsequent pulmonary
197 inflammation [19, 20].

198 Pulmonary inflammation against foreign substances impairs pulmonary oxygen exchange [21], while it is an
199 essential immune reaction for healing from foreign substances invaded into the living body [22]. Activated
200 neutrophils and macrophages cause inflammation to tissues and, at the same time, phagocytose and remove
201 foreign substances [23]. Secondary to the disappearance of foreign substances, lung inflammation also converges,
202 and tissue repair of the damaged alveolus and vascular structure occurs. In a typical acute lung injury, the intense
203 inflammatory response subsides within 7 days of onset and then shifts to the proliferative phase [24]. In this

study, which investigated the non-infectious inflammatory response to jelly foods administered intratracheally, same as previous studies [25, 26], inflammatory reaction was observed at 1 and 2 days after tracheal administration only with HC jelly. After 7 days of administration, pulmonary inflammation was converged in all groups.

In our study, the inflammatory response caused by the aspiration of jelly products were evaluated with the acute lung injury (ALI) score that utilized histological evaluation of inflammatory changes in the lungs. This scoring method has been used in previous animal studies investigating pulmonary inflammation induced by the administration of foreign substances and the tissue injury induced by hyperoxia [15-18, 27, 28]. A study that examined the relationship between the ALI score, blood gas analysis, and the expression of inflammatory cytokines in lung tissue, reported that the pulmonary gas exchange capability was disrupted and the expression of inflammatory cytokines increased when the ALI score was high [27]. In our study, the ALI score was high at 1 and 2 days after intratracheal administration of HC jelly, which contains proteins, lipids and, carbohydrates, when compared to that of KURIN jelly, which contains carbohydrates only, and that of saline alone. This result reveals that aspiration of HC jelly induces more severe pulmonary inflammation than that of KURIN jelly.

Several cytokines have been identified as mediators in the onset of pulmonary inflammation [29, 30]; especially, in this study, we focused on the expression of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 in the lung tissue. These pro-inflammatory cytokines were essential mediators in the pathogenesis of acute

221 respiratory response for foreign materials [22, 24, 31], and evaluated in studies of aspiration pneumonitis, acute
222 lung injury and acute respiratory distress syndrome [12, 30, 32-34]. Especially, these three mediators, IL1-β,
223 TNF-α and IL6, were called “classic cytokines” [35] and evaluated in studies of pulmonary inflammatory
224 responses [12, 18, 30, 32-37]. IL-1β is secreted by activated macrophages and important bioactive cytokine in
225 the early phase of lung injury [24]. In IL-1β transgene-positive mice, chronic production of IL-1β observed in
226 respiratory epithelial cells and caused lung inflammation [38]. Moreover, secreted IL-1β have ability to activate
227 neutrophils and the migration of neutrophils into the alveoli, suggesting IL-1β contributes to the pathogenesis of
228 acute lung inflammation [30]. TNF-α is produced by macrophages and an important cytokine in the development
229 of inflammatory diseases [39]. Analysis of the inflammatory mediators in the sepsis animal model, induced by
230 the administration of lipopolysaccharide, revealed that TNF-α is elevated ahead of other cytokines, subsequent
231 TNF-α signaling induces other cytokines [35]. In the onset of pulmonary inflammation, TNF-α activates
232 neutrophils and induces inflammation in alveolar epithelial cells, this affects the interaction between neutrophils
233 and alveolar epithelial cells, thus inducing the migration of neutrophils into the alveoli [40]. IL-6 is an
234 inflammatory cytokine that is also produced by the macrophages and alveolar epithelium. The expression of IL-
235 6 increases in macrophages and epithelial cells in response to stimulation by TNF-α, resulting in increased
236 neutrophil accumulation and activation [30]. Furthermore, in some animal studies, IL-6 is used as a marker for
237 acute phase of airway inflammation [12, 30]. In our study, positive cells for these pro-inflammatory cytokines

238 were observed wider only in the HC group compared to the other groups. These results of this study, in addition
239 to the evaluation with ALI score using HE-stained specimens, clarified also through immunohistochemical
240 analysis that HC jelly induced severe pulmonary inflammation than KURIN jelly.

241 In the characteristic of aspirated materials, the acidity of substances can affect lung inflammation, and
242 substances with low pH cause severe lung injury [11, 41]. Comparing the pH of prepared solutions, diluted HC
243 jelly had weakest acidity compared to the others; therefore, the severe inflammatory response observed after
244 administration with HC jelly could be caused by factors other than acidity.

245 Comparing the nutrients contained in the two jelly products, HC jelly has protein and lipids added to
246 carbohydrates to increase the calorific content. Though detail studies looking into the difference between dietary
247 nutrients on aspirated materials and pulmonary inflammation are lacking, previous animal studies have reported
248 that the nutrients contained in foods can exacerbate lung injury [41] and possibility has been suggested that the
249 severity of lung inflammation caused by each of the three major nutrients in food might be different [12]. In
250 animal studies investigating the relationship between lipids contents in enteral solutions and aspiration related
251 lung inflammation, animal fat might exacerbate pulmonary inflammation due to differences in fat decomposition
252 mechanism [42]. However, because the amount of fat contained was different, it was concluded that various other
253 factors than lipids, such as proteins contained in the enteral solution, influenced to the aspiration related
254 pulmonary inflammation. Compared with the nutritional components of two jelly products, vegetable oils and

255 vegetable proteins that may have relatively weak toxicity for aspiration related lung injury were contained only
256 in HC jelly [42, 43]. Because of the difference in the nutritional components of the jelly products used in this
257 study, protein and lipids contained only in HC jelly can exacerbate pulmonary inflammation caused by the
258 aspiration.

259 In summary, this study demonstrated the pulmonary response was different depending on the nutrients of
260 aspirated foods and, in the management of dysphagia, the nutrients in the modified foods applied for the patients
261 with dysphagia could be considered in aspect of the inflammatory response to the aspirated foods.

262 Our study has some limitations. Firstly, many of the lung inflammations caused by aspiration of food are
263 pneumonia associated with oral bacterium [44]. In this study, bacteria were not administered and lung
264 inflammation were caused by intratracheal administration only of sterile jelly products. The non-infectious
265 pulmonary inflammation observed in this study is defined as pneumonitis, which is a condition distinct from
266 aspiration pneumonia associated with infections [11]. In order to create an animal model of aspiration pneumonia,
267 both jelly and oral bacteria should be intratracheally administered.

268 Secondary, Aspiration of meals causes pneumonia with a small number of frequent aspirations [45]. With
269 reference to the procedures of past animal experiments [12, 42], this study investigated the pulmonary
270 inflammation with a single intratracheal administration of jelly products. In order to create an animal model of

aspiration pneumonia, in addition to the combined administration of bacteria, future studies with intratracheal administration of frequent small doses were considered necessary.

Conclusions

This study revealed the differences in the severity of pulmonary inflammation caused by the aspiration of two types of jelly products with different nutritional components using histology and immunohistology. Compared to the jelly product composed only of carbohydrates, the jelly product containing proteins, lipids, and carbohydrates caused severe pulmonary inflammation when administrated to the air tract. This study suggests that the dietary management for patients with dysphagia might take account of not only the viscosity and syneresis but also the nutrients contained in their diets.

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Figure legends

Fig 1

Title: Experimental Protocol of this study

Text: In each group, intratracheal injection with prepared solutions was performed for fifteen SD rats (Black circles), and a total of forty-five rats was enrolled for this study. In each group, lungs were harvested from five rats at 1, 2, and 7 days after intratracheal administration (Black triangle) for the histopathological and Immunohistochemical evaluations.

Fig 2

Title: Observation of lung tissue with weak magnification at 1, 2 and 7 days after the intratracheal administration

Text: The KURIN group and Saline group did not show strong inflammatory change, while the HC group showed severe inflammatory change at 1 and 2 days after aspiration.

Hematoxylin and eosin stain, ×4, scale bar = 2 mm

KURIN: Injected with diluted Isocal jelly KURIN®; HC: Injected with diluted Isocal jelly HC®, Saline: Injected with physical saline alone

Fig 3

Title: Observation with high magnification of lung tissue

Text: Numerous neutrophils were observed in the alveoli (arrow head) in the HC group compared with the KURIN group and Saline group at 1 day after intratracheal administration. Neutrophil infiltration was observed in the alveoli (arrow head) and the interstitial spaces in the lung (arrow) in the HC group at 2 days after intratracheal administration. There was no inflammatory cell infiltration in either group at 7 days after intratracheal administration.

Hematoxylin and eosin stain, ×400, scale bar = 100 μm

KURIN: Injected with diluted Isocal jelly KURIN®; HC: Injected with diluted Isocal jelly HC®, Saline: Injected with physical saline alone

Fig 4

Title: Immunohistochemical stained with anti-IL-1β antibody

Text: IL-1β-positive mononuclear cells (arrow head) observed more widely in the HC group than in the other groups at 1 and 2 days after intratracheal administration.

×400, Scale bar = 100 μm.

KURIN: Injected with diluted Isocal jelly KURIN®; HC: Injected with diluted Isocal jelly HC®, Saline: Injected with physical saline alone

Fig 5

Title: Immunohistochemical stained with anti-TNFα antibody

Text: Infiltration of TNFα-positive mononuclear cells (arrow head) into the alveoli was observed at 1 and 2 days after intratracheal administration. TNFα-positive mononuclear cells observed in the HC group more widely than in the other groups.

×400, Scale bar = 100 μm.

KURIN: Injected with diluted Isocal jelly KURIN®; HC: Injected with diluted Isocal jelly HC®, Saline: Injected with physical saline alone

Fig 6

Title: Immunohistochemical stained with an anti-IL6 antibody

Text: Neutrophils, mononuclear cells, and alveolar epithelial cells were positive for IL6, a marker of airway inflammation. At 1 and 2 days after intratracheal administration, IL-6-positive cells were expressed in a wider

446 range in the HC group than in the other groups. Only alveolar epithelial cells were positive for IL6 ~~in both groups~~

447 at 7 days after intratracheal administration in all groups.

448 ×400, Scale bar=100μm.

449 KURIN: Injected with diluted Isocal jelly KURIN®; HC: Injected with diluted Isocal jelly HC®, Saline: Injected

450 with physical saline alone

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Table 1

Title: Nutritional contents of jelly products (in one package (66g))

	Carbohydrate	Protein	Lipid	Energy
KURIN Jelly	16.4g	0g	0g	45kcal
HC jelly	16.8g	3.0g	7.9g	150kcal

Footnote: KURIN Jelly: Isocal Jelly KURIN®, HC Jelly: Iocal Jelly HC®

Table 2

Title: The pH of prepared solutions

	Diluted Isocal jelly KURIN®	Diluted Isocal jelly HC®	Saline
pH	4.02±0.08*	6.78±0.08†	5.90±0.07

Footnote: * $P < 0.05$ compared with the saline and the diluted Isocal jelly KURIN®; † $P < 0.05$ compared with the saline and diluted Isocal jelly HC®.

Table 3

Title: Histological scores of the pulmonary inflammation against aspirated materials

	Acute Lung Injury score		
	Day 1	Day 2	Day 7
KURIN	0.30±0.07	0.46±0.04†	0.34±0.04
HC	0.63±0.07*	0.82±0.04*	0.35±0.04
Saline	0.29±0.04	0.34±0.06	0.33±0.03

Footnote: KURIN: Injected with diluted Isocal jelly KURIN®; HC: Injected with diluted Isocal jelly HC®, Saline: Injected with physical saline alone. * $P < 0.05$ compared with the KURIN group and the saline group; † $P < 0.05$ compared with the HC group and saline group.