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**Original Article**

# Pulmonary Edema Due to Oral Gavage in a Toxicological Study Related to Aquaporin-1, -4 and -5 Expression

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**Abstract:** A one-time oral gavage can be enough to cause of alveologenic edema with higher expression of AQP-1 and -4 than that with repeated-dose oral gavage, which caused both profound perivascular edema and hydrostatic pressure edema, while AQP-5 was similarly expressed. The alteration of AQPs expression was probably related to alveolar fluid clearance across the alveolar and bronchiolar epithelium in different stages of lung injury. The results clarified the type of lung edema in acute and sub-chronic toxicity studies without treatment related effect of tested material. The pathogenesis of pulmonary edema due to oral gavage toxicological study is associated with the cellular immune response to the reflux materials. Mast cell and leukocyte accumulation may contribute to increase vascular permeability leading to permeability edema. The increase in alveolar septum epithelium, perivascular and peribronchial cuffing, accumulation alveolar lipid containing macrophage and medial hyperplasia of the pulmonary artery might have been caused to increase airway resistance, which resulted in hydrostatic pressure edema. (DOI: 10.1293/tox.26.283; J Toxicol Pathol 2013; 26: 283–291)

**Key words:** pulmonary edema, gavage toxicology study, aquaporin-1, -4 and -5

## Introduction

In the assessment and evaluation of the toxic characteristics of a substance, oral gavage is a necessary technique in acute, sub-chronic and chronic oral toxicity studies[1–3]. Unexpected respiratory symptoms in individual animals are recurrently observed during dosing for several weeks or at the end point of studies, which may be regarded as gavage-related complications, especially reflux and technical gavage errors[4]. Some studies have reported the prominent histopathological findings in these complication, such as respiratory tract erosion or ulceration, pulmonary edema, bronchiolar or alveolar hypertrophy and lesion of esophagogastric tract[4–8].

One of the most important life-threatening gavage complication is lung edema, which is categorized as an acute lung injury. In general, lung edema is composed of hydrostatic pressure edema and permeability edema, which cause disturbance of pulmonary pressure and permeability of the blood-gas barrier, respectively[9]. The Aquaporins (AQPs) are membrane water-transporting proteins and examples of edema-related molecules that have been identified in the lung, including AQP-1, -3, -4 and -5[10]. AQP-1 was identified in microvascular endothelia and the pleura, AQP-3 identified in large airways, AQP-4 was identified in large- and small-airway epithelia, and AQP-5 was identified in type I alveolar epithelial cells. A number of reports have indicated that the expression of AQP-1, -4 and -5 changes in associated with lung edema, lung injury and pulmonary hypertension[10–22].

To increase the understanding of pathological processes of lung edema due to gavage-related complications and emphasize its effect on interpretation of treatment-related effects in toxicologic studies, in the present study, we categorized the type of lung edema and lung water clearance in associated with AQP-1, -4 and -5 expression between 2 groups of acute single-dose and sub-chronic repeated-dose toxicity studies (90-day). To demonstrate hydrostatic pressure edema, the occurrence of micro airway obstruction or endobronchial obstruction which exhibited by macrophages containing lipid vacuoles[23,24] and the occurrence of chronic pulmonary hypertension exhibited by tunica medial hy-

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perplasia of the pulmonary artery and peribronchial cuffing[25] were evaluated. To demonstrate permeability edema, the occurrence of perivascular edema, alveologenic edema and inflammatory response, especially mast cells, was measured[23]. To demonstrate AQP-1, -4 and -5 expression in the lung, immunohistochemistry was performed. Moreover, to demonstrate cardiac histopathology associated with gavage-related reflux, the occurrence of early stages of cardiomyopathy[26] was also evaluated.

## Materials and Methods

*Oral acute and sub-chronic toxicity tests*

All of the animal studies were performed in accordance with the Mahidol University policy for the care and use of animals for scientific purposes and approved by the institutional ethics committee. Forty samples of each lung and heart were randomly collected from a total of 170 samples from 4 oral toxicological studies of the extracts from a Thai herb (Pikhud Navakot) and fruit (Mangoesteen) at the National Laboratory Animal Center, Mahidol University, during 2011; ten samples were from acute test groups and 30 samples were from sub-chronic test groups. Healthy young adult Sprague Dawley rats (8 weeks old) were used at the beginning of those studies. The animal housing environment was controlled with a heating, ventilating and air conditioning (HVAC) system to achieve a temperature of 23 ± 2°C, 55 ± 15% relative humidity and, 10 to 15 air changes per hour ventilation and had a 12:12-h dark-light cycle; a pasteurized standard diet and 7 to 10 ppm chlorinated water were provided *ad libitum*. All rats were allowed an acclimatization period prior to being used for a test. The toxicity studies followed OECD guidelines 420 and 408 for acute and sub-chronic test, respectively[1,2]. Briefly, in the acute test, the rats were administered an oral dose of 4,000 mg/kg and then observed individually at 0.5, 4, 8, 12 and 24 h post dosing and at least once daily for 14 days. In the sub-chronic test, the rats were administered daily oral doses of 10, 100 and 1,000 mg/kg for 90 days and carefully observed individually each day for clinical signs.

*Test substance*

The extracts of Pikhud Navakot were kindly provided by Associate Professor Dr. Uthai Sotanaphun, Department of Pharmacognosy, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand. The extracts of Mangoesteen were kindly provided by Assistance Professor Dr. Aikkarach Kettawan, Institute of Nutrition, Mahidol University, Nakhon Pathom, Thailand. The extracts were prepared as follow. Briefly, the raw materials of the herbs were ground into powder. The powder was immersed in ten times its volume of 80% ethanol overnight, boiled for 3 h and then filtered to remove the residue. Next, the aqueous extracts were repeatedly boiled for 3 h and filtered two times. The aqueous extracts were spray dried to remove trace solvent. The percentage yield of the crude extract was roughly 20 to 25% of the raw material.

*Oral gavage*

Oral gavage was performed by well-trained and experienced staff. A stainless steel feeding needle (1.5 in., 20 gauge, 2.25-mm ball) was used. Each gavage treatment was given in a 1.0–3.0 mL bolus (10 mL/kg) of tested material. Fasting was conducted prior to oral dosing in all studies.

*Histopathology*

At the end of the test, which lasted 14 days for the acute test and 90 days for the sub-chronic test, surviving rats were euthanized by overdose inhalation of carbon dioxide. All tested rats were subjected to gross necropsy. The lungs and hearts were removed and fixed in 10% neutral buffer formalin for 48 h. Fixed specimens were embedded in paraffin, cut into 4-µm sectioned and then therefore stained with hematoxylin and eosin (H&E).

The lungs were examined histopathological as follows: alveolar septum thickness was measured as the ratio of the septal area to the alveolar sac, while peribronchial and perivascular cuffing, perivascular edema, alveologenic edema, tunica media hyperplasia of the pulmonary artery, mast cell accumulation and accumulation of alveolar lipid containing macrophage were evaluated by severity scoring. The severity scores were classified as follows: absent, 0; focal, 1; moderate, 2; and severe, 3. Moreover the hearts were examined histopathologically for the early stage of cardiomyopathy based on aggregation of mononuclear inflammatory cells with fibrosis, which was also graded by method above. Area measurements were performed using an image analysis program (ImageJ®, NIH, version 1.36, by Wayne Rassband) with 10 fields of the area of interested/sample.

*Immunohistochemical staining*

Ten lungs of each group (acute and sub-chronic tests) were randomized for immunohistochemical study. The immunohistochemical staining method was modified from of Papadopoulos and Verkman[27] and Ampawong *et al.*[28] Briefly, described (1) sample were deparaffinized with xylene and then rehydrated a graded of ethanol, (2) heat-induced antigen retrieval was performed in citrate buffer (pH 6), (3) endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol, (4) non specific binding was blocked with 10% fetal calf serum, (5) incubation was performed with 1:100 polyclonal rabbit anti-rat AQP-1, 4 and -5 (Millipore®) in PBS with 1% fetal calf serum at 4°C overnight, (6) incubation was performed with an EnVision Kit (DAKO®), (7) staining was visualized with diaminobenzidine (DAB; DAKO®), (8) the samples were counterstained with hematoxylin, (9) the samples were permanently mounted with DePX. The levels of AQP-1, -4 and -5 expressions were scored by the previously described method, particularly in the alveolar epithelium and bronchiolar epithelium.

*Statistical analysis*

Quantitative results were expressed as the mean ± standard error of mean. Data were statistically analyzed with IBM® SPSS® Statistics software version 20 using a one-way

**Table 1.** Differentiation of Pulmonary Lesions in Acute and Sub-chronic Toxicity Studies

| Pulmonary lesions | % Present | | P-value |
|---|---|---|---|
| | Acute (n=10) | Sub-chronic (n=30) | |
| Alveolar septal thickness | 25.0 | 100.0 | 0.000 |
| Peribronchial cuffing | 70.0 | 100.0 | 0.017 |
| Perivascular cuffing | 10.0 | 62.5 | 0.005 |
| Perivascular edema | 16.7 | 95.8 | 0.000 |
| Alveologenic edema | 80.0 | 4.2 | 0.000 |
| Alveolar lipid containing macrophage | 10.0 | 54.2 | 0.002 |
| Medial hyperplasia | 0.0 | 95.8 | 0.000 |
| Mast cell to perivascular edema | 0.0 | 75.0 | 0.000 |
| Early stage of cardiomyopathy | 10.0 | 21.8 | 0.694 |

P-value: Pearson's Chi-square.

analysis of variance (ANOVA) followed by Levene's test. To differentiate differences between groups, multiple comparisons with the Bonferroni test and Dunnett's test were performed for equal and non-equal variance assumptions, respectively. Bivariate correlation was examined by Spearman's rho correlation test. Proportional statistics were examined by Chi-square test.

## Results

*Mortality*

There were no treatment-related mortalities and clinical signs of toxicology in both the acute and sub-chronic studies. The treated and concurrent control groups were similar in terms of clinical manifestations.

*Lung histopathological description*

There were no pathology-related effects from any of the kinds of gavage material in this study when compared with the sham (normal saline solution) group. The lung lesions in the of sub-chronic test group were composed of thickening of the alveolar septum, peribronchiolar/vascular cuffing, perivascular edema, accumulation of alveolar lipid containing macrophage, medial hyperplasia of the pulmonary artery and mast cell accumulation to perivascular edema, while the acute test group exhibited a low severity of those lesions except for alveologenic edema as shown in Table 1.

*Alveolar septal thickness*

The ratio of the septal area to the alveolar sac in the sub-chronic test group (1.90 ± 0.08) was significantly higher than in the acute test group (1.05 ± 0.05) (P=0.000), which reflected the increasing alveolar septum areas related to leukocyte infiltration (Fig. 1A-B).

*Peribronchial and perivascular cuffing*

The occurrence of leukocyte infiltration into peribronchial (hyperplasia of bronchiolar associate lymphatic tissue, BALT) (Fig. 1C) and perivascular tissue (Fig. 1D) was significantly higher in the sub-chronic test group (1.08 ± 0.05 and 0.79 ± 0.08, respectively) than in the acute test group (0.70 ± 0.15 and 0.10 ± 0.05, respectively) (P=0.007 and 0.000, respectively).

*Perivascular/ Alveologenic edema*

The sub-chronic test group (1.33 ± 0.13) predominately exhibited perivascular edema (Fig. 1F) when compared with the acute test group (0.30 ± 0.15) (P= 0.000). On the other hand, alveologenic edema (Fig. 1E) found much more frequently in the acute test group (0.90 ± 0.17) than in the sub-chronic test group (0.16 ± 0.07) (P=0.000).

*Alveolar lipid containing macrophage/Medial hyperplasia*

Alveolar lipid containing macrophage (Fig. 1G) was again predominately exhibited in the sub-chronic test group (0.75 ± 0.16), while the acute test group (0.10 ± 0.10) exhibited a smaller amount (P=0.018). Medial hyperplasia (Fig. 1H) was absent in the acute test group, while it was generally observed (P=0.000) in the test group (1.62 ± 0.17).

*Mast cell to perivascular edema*

There were no mast cells in the perivascular area (Fig. 1I-J) of the acute test group but they were predominately found in the sub-chronic test group (1.08 ± 0.14). Moreover, the results demonstrated positive correlation between the number of mast cells and the severity of perivascular edema (P=0.017); Spearman's Rho correlation coefficient was 0.964 (Fig. 2).

*Early stage of cardiomyopathy*

The early stage of cardiomyopathy was not observed. However, the presence of focal mononuclear inflammatory cell aggregation with fibrosis, which is found in an early stage of cardiomyopathy (Fig. 1K) was similar in both acute (0.10 ± 0.10) and sub-chronic (0.37 ± 0.11) test group.

*Aquaporin-1, -4 and -5 expression*

AQP-1 is expressed on the pleura and vascular endothelium, and semiquantitative results demonstrated that the expression in the acute test group (2.08 ± 0.02) (Fig. 1M) was higher than in the sub-chronic test group (0.75 ± 0.04) (Fig. 1N) (P=0.001). Interestingly, expression of AQP-1 was decreased in edematous vessels (1.78 ± 0.25) (Fig. 1P) and

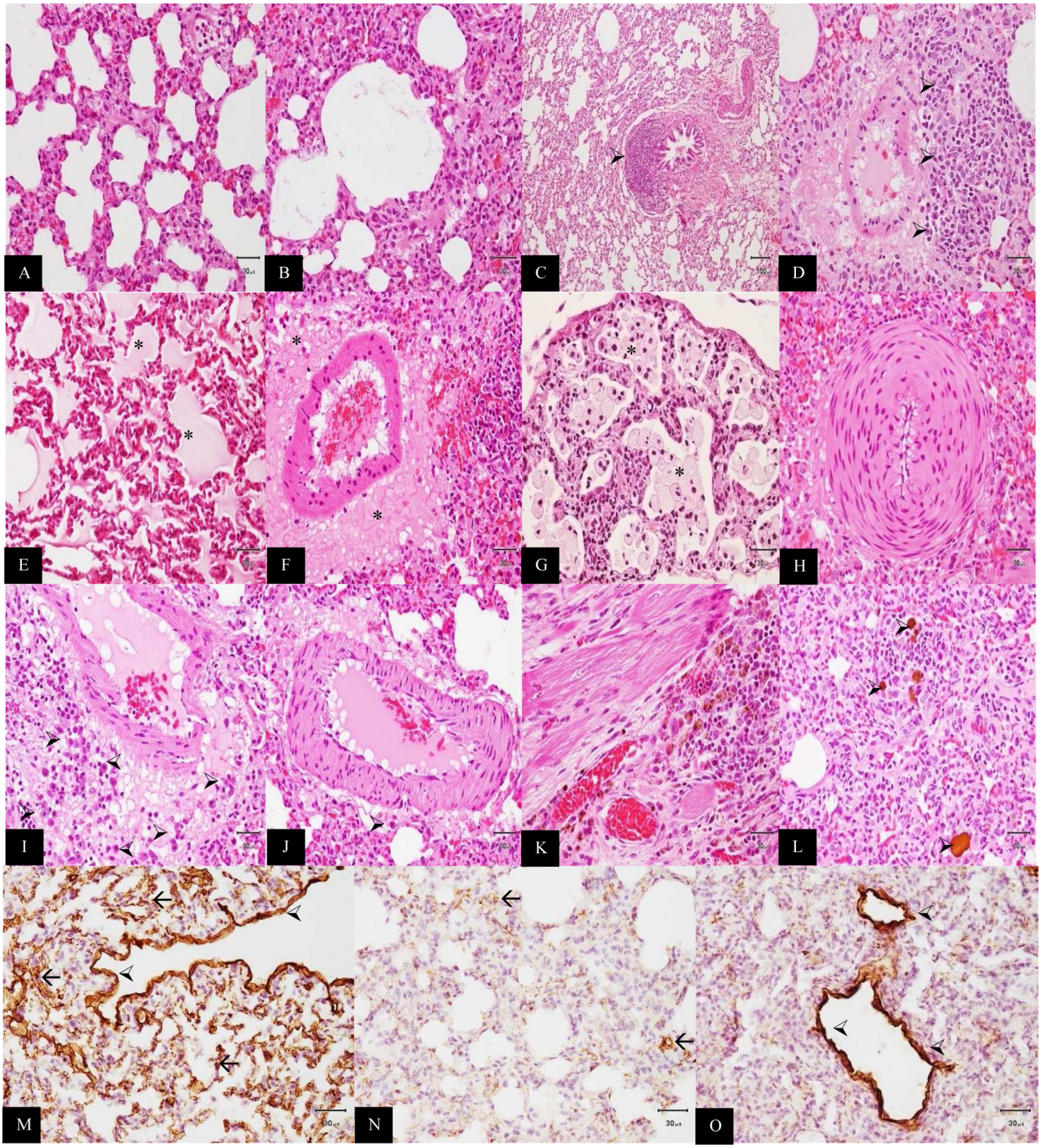


**Fig. 1.** Alveolar thickness (A) acute study, (B) chronic study, (C) peribronchial cuffing, (D) perivascular cuffing, (E) alveologenic edema (* eosinophilic material), (F) perivascular edema (* eosinophilic material), (G) aggregation of alveolar macrophage (*), (H) medial hypertrophy of pulmonary artery, (I) mast cell (arrow head) aggregation in an extensive perivascular edema area, (J) fewer mast cells (arrow head) in a smaller perivascular edema area, (K) focal lymphocyte aggregation with fibrosis in the myocardium, (L) phagocytized gavage material (arrow head) in multinucleated giant cells, hematoxylin & eosin staining, ×400, (M–W) localization and expression of AQP-1, -4 and -5 in the lung, (M–N) AQP-1 in microvascular endothelia (arrow) and the pleura (arrow head), higher expression in the acute test (M) than in the sub-chronic test group (N), (O) AQP-1 in normal vessels (arrow head), (P) AQP-1 in edema vessel (arrow head), (Q) AQP-1 in medial hyperplasia of the pulmonary artery (arrow head), (R–U) AQP-4 in small-airway epithelia (arrow head) and alveolar epithelia (arrow), higher expression in the acute tested group (R & T) than in the sub-chronic test group (S & U), (V–W) equal expression of AQP-5 in alveolar epithelial cells of the acute test group (V) and sub-chronic tested group (W), immunohistochemistry staining, ×1,000.

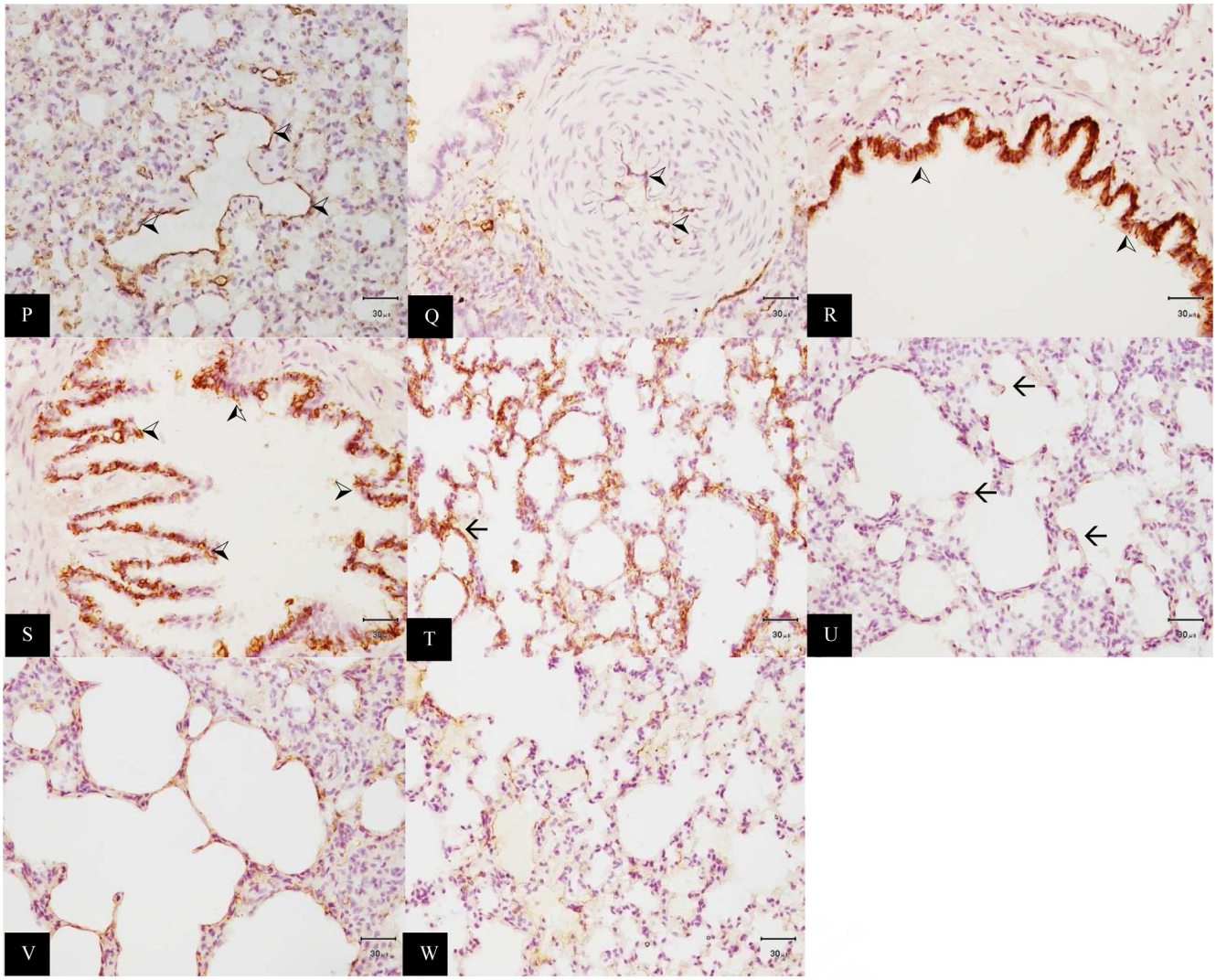


**Fig. 1.** Continued.

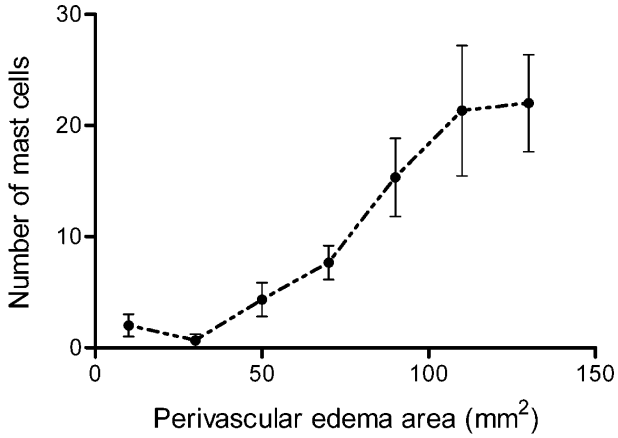


**Fig. 2.** Mast cells are increased proportional to the severity of perivascular edema.

medial hyperplasia vessels (0.54 ± 0.03) (Fig. 1Q) when compared with intact veins (3.54 ± 0.12) and arteries (2.48 ± 0.04), respectively (P=0.029, P=0.009) (Fig. 1O). AQP-4 was expressed on the bronchial and alveolar epithelium, and similar to the AQP-1 expression (1.54 ± 0.17 and 0.95 ± 0.04, respectively) (Fig. 1R & T), the acute test group exhibited than the sub-chronic test group (0.72 ± 0.24 and 0.15 ± 0.14, respectively) (Fig. 1S & U) (P=0.000 and 0.000, respectively). AQP-5 was expressed on the bronchiolar epithelium, and the results indicated that there was no difference in of expression between the acute (0.34 ± 0.14) (Fig. 1V) and sub-chronic (0.40 ± 0.25) test group (Fig. 1W).

The correlation between AQPs immunohistochemistry and individual pulmonary alteration in the acute and sub-chronic toxicity studies is presented in Table 2. Negative correlation was found between AQP-1 and -4 and alveolar septal thickness, peribronchial/vascular cuffing, perivascular edema, alveolar lipid containing macrophage, medial hyperplasia, and mast cell to perivascular edema. There was no correlation between AQP-5 and individual pulmonary alteration. Positive correlation was found between alveologenic edema and AQP-4.

**Table 2.** Correlation of AQPs Immunohistochemistry and Individual Pulmonary Alterations in Both Acute and Sub-chronic Toxicity Studies

| Pulmonary lesions | AQP-1 | | Bronchiolar AQP-4 | | Alveolar AQP-4 | | AQP-5 |
|---|---|---|---|---|---|---|---|
| | P-value | Coefficient | P-value | Coefficient | P-value | Coefficient | P-value |
| Alveolar septal thickness | **0.000** | **–.521** | 0.657 | –.089 | **0.000** | **–.545** | 0.987 |
| Peribronchial cuffing | **0.022** | **–.392** | 0.304 | –.182 | **0.019** | **–.401** | 0.828 |
| Perivascular cuffing | 0.228 | –.188 | **0.025** | **–.385** | **0.007** | **–.454** | 0.936 |
| Perivascular edema | **0.000** | **–.583** | 0.069 | –.281 | **0.001** | **–.549** | 0.460 |
| Alveologenic edema | **0.000** | **–.668** | 0.065 | 0.321 | **0.001** | **0.554** | 0.588 |
| Alveolar lipid containing macrophage | 0.090 | –.295 | 0.169 | –.242 | **0.023** | **–.389** | 0.844 |
| Medial hyperplasia | **0.000** | **–.661** | **0.002** | **–.509** | **0.000** | **–.695** | 0.614 |
| Mast cell to perivascular edema | 0.096 | –.290 | 0.309 | –.180 | **0.001** | **–.543** | 0.925 |

P-value: Spearman's rho (nonparametric correlation).

## Discussion

Pulmonary edema is a component of many lung conditions, such as inflammation, congestive heart failure, pulmonary neoplasm, agonal changes, or drug-induced conditions; however, most importantly, it may be a manifestation of acute lung injury[23]. The term edema is reserved for a poor cellular exudate characterized by the presence of pale, homogenous eosinophilic material in the alveoli, lung septate, and perivascular connective tissue. Noncardiogenic pulmonary edema, one of the most important complications of this procedure, consists of hydrostatic pressure edema and permeability edema which occur as a result of altered hemodynamics and increased permeability or rupture of the blood-gas barrier respectively, then create a protein-rich transexudate[9].

Irritant foreign bodies may induce severe pulmonary edema and a variable granulomatous inflammatory response, in which foreign material may be visible within well-developed granulomas[29] as shown in the sub-chronic toxicological study exhibiting phagocytized gavage reflux material (Fig. 1L). Oral administration of hexachlorobenzene in Brown-Norway rats results in development of a high background incidence of spontaneous granulomatous pulmonary lesions and is often used for the study of allergic airway disease[30] while no treatment-related effect of lung pathology was found in the present study.

In this study, it was surprising that only a single dose of oral gavage could cause permeability edema that exhibited alveologenic edema and an increase in of alveolar septal thickness with leukocyte infiltration (Fig. 1A-B) and bronchial/vascular cuffing (Fig. 1C-D) when compared with a normal lung. Apart from alveologenic edema (Fig. 1E), repeated-dose oral gavage caused extreme versions of both types of edema, the permeability type indicated by predominately perivascular edema (Fig. 1F) and the hydrostatic type indicated by micro-airway obstructive lesions particularly pulmonary arterial hyperplasia (Fig. 1H), and peribronchial/vascular cuffing (Fig. 1C-D) together with an increase in alveolar thickness and alveolar lipid containing macrophage (Fig. 1G). These results could be clarified to differentiate the type of edema correlated with acute and chronic oral gavage toxicity studies with correspondence to the lung histopathological finding in several reports[23–25,31]. The accumulation of mononuclear cells in perivascular cuffing and peribronchiolar cuffing is also related to the increasing alveolar thickness and contributed to the change in the blood gas barrier leading to edematous formation[25]. All of these histopathological changes might be coordinated affected to increase airway resistance, particularly at the level of peripheral airways, which causes hydrostatic pressure edema.

Permeability edema related to the evidence of perivascular edema (Fig. 1F) may be associated with perivascular mast cells (Fig. 1I). The present study showed that the number of mast cell was proportionally correlated with the severity of perivascular edema (Fig. 2). Histamine, an amine stored in mast cells, is a well-known activator of vascular permeability leading to permeability edema by changing the formation of interendothelial junction gaps and the tight junctional proteins[32,33]. Moreover, microvascular endothelial cells have the ability to contract when stimulated, particularly when stimulated by aspirated/reflux material. These cause the formation of endothelial pores that are accountable for the leakage and extravasation of plasma proteins, contributing to edematous formation[34].

Alveolar fluid clearance across the alveolar epithelium is a mechanism of fluid removal from the lung[35]. The bronchial circulation plays a significant role in the formation and reabsorption of both hydrostatic and permeability edema[36]. AQP-1 is expressed in microvascular endothelia throughout the lung and airways, AQP-4 is expressed in epithelia throughout the airways, and AQP-5 is expressed in type I alveolar epithelial cells. Many reports have demonstrated that AQP-1, AQP-4 and AQP-5 are not required for the physiological clearance of lung water in the lung or for the accumulation of extravascular lung water in the injured lung[10,11,15].

However, some reports have demonstrated that upregulation and downregulation of AQPs are closely related to pulmonary edema in different kinds of lung injury. AQP channels may have a protective effect in ventilator-induced lung injury[14]. This study demonstrated that pulmonary ede-

ma associated with oral gavage in the acute toxicity test was categorized as alveologenic edema exhibiting higher AQP-1 and -4 expression (Fig. 1M-W) than in the sub-chronic test (Table 2). Lung AQP-1 is markedly upregulated in animals exposed to hypoxia, suggesting that AQP-1 has $O_2$ permeability and thus could facilitate $O_2$ diffusion across the cell membrane[37]. AQP-4 mRNA expression is upregulated on the alveolar type II cell membrane to regulate the exchange of fluid between the alveolar space and alveolar epithelium barrier and play an important compensational role in pulmonary liquid clearance in the event of sodium transport damages in acute lung injury[19,45]. While the chronic toxicity study was categorized as exhibiting both hydrostatic pressure edema and perivascular edema, which lowered of the expression of AQP-1 and -4 (Fig. 1M-W) compared with the acute test (Table 2), downregulation of AQP-1 and -4 in the alveolar microvessels may act as a compensatory mechanism to protect against formation of excessive pulmonary edema[21]. Hypertonicity aspiration could induce the expression of AQP-1 and AQP-5[38,39]. They may facilitate removal of water from airspaces after accidental aspiration of materials[40]. The hyperisotonic pressure might be an important activator of AQP-1 and AQP-5 in the rat airway epithelium[19,20]. In addition, AQP-5 plays a protective role in the maintenance of pulmonary barrier function against *Pseudomonas aeruginosa* infection[22,44]. In some stages of pulmonary edema, the decreased expression of AQP-5 mRNA may be related to the severity of airflow obstruction[18]. This is similar to the finding of Dong *et al.*, who showed that AQP-1 and AQP-5 were significantly reduced after 24 h of foreign material-induced asthma and that anti-asthmatic agents could alleviate pulmonary edema through upregulation of the expression of AQP-1 and AQP-5 in mouse lungs.

The myocardium can be damaged by a variety of insults such as anoxia, ischemia, infectious agents and physical and chemical agents, and its pattern of response is limited[23]. In the rat, small foci of necrosis, focal inflammation and fibrosis are occasionally observed in young untreated rats of most strains and become more common with increasing age[41]. This study demonstrated that the oral gavage toxicity study did not caused the early stage cardiomyopathy (Fig. 1K).

Regarding to gavage-related reflux and technical gavage errors, even very small amounts of the treated material, 20 µL, were able to induce serious irritation in the respiratory tract and mortality[5]. The pathogenesis of a gavage-related reflux pathway of respiratory effects is described as mechanical and spontaneous refluxes by Damsch *et al.* Mechanically induced reflux is the most likely cause of reflux, occurring directly after gavage when withdrawing the tube from the animal. This is also called retrograde aspiration. On the other hand, spontaneous reflux may be related to gavage administration of a large volume resulting in gastric overflow. To reduce or protect against complications from oral gavage in chronic studies such as in pharmacological and toxicological studies, gavage modification methods have been used. Moreover, the use of brief inhalational anesthesia reduces gavage-associated death and euthanasia due to esophageal trauma and minimizes stress-related weight loss[42]. The animal stress and mortality related to oral gavage can be minimized when the procedure is carried out by an experienced technician[43].

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