DOI: 10.4149/BLL_2015_036

*Bratisl Lek Listy 2015; 116 (3)*
177 – 183

EXPERIMENTAL STUDY

# Ameliorative effect of Leflunomide on lung injury following an aspiration

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**Abstract:** *Background:* We aimed to investigate the therapeutic effectiveness of leflunomide (LEF) in lung injury after an aspiration of unknown pathophysiology.
*Material and methods:* Forty-two healthy Sprague Dawley rats were anesthetized and allocated to six experimental groups: saline (S) aspirated, S+LEF, hydrochloric acid (HCl) aspirated, HCl+LEF, formula aspirated (FOR), and FOR+LEF. The treatment groups (S+LEF, HCl+LEF, and FOR+LEF) received 20 mg/kg/day intraperitoneal (i.p.) injection of LEF for seven days. At the end of the seven days, blood and tissue samples were taken from the rats for histopathological, biochemical, and immunohistochemical examination.
*Results:* There was a significant increase in serum levels of YKL-40, a chitinase-like protein, in the HCl group after the aspiration ($p<0.01$). The increase in serum YKL-40 levels decreased significantly with LEF treatment ($p<0.01$). There was no significant difference in serum YKL-40 levels in the FOR group compared to the control group at pretreatment and in the FOR+LEF group at post-treatment. There was a significant increase in serum thiobarbituric acid-reactive species (TBARS) values in the HCl and FOR groups compared to the control group ($p=0.001$ and $p<0.01$, respectively). In both treatment groups, the serum TBARS values significantly decreased after treatment with LEF ($p=0.001$ and $p<0.05$, respectively). There was a significant improvement in the histopathological scores, which deteriorated after the aspiration, and in the number of inducible nitric oxide synthase (iNOS)-positive cells after treatment with LEF.
*Conclusions:* LEF can be a useful agent in the treatment of gastric content aspiration-induced acute lung injury (ALI) *(Tab. 1, Fig. 5, Ref. 43)*. Text in PDF *www.elis.sk.*
Key words: aspiration, lung injury, Leflunomide, iNOS, oxidative markers.

## Introduction

Foreign substances taken into the lungs through inhalation are described as aspiration. Aspiration pneumonitis (AP) refers to the inhalation of sterile gastric contents due to acute chemical lung injury induced by a noninfectious inflammatory response (1). Aspiration of acidic gastric content is a serious aspiration syndrome, with mortality rates up to 30–70 % (2). Changes in the level of consciousness, neuromuscular and gastrointestinal diseases, and diseases requiring intubation in the intensive care unit increase the risk of aspiration (3). In anesthesia practice, surgery is a predisposing factor for the development of AP in preoperative and postoperative periods. Thus, developing diagnostic and treatment modalities for AP is very important in the practice of anesthesia.

Most cases of chemical aspiration proceed to acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), with a high rate of morbidity and mortality and end-organ damage due to hypoxia (4). Factors contributing to hypoxemia include pulmonary edema, decreased surfactant activity, reflex airway closure, alveolar hemorrhage, and hyaline membrane formation (5). Increases in inflammation-induced free oxygen radicals cause additional damage by oxidation of cellular components such as lipids, proteins, and DNA. The most important damage is caused by lipid peroxidation, which results in distortion of the cell membrane function and structure. Malondialdehyde (MDA), the end product of lipid degradation, is considered to be an indirect indicator of lipid peroxidation. Levels of MDA are determined in blood by measurement of biochemical thiobarbituric acid reactive species (TBARS) (6).

Nitric oxide (NO), used as a signaling molecule in intracellular physiological and pathological processes, reacts with the increased levels of superoxide radicals induced by inflammation and forms peroxynitrite, which is a more potent oxidant (7, 8). As shown in many studies, levels of NO, produced by inducible NO synthase (iNOS), and iNOS in tissues and blood can be used to detect oxidation in inflammatory processes (9–11).

In recent years, many studies have investigated biomarkers that could be used in the diagnosis and monitoring of diseases,

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that are easy to use, and that provide more accurate results when measured in serum and tissues. Studies showed that YKL-40, a chitinase-like protein, was increased in serum and tissue, especially in chest diseases accompanied by inflammation, infection, and remodeling, such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, and pleural effusion (12–15). They also showed that YKL-40 Can be used as a biomarker for monitoring the severity of these diseases (12–15).

Leflunomide (LEF) is an effective drug used in the treatment of many diseases, particularly rheumatic conditions, due to its anti-inflammatory, antiproliferative, and immunosuppressive effects (16). It also has therapeutic uses in many other diseases due to its ability to protect against oxidative damage and its antifibrotic properties (20). It acts by inhibiting dihidro-orotate dehydrogenase and protein tyrosine kinase enzymes, which are important in the immune response (16–18).

In this study, we examined the therapeutic efficacy of LEF in lung injury caused by gastric aspiration, which is a particularly significant problem in the intensive care units, using oxidative markers to investigate the relationship between serum YKL-40 and the severity of damage in ALI due to pulmonary aspiration.

## Materials and methods

*Experimental protocol and animals*

This study was approved by the Experimental Animal Studies Ethics Committee of Ondokuz Mayis University, Samsun, Turkey. A total of 42 healthy female Sprague Dawley rats weighing 250–300 g were enrolled in this study and provided by the Experimental Research Center of the medical faculty of Ondokuz Mayis University. All the rats were maintained in windowless animal quarters at a temperature of 24 °C and humidity of 55–6 % under a 12 h light/12 h dark cycle, with free access to food and water.

The rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). They were allowed to breathe spontaneously during the surgical procedure. After a neck incision, a direct puncture with a 21-gauge needle was performed 2–4 tracheal rings below the larynx, and saline, hydrochloric acid (HCl), and formula (FOR) were injected into the larynx at a volume of 1 ml/kg. All the rats were allocated to six experimental groups: saline (S) aspirated, S+LEF, HCl (0.1 M, pH 1.25) aspirated, HCl+LEF, FOR aspirated (Ensure® Nutrition Shake, Abbott Nutrition), and FOR+LEF. Each group contained seven animals. After administration, the incision was repaired with a 6-0 ethilon suture.

After seven days of treatment of the S+LEF, HCl+LEF, and FOR+LEF groups with 20 mg/kg/day i.p. injection of LEF (19), all the rats were killed with an i.p. injection of ketamine hydrochloride and 10 mg/kg of xylazine. To obtain a sample of lung tissue, lungs were removed with bilateral anterior thoracotomy and median sternotomy after euthanasia. Samples were taken from both lungs for histopathological and immunohistochemical examination. Bloods samples were acquired from the hearts.

*Histopathological studies*

Immunohistochemical and histopathological analyses of the lung tissue samples were conducted to determine the effects of aspiration and treatment with LEF. The lungs were rapidly excised and fixed in 10 % buffered formalin for 24–72 h and embedded in paraffin as per routine procedures. Sections 5 μm thick were prepared from the blocks and stained with hematoxylin eosin for routine microscopic examination. A histopathological evaluation was performed in at least eight randomly selected microscopic high-power fields from each tissue sample. Subsequently, all slides were examined by a pathologist blinded to the study groups, and they scored all the microscopic slides according to the degree of peribronchial inflammatory cell infiltration (PICI), alveolar septal infiltration (ASI), alveolar edema (AED), alveolar exudate (AEX), and alveolar histiocytes (AHI) using the 4-point scale developed by Takil et al (Tab. 1) (20).

*Immunohistochemistry procedure (LEF, aspiration pneumonia)*

The lung tissue samples were fixed in 10 % neutral buffered formalin and embedded in paraffin. All the samples were sectioned at a thickness of 5 μm, deparaffinized, and rehydrated. The streptavidin-biotin-peroxidase complex technique (Histostain Plus Kit; Zymed, cat no: 85-8943, CA, USA) was used for the immunohistochemical study. Endogenous peroxidase activity was removed by incubation with 2 % hydrogen peroxidase in methanol for 30 min at room temperature. Antigen retrieval was performed by microwave treatment for 15 min in citrate buffer (pH 6.0). Rabbit polyclonal anti-inducible iNOS antibody (1/250; Abcam, cat no: ab3523, UK) was used as the primary antibody. Aminoethylcarbazole was used as the chromogen in H2O2 for 10 min. The sections were counterstained with Lillie Mayer's hematoxylin for 1 min and rinsed with tap water. Subsequently, the sections were mounted with an aqueous mounting medium. Immunohistochemical iNOS staining of the lung tissue slides were evaluated semiquantitatively according to the intensity of the differences between each experimental group. The staining intensity of iNOS was recorded as faint (−/+), mild (+), moderate (++), and strong

**Tab. 1. The 4-point scale used for histopathologic assessment.**

| | 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| PICI | No | Prominent germinal centers of lymphoid follicules | Infiltration between lymphoid follicules | Confluent bandlike form |
| ASI | No | Minimal | Moderate | Severe, impending of lumen |
| AED | No | Focal | In multiple alveoli | Widespread, Involving lobules |
| AEX | No | Focal | In multiple alveoli | Prominent, widesp |
| AHI | No | Scattered ina few alveoli | Forming clusters in alveolar spaces | Filling the alveolar spaces |

PICI – Peribronchial inflammatory cell infiltration; ASI – Alveolar septal infiltration; AED – Alveolar edema; AEX – Alveolar exudate; AHI – Alveolar histiocytes; IF – Interstitial fibrosis; GRA – Granuloma; NEC – Necrosis.

(+++). The evaluation of the immunostaining was performed in at least eight randomly selected areas per lung section, using two sections from each animal at 400× magnification. The final score calculated in each category for each individual rat was the mean of the scores from the sections of the lungs examined.

*Biochemical evaluation*

The blood samples were transferred to tubes without anticoagulant after the rats were sacrificed. The blood was incubated for 30 min at room temperature to allow clotting. Then, serum was obtained by centrifuging at 40 °C, 3000 g for 10 min. The supernatant was stored at –80 °C in another tube until the study was made. All the samples were dissolved at 2–8 0C for one day prior to the biochemical analysis.

The TBARS levels in the serum samples were measured with a TBARS assay kit (10009055, Cayman Chemical Company, Ann Arbor, MI, USA) in accordance with the procedures specified by the manufacturer. Malondialdehyde (MDA)-TBA products that formed in this assay, which are based on the reaction of MDA with TBA in an acidic environment, were measured colorimetrically at 540 nm. Test results were given as mmol/L.

Chitinase 3-like-1 (YKL-40) levels in one of the serum samples was measured with a YKL-40 ELISA kit (CK-E90203, Hangzhou Eastbiopharm Co. Ltd., Hangzhou, China) in accordance with the procedures of the manufacturer. The results of this test, run in conjunction with the sandwich ELISA method, in which a double-antibody was used, were given in ng/ml.

*Statistical methods*

All the histopathological and biochemical parameters were analyzed with the SPSS 21.0 package software for Windows. The biochemical measurements are shown as the mean (±) standard deviation (SD). The histopathological scores are presented as the median (min-max). All the measurements and scores were compared to Mann–Whitney U tests. The p value of $< 0.05$ was considered statistically significant.

## Results

*Biochemical findings*

Serum YKL-40 levels

There was no statistically significant change in the serum YKL-40 levels between the sham (18.33 ± 3.56 ng/ml) and the S+LEF groups (18.74 ± 0.64 ng/ml) ($p > 0.05$). Serum YKL-40 levels were significantly higher in the HCl group (32.61 ± 8.19 ng/ml) than in the sham group ($p < 0.01$), but there was no statistically significant difference in the serum YKL-40 levels between the FOR (24.81 ± 9.41 ng/ml) and sham groups ($p > 0.05$) (Fig. 1).

After the LEF treatment, serum YKL-40 levels were decreased in the HCl+LEF group (20.35 ± 3.13 ng/ml) compared to the HCl group ($p < 0.01$). There was no statistically significant change in the serum YKL-40 levels in the FOR+LEF group (19.15 ± 2.0 ng/ml) compared to the FOR group ($p > 0.05$) (Fig. 1) in response to the LEF treatment.

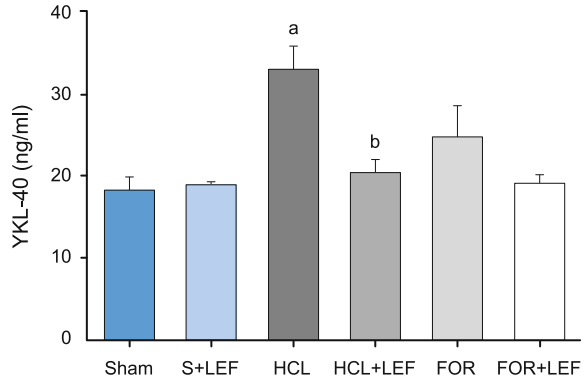


**Fig. 1. Serum YKL-40 levels in all study groups were expressed. a – $p < 0.01$ compared to Sham group; b – $p < 0.01$ compared to HCL group. Sham (S); Normal saline aspirated, S+LEF; S group treated with Leflunomide, HCl; Hydrochloric acid aspirated, HCl+LEF; HCl group treated with Leflunomide, FOR; Formula aspirated, FOR+LEF; FOR group treated with Leflunomide.**

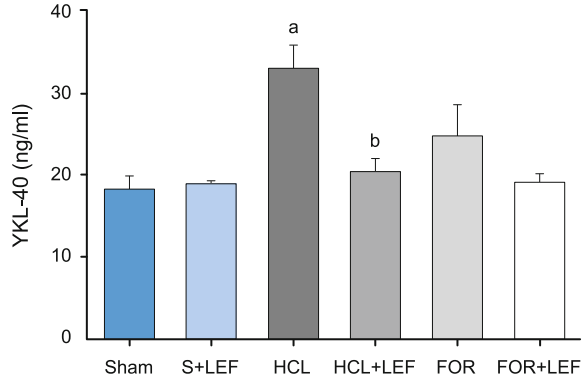


**Fig. 1. Serum YKL-40 levels in all study groups were expressed. a – $p < 0.01$ compared to Sham group; b – $p < 0.01$ compared to HCL group. Sham (S); Normal saline aspirated, S+LEF; S group treated with Leflunomide, HCl; Hydrochloric acid aspirated, HCl+LEF; HCl group treated with Leflunomide, FOR; Formula aspirated, FOR+LEF; FOR group treated with Leflunomide.**

Serum TBARS levels

There was no statistically significant difference in the serum TBARS levels between the sham (6.85 ± 1.37 µmol/L) and the S+LEF groups (7.43 ± 1.09 µmol/L) ($p > 0.05$). Serum TBARS levels were significantly higher in the HCl (19.94 ± 5.22 µmol/L) and FOR (17.88 ± 7.33 µmol/L) groups than in the sham group ($p = 0.001$ and $p < 0.01$, respectively) (Fig. 2).

The serum TBARS levels in the HCl+LEF group (7.32 ± 0.69 µmol/L) were statistically lower than in the HCl group ($p = 0.001$). In contrast, the serum TBARS levels in the FOR+LEF group (7.56 ± 0.64 µmol/L) were statistically lower compared to those in the FOR group ($p < 0.05$) (Fig. 2).

*Histopathological results*

The histopathological scores in the study groups are shown in the Figure 3. In the HCl group, PICI and lymphoid follicles were

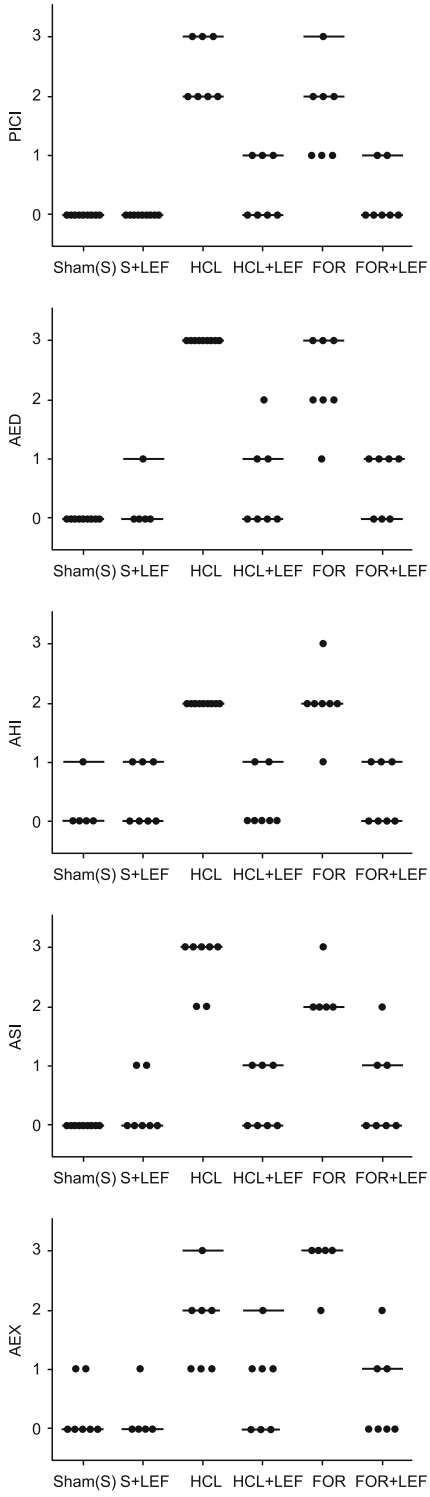


**Fig. 3. All histopathological 4-point scale scores of all study groups were represented. Peribronchial inflammatory cell infiltration (PICI); Alveolar septal infiltration (ASI); Alveolar edema (AED); Alveolar exudate (AEX); Alveolar histiocytes (AHI). Sham (S); Normal saline aspirated, S+LEF; S group treated with Leflunomide, HCl; Hydrochloric acid aspirated, HCl+LEF; HCl group treated with Leflunomide, FOR; Formula aspirated, FOR+LEF; FOR group treated with Leflunomide.**

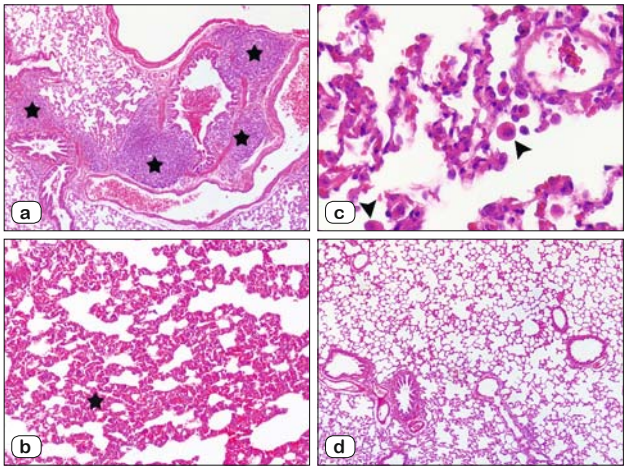


**Fig. 4. Histopathological evaluation of lung tissues in study groups were represented. a – Peribronchial inflamatory cell infiltration, showing infiltration between lymphoid follicules (star) in HCL group, HE x4, b – Moderate alveolar septal infiltration (star) in FOR group, HEx10, c – Multivacuolated alveolar histiocytes in alveolar space (arrow heads), FOR+LEF group, HEx40, d – There is no peribronchial and interstitial cell infiltration in Sham group, HEx4.**

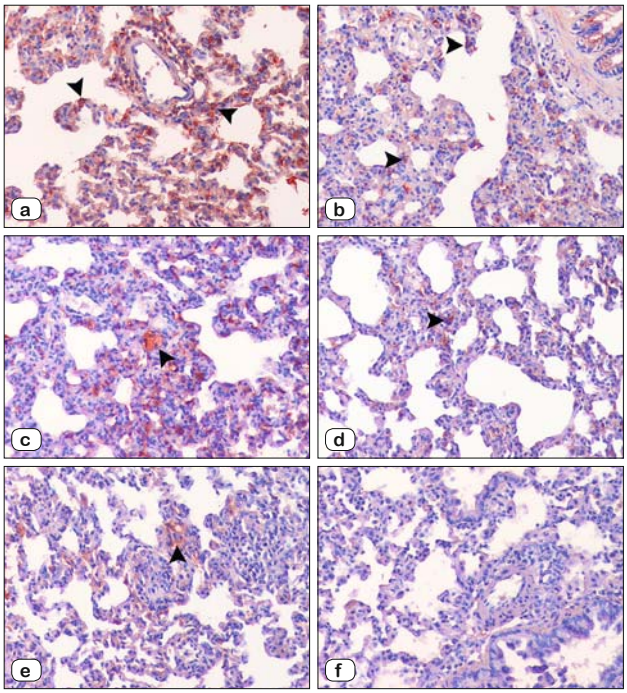


**Fig. 5. Immunohistochemical expression of iNOS in all study groups (a, c, e – study group, b, d, f – treatment group). Immunoperoxidase technique, Harris hematoxyline counter staining, AEC as a chromogen, x20. a – Strong immunopositive reaction of iNOS in the HCL group. Cytoplasmic and membranous staining of the cells in the interalveoler septal tissue was represented (arrow heads), b – Moderate immunopositive reaction of iNOS in HCL+LEF group, intracytoplasmic staining in the thickened alveolar septum (arrow heads), c – Moderate İNOS immunoreactivity in FOR group (arrow head), d – Weak immunohistochemical reaction in the interalveolar septal tissues (arrow head) of FOR+LEF group, e – Weak iNOS positive reaction in the interstitial cells in the interalveolar septal tissues of Sham group (arrow head), f – There is no immunopositivive reaction of İNOS in the SF+LEF group.**

prevalent in lung tissue (Fig. 4a). The histopathological scores for PICI, ASI, AED, AEX, and AHI were higher in the HCl group than in the sham group ($p = 0.001$, $p = 0.001$, $p = 0.001$, $p < 0.01$, and $p = 0.001$, respectively) (Fig. 3). All the scores in the FOR groups were increased compared to the sham group ($p = 0.001$). After FOR aspiration, moderate ASI was observed in the lung tissue (Fig. 4b). In the HCl+LEF group, all the histopathological scores, except for AHI ($p > 0.05$), were lower than in the HCl group ($p = 0.001$). There was a significant decrease in the PICI, ASI, AED, AEX, and AHI scores in the FOR+LEF group compared to the FOR group ($p < 0.01$, $p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively) (Fig. 3). There was no statistically significant change in any of the histopathological scores in the sham and S+LEF groups. The lung tissue in the sham group showed a normal structure (Fig. 4d).

*Immunohistochemical results*

In the HCl group, strong iNOS-positive cells were observed in the interalveoler septal tissue (median 3+ [3+/3+]) (Fig. 4a). iNOS-positive cells were significantly lower in the HCl+LEF (median 1+ [0+/1+]) (Fig. 4b) and FOR+LEF groups (median 1+ [0+/2+]) (Fig. 4d) compared to the HCl (median 3+ [3+/3+]) (Fig. 4a) and FOR groups (median 2+ [1+/3+]) (Fig. 4c). In the sham group, a weak iNOS-positive reaction (median -/+ [0+/1+]) (median) was observed in the interstitial cells in the interalveolar septal tissues (Fig. 4e). There was no immunopositive reaction of iNOS in the SF+LEF group (median 0+ [0+/0+]) (Fig. 4f).

**Discussion**

In this study, the pathogenesis of aspiration syndrome, which is a particularly significant problem in anesthesia and intensive care units, was evaluated in an experimental rat model. HCl aspiration of gastric contents and FOR, an important component of lung injury, was used in the experimental model. After HCl aspiration, a statistically significant increase was found in serum YKL-40 levels in parallel with lung injury. The increase in serum YKL-40 values after FOR aspiration was not statistically significant. LEF treatment was effective, especially in lowering the increased level of TBARS in acid aspiration-induced lung injury. Although there was no significant increase in serum YKL-40 levels in FOR aspiration-induced lung injury, TBARS levels, which were increased after lung injury, significantly decreased after the LEF treatment. LEF was effective in particular in reducing the expression of iNOS in tissue in the lung injury induced by HCl and FOR aspiration.

ALI is a diffuse form of heterogeneous lung injury, which develops due to primary (e.g., pneumonia) and secondary (e.g., pancreatitis) factors. It may cause life-threatening complications, such as ARDS (21). AP is one of the most important causes of primary lung damage. AP is common during anesthesia and intensive care and develops due to aspiration of gastric contents. Aspiration of gastric contents during the induction of anesthesia and recovery from anesthesia and aspiration of food during long-term enteral feeding in intensive care units give rise to serious complications (22). Although many studies have investigated the complex inflammatory mechanisms of ALI, there is no specific treatment that provides therapeutic efficacy (23, 25). Research is ongoing on reactive oxygen species (ROS), nitrogen species, and proinflammatory cytokines that contribute to the inflammatory process. Alveolar-capillary damage, leakage of protein-rich fluid into the interstitium and alveolar space, excessive release of cytokines, and neutrophil migration cause hypoxia and systemic damage in the acute phase of ALI (23). In our study, we observed a significant increase in PICI, ASI, AED, AEX, and AHI in lung tissue in the histopathological examination after FOR and HCl aspiration. This finding is similar to that reported in other working models, which demonstrated a comparable damage after gastric content aspiration (20, 24, 25).

One of the most important mechanisms responsible for lung injury is a direct cell damage due to oxidation of the lipid component in the cell membrane caused by ROS, the production of which increases in response to activated macrophages, endothelial cells, and polymorphonuclear neutrophils (25). The degree of lipid peroxidation, which disrupts the permeability and the integrity of the membrane, is determined by TBARS levels in blood and tissue (26). Many experimental studies have used serum TBARS levels in the follow-up of inflammation, which forms as a result of tissue damage (27, 6). In an experimental ischemia-reperfusion study of rats, Yildiz et al. stated that serum TBARS levels are quite an effective marker in the determination of the severity of the oxidative stress that increases after injury (16). We found that serum TBARS levels increased in line with the histopathological findings of aspiration-induced lung injury. We observed a decrease in the serum levels of TBARS after the treatment with LEF. The findings highlight the importance of lipid peroxidation, especially in the damage that occurs in lung tissue after aspiration.

Many studies have demonstrated an increase in NO metabolites in body fluids in various models of ALI and ARDS (28, 29). NO is the inflammatory mediator synthesized from L-arginine, an amino acid (8) and peroxynitrite, which is formed by NO, thought to play an important role in the advancement of ALI (9). Studies have demonstrated that selective inhibition of iNOS decreases tissue damage (11, 30). In our study, we observed that immunohistochemical iNOS expression and lipid peroxidation were significantly increased in the HCl and FOR aspiration groups and that LEF treatment significantly decreased this expression.

Various rating systems are available for diagnosis of ALI. However, none are superior in disease follow up. New evaluation methods are required which show the type, degree, and severity of inflammation (9). Studies have used various biomarkers in epithelial fluid obtained from bronchoalveolar lavage, serum, and tissue to investigate lung inflammation (31, 32). Examples include inflammatory cytokines and molecules produced by epithelial and inflammatory cells, such as cellular growth factors, enzymes, and plasma-derived proteins (33–35). Endothelial and alveolar epithelial damage in ALI results in a systemic inflammatory response. (23). Much research is focused on identifying serum and tissue biomarkers in ALI that can be used to follow up inflammation. YKL-40 383 is a glycoprotein composed of amino acids. YKL-40 controls basic functions in mammalian cells, such as mitogenesis, differentiation, and extracellular matrix homeostasis. It is produced in many cells of the body, such as macrophages, neutrophils, chon-

drocytes, fibroblasts, vascular smooth muscle, endothelial, hepatic stellate, colonic, and airway epithelial ductal cells, and serum levels of YKL-40 increase significantly at sites of tissue damage (36, 37). Recent studies reported that YKL-40 levels are increased in bronchial asthma and chronic obstructive pulmonary disease and that they can be used as a biomarker for these diseases (37–39). Guerra et al reported that YKL-40 is an indicator of reduced lung function and that it can be used as a biomarker in the monitoring of the pulmonary function of chronic cigarette smokers (41). Kim et al measured the level of YKL-40 in patients with pulmonary or pleural diseases (12). They found a higher level in patients with pneumonia compared to the control group and patients with pleural disease, asthma, or cancer. They stated that YKL-40 is a more valuable biomarker in patients with pneumonia than in patients with asthma (12). In our study, we found serum YKL-40 levels increased in lung injury occurring after the aspiration consistent with that reported in the literature.

LEF is an anti-inflammatory agent used in particular for the treatment of autoimmune diseases. It is thought to be effective in inhibiting proinflammatory cytokines by active metabolite A771726 (teriflunomide) (42, 16). Many studies have reported that the anti-inflammatory and immunoregulatory effects of LEF suppress the formation of ROS and tumor necrosis factor induced by lipid peroxidation and nuclear factor-kappa B activation, respectively, both of which play an important role in inflammation (18, 19, 42). In an intestinal ischemia-reperfusion study performed on rats, Yildiz et al demonstrated that LEF exhibited anti-inflammatory and antioxidant effects on damaged intestinal tissue (16). Similarly, in a renal ischemia/reperfusion study performed on rats, Karaman et al. demonstrated the efficacy of LEF in combatting oxidative damage following ischemia-induced ROS production in kidney (43).

In summary, the inhalation of gastric contents plays an important role in aspiration-induced lung damage. HCl is responsible for the majority of this damage. LEF therapy appears to be effective in combatting lung injury that develops secondary to aspiration. The current study can lay the groundwork for clinical studies.

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Received October 30, 2013.
Accepted November 12, 2013.