

Prophylactic Inhalation of *L-alanyl-L-glutamine* Enhances Heat Shock Protein 72 and Attenuates Endotoxin-Induced Lung Injury in Rats

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Short title: GLN inhalation enhances HSP72

Summary

Studies have demonstrated that heat shock protein70 (HSP70) plays an important role in the protection of stressed organisms. The development of strategies for enhancing HSPs expression may provide novel means of minimizing inflammatory lung conditions, such as acute lung injury. This study aimed to examine the effect of L-alanyl-L-glutamine (GLN) inhalation in enhancing pulmonary HSP72 (inducible HSP70) expression and attenuating lung damage in a model of acute lung injury induced by Lipopolysaccharide (LPS) inhalation. The experimental rats were randomly assigned to one of four experimental groups: (1) NS: saline inhalation; (2) NS-LPS: pretreatment by saline inhalation 12 hours before LPS inhalation; (3) GLN: glutamine inhalation; (4) GLN-LPS: pretreatment by glutamine inhalation 12 hours before LPS inhalation. The results show that GLN compared with saline administration, led to significant increase in lung HSP72 both in non LPS-treated rats and LPS-treated rats. In LPS-treated rats, pretreatment by GLN inhalation produced less lung injury as evidenced by the decrease in lung injury score and dramatic decrease in lactate dehydrogenase (LDH) activity and polymorphonuclear leukocyte cell differentiation counts (PMN %) in the bronchoalveolar lavage fluid. The study indicates that prophylactic glutamine inhalation associated with the enhancement of HSP72 synthesis attenuates tissue damage in experimental lung injury.

Key Words: glutamine, heat shock protein72, acute lung injury, aerosol therapy

Introduction

A self-protective phenomenon, a so-called heat shock response (HSR) was first observed in *Drosophila* embryos by Ritosa *et al.* in 1962 (Ritossa 1962). Sudden heat stress can induce the expression of a set of proteins in living cells referred to as stress or heat shock proteins (HSPs). They are synthesized constitutively or inducibly under a wide variety of stresses. Among the 70-kDa family of HSP (HSP70s), inducible HSP70, also named as HSP 72, is the most important protein in the HSP family playing a vital role in cellular protection against injuries in the presence of various stresses (Dong *et al.* 2005, De Maio 1999, Karinch *et al.* 2001, Schmitt *et al.* 2007) . Several reports have demonstrated that external heating or administration of sodium arsenite results in expression of HSP72 and significantly reduces end-organ injury and mortality rate resulting from endotoxemia caused by *Escherichia coli* lipopolysaccharide (LPS) or cecal ligation and puncture (CLP) (Bruemmer *et al.* 2001, Hotchkiss *et al.* 1993, Villa *et al.* 1994, Ribeiro *et al.* 1994, Chen *et al.* 2003, Huang *et al.* 2012). However, neither heat stress nor sodium arsenite are clinically relevant. The development of practical

strategies for enhancing HSP72 expression may provide novel means of therapeutic intervention in critically ill patients.

Glutamine (GLN), a nonessential amino acid, is the most abundant in the healthy human being and has also been considered a “conditionally essential” amino acid in states of serious illness or injury (Roth 2008, Bongers *et al.* 2007). More recently, studies have supported the evidence that GLN mediated enhancement of HSP72 works against inflammatory injury and illness in experimental settings (Morrison *et al.* 2006, Singleton *et al.* 2005). Studies have implied that septic shock and acute lung injury lead to a down regulation of HSP70 expression in the lung. The decrease in HSP70 may lead to a worsening of lung tissue metabolism, subsequent lung injury, and organ failure. This decreased HSP70 expression can also be corrected by administration of GLN (Wischmeyer *et al.* 2001, Singleton *et al.* 2007). Research in animal models of sepsis and lung injury demonstrates the beneficial effect of supplemental GLN on survival, metabolic function, immune function and tissue protection (Zhang *et al.* 2009, Jing *et al.* 2007, Singleton *et al.* 2005). Clinical trials in human subjects have also demonstrated that GLN reduces infectious complications, inflammatory response and ICU mortality (Vermeulen *et al.* 2007, Wischmeyer 2008, Sufit *et al.* 2012). Now, increasing evidence suggests GLN as a nontoxic, clinically relevant enhancer of HSP72 expression in critically ill patients. GLN appears to be a possible candidate for

novel cytoprotective drugs by enhancement of HSP72 expression. Moreover, reported routes of administration for exogenous glutamine to prevent glutamine-deficiency in critically ill patients are mainly oral, parenteral, enteral and intravenous. Therefore, we designed a practical method to administer GLN via airway inhalation. Inhalation therapy refers to the delivery of drugs directly to the lungs with minimal risks of systemic effects (Rubin 2010). Besides, the onset of action for nebulized GLN is substantially faster than for oral or parenteral formulation. Apart from the route of administration, other points of interest are the expression of HSP72 evoked by a single dose of GLN inhalation in the lung tissue and its beneficial effects. This study aimed to examine the hypothesis that inhalation of nebulized GLN could enhance pulmonary HSP 72 expression and prophylactically attenuate lung damage in a rat model of acute lung injury induced by Lipopolysaccharide (LPS) inhalation.

Materials and Methods

Animals

Experiments were performed on adult male Sprague-Dawley (SD) rats weighing 280-380 g. Room temperature was maintained at $21 \pm 1^\circ\text{C}$ under a 12 h light-dark regimen. Food and water were provided *ad libitum*. Our study adhered to the

guidelines of the National Institutes of Health for the use of experimental animals and all experiments were approved by the Committee for the Use of Experimental Animals of Kaohsiung Medical University (IACUC Approval NO: 99062).

Time course effect of GLN inhalation on HSP 72 expression

The time-response curve from 0 to 20 h (0 h, 4 h, 8h, 12h, 16h, 20h) after GLN inhalation was examined at a dose of 1.5g GLN. Lung tissues were harvested at various time points after GLN inhalation. HSP 72 expressions were analyzed by western blot and the 12-h time point was chosen to obtain the maximal response of HSP72 protein expression.

Experimental protocol:

Rats were randomly assigned to one of four experimental groups, (1) NS (n=8): saline placebo inhalation; (2) NS-LPS (n=8): pretreatment by saline inhalation 12 hours before LPS inhalation; (3) GLN (n=8): glutamine inhalation; (4) GLN-LPS (n=8): pretreatment by glutamine inhalation 12 hours before LPS inhalation. For all manipulations, rats were given intraperitoneal injections of ketamine (80 mg/kg) and xylazine (12 mg/kg). **At the end of the experiment, the rats were sacrificed and samples were harvested under a lethal dose of ketamine (100 mg/kg). The NS and GLN groups**

were devitalized at 12 h after inhalation treatment whereas rats from NS-LPS and GLN-LPS groups receiving NS or GLN prophylactic inhalation respectively were sacrificed at 8 h after LPS inhalation. The degree of lung injury was assessed by various parameters including pathologic change in lung tissue, and lactate dehydrogenase (LDH) activity and polymorphonuclear leukocyte cell differentiation counts (PMN %) in the bronchoalveolar lavage fluid (BALF). The HSP 72 of lung tissue was examined by Western blot analysis.

Drug preparation and Delivery

Lipopolysaccharides and L-Alanyl-L-glutamine were purchased from Sigma Chemical (St. Louis, MO, USA). GLN was prepared as a 38% solution dissolved in 0.9% saline solution. Approximately four milliliters of GLN solution were administered via nebulization to yield a single dose of 1.5g GLN. The LPS stock aliquot (2mg/ml) was reconstituted by dissolving *Escherichia-coli*-derived LPS in endotoxin free sterile 0.9% saline. The LPS solution was prepared by diluting 1 ml LPS stock in 1ml 0.9% saline for nebulization. Saline solution was used as a placebo. Nebulization of inhaled agents was performed by Aeroneb Pro (Aerogen) and delivered via a nose cone (Rodent anesthesia mask, Braintree Scientific, Inc.). The treatment time was approximately 15-20 minutes for GLN and 5-10 minutes for LPS.

BALF analysis

The left lung was intratracheally lavaged twice with 0.9% saline (2.5ml per lavage).

The effluents were centrifuged at 3,000 rpm at 4°C for 10 min. The BALF supernatant was immediately examined for LDH activity. PMN % was used to measure migration of PMN into the alveoli. To perform cell differentials, cells were fixed on glass slides by using cytospin and were stained with Wright-Geimsa. The ratio of PMN to total cell (PMN %) was counted to be at least 200 cells/smear (Ong *et al.* 2003).

HSP 72 detection in lung tissue

Western blotting was performed as previously described (Wang *et al.* 2012). The monoclonal anti-HSP72 antibody (Enzo Life Science, New York, USA) were utilized as the primary antibody. The anti-mouse immunoglobulin G (GE Healthcare) conjugated with peroxidase was used as the secondary antibody. Densitometric analysis was performed using Bio-1D V.97 software (Vilber Lourmat, Deutschland).

Lung Pathology

Histology was assessed by hematoxylin and eosin staining. For quantification of lung injury score, various degrees of lung injury score were assessed as degree 0 to 4 in two categories: edema and cellular infiltration. A score of 0 represented normal findings

and scores of 1, 2, 3 and 4 represented mild (<25%), moderate (25-50%), severe (50-75%) and very severe (>75%) lung involvement. The histopathological assessment was performed in a blind fashion by several laboratory assistants. Each one gave a score for edema and cell infiltration from 0 to 4. The individual scores were added together to obtain a final score, ranging from 0-8 (Chuang *et al.* 2007, Hironori *et al.* 2010).

Statistical Analysis

Values are expressed as means \pm standard deviation (SD). Statistical evaluation of the group comparison was made with one-way ANOVA and paired Student t test.

Differences were considered statistically significant at $P < 0.05$.

Result

Time course effect of GLN inhalation on HSP 72 expression

Figure 1 shows the time-response graph for experiments examining multiple time points from 0 to 20 h at a dose of 1.5g GLN inhalation for HSP72 induction. There was a significant variation in HSP 72 expression in all GLN treated animals at different time points (compared with 0 h, $P < 0.05$). Notable findings include a peaking at 12 h (compared with 0 h, $P < 0.0001$) and a non-significant decay after 16 h and 20 h

(compared with 12 h, $P > 0.5$, $P > 0.3$).

GLN enhances HSP72 expression in non LPS-treated and LPS-treated rats

As determined by western blot analysis (Fig. 2), glutamine compared with saline inhalation led to a significant increase in lung HSP 72 both in non LPS-treated rats (NS compared with GLN, 1 ± 0.15 Vs. 2.10 ± 0.46 , $P < 0.0001$) and LPS-treated rats (NS-LPS compared with GLN-LPS, 0.68 ± 0.16 Vs. 1.33 ± 0.27 , $P < 0.001$). LPS inhalation down-regulated HSP-72 expression compared with saline inhaled animals (NS vs. NS-LPS, $P < 0.05$).

GLN's protective effects on LPS induced lung injury

To examine the effect of GLN inhalation on protection from LPS induced lung injury, a single dose of 1.5g GLN or saline solution was used to pretreat the subject rats 12 hours before LPS inhalation. In LPS-treated rats, pretreatment by glutamine inhalation (GLN-LPS) significantly increased lung HSP 72 expression compared with the saline pretreatment group (NS-LPS) (Fig. 2, $P < 0.001$). The degree of lung injury was assessed by various parameters including pathologic change in lung tissue, and LDH activity and differential PMN % in the BALF.

Lung pathology

Inhaled LPS elicited marked pulmonary edema and infiltration of PMN into bronchoalveolar space (Fig. 3A.). The histomicrograph and quantification of lung injury score revealed that GLN pretreatment significantly attenuated the extent of lung injury (NS-LPS compared with GLN-LPS, 6.5 ± 1.4 vs. 3.9 ± 0.9 , $P < 0.0001$, Fig. 3B). Animals receiving GLN only did not show pathological changes in lung tissue.

PMN% and LDH in bronchoalveolar lavage fluid

Inhaled LPS led to significant increases in PMN% and LDH in all LPS-treated animals (NS-LPS and GLN-LPS) compared with the NS group ($P < 0.0001$; Fig.4 & Fig.5). In the GLN-LPS group, pretreatment with GLN dramatically reduced PMN% versus the NS-LPS group receiving saline pretreatment ($49.5 \pm 12.3\%$ vs. $85.9 \pm 9.2\%$, $P < 0.0001$; Fig. 4) and also decreased LDH activity (79 ± 14.4 vs. 129.2 ± 24.5 mAbs/min, $P < 0.0001$; Fig.5) in the BALF. Animals receiving GLN only did not show any increase in either PMN% or LDH.

Discussion

In the present experiment, we used a rat model of acute lung injury induced by LPS inhalation. The degree of lung injury was assessed by various parameters including pathologic change in lung tissue, and LDH activity and PMN % in BALF. The results imply that prophylactic inhalation of nebulized glutamine 1) enhanced lung tissue HSP72 synthesis, 2) decreased lung injury score, 3) reduced LDH activity and neutrophils in BALF. The beneficial effect of inhaled GLN is associated with its capacity to induce HSP72 expression in rats' lungs.

In previous animal and human studies, GLN has been administered as a continuous infusion, usually as a supplement to parenteral or enteral nutrition. Here, we report for the first time on GLN administered pharmacologically as a single dose via inhalation. The optimal dose of inhaled 1.5g GLN for these experiments is based on previous titration data (not shown) indicating the maximal HSP72 expression occurs at the lung tissue. Because GLN is given as a single dose over a short period, lung tissue levels of HSP72 may increase transiently; therefore we also examined multiple time points from 0 to 20 h after GLN inhalation and the 12-h time point was chosen to obtain the maximal response of HSP72 expression (Fig 1).

In the experimental model of acute lung injury, the most widely used method to quantify lung injury histologically is lung injury score. However, there is no

agreement on which specific parameter should be adapted for scoring because the histological features varied with different models and different phases during acute lung injury (Matute-Bello *et al.* 2008, Hironori *et al.* 2010). In our preliminary studies, we found that inhalation of nebulized LPS at a dose of 2 mg was followed by an early phase response characterized by increases in BALF PMNs and LDH; and down-regulation of HSP72 at 8h after LPS inhalation. The late phase response that followed was characterized by gradual normalization of BAL PMNs; and up-regulation of HSP72 at 16~32 after LPS inhalation. It is noteworthy that during late phase response there was a hyaline membranes formation which was absent during early phase response. These findings indicate that certain parameters such as hyaline membrane formation for lung injury scoring were not uniform during early versus late phases of response. Accordingly, in our study we selected particular histological features such as edema and cell infiltration at time-point of 8-h which is regarded as most reliable during acute lung injury.

Studies have documented that significant neutrophil recruitment may be responsible for the production of inflammatory mediators after an LPS challenge. GLN supplementation markedly attenuates the increased bronchoalveolar epithelial permeability (Hulsewe *et al.* 2003, Tjader *et al.* 2007). Singleton *et al.* (2007) also report that GLN has been shown to act against lung injury with the beneficial effect of

healing lung parenchymal injuries and reduction of air leakage in rats. In this regard, GLN tends to play a protective role in alveolar barrier injury and inflammatory exudation. In connection with those studies, our results have also indicated that pretreatment by inhaled GLN could protect rats from LPS-induced ALI. Inhaled GLN markedly improved lung morphology, inhibited pulmonary edema formation and prevented infiltration of PMNs into alveoli (Fig.3). The increases in BALF PMN counts and LDH activity induced by LPS were also abated by the prophylactic glutamine inhalation (Fig.4 & 5). It should be point out that the distribution of LDH isoenzymes varied in different tissues and the LDH-3 is the predominate isoform in the lungs (Drent *et al.* 1996). Although we have not determined the isoform of LDH, we have examined changes in total LDH in the circulating serum versus BALF. We found that total LDH in BALF was increased while serum LDH failed to change. These findings suggest that the LDH detected in BALF was most likely released locally from injured lung, and preassembly it consisted predominately the LDH-3 isoform.

Glutamine has a wide range of effects, including tissue protection 、immune regulation 、antioxidant capacity and stabilization of metabolism. Among these, enhanced HSP 72 expression plays an important role in the anti-inflammatory and tissue protection effects. The enhancement of HSP72 expression is not the only effect of GLN mediated protection but it is certainly the most significant and promising effect

in the treatment of lung injury (Roth 2008, Singleton *et al.* 2005, Wischmeyer *et al.* 2001, Vermeulen *et al.* 2007). The remarkable study done by Singleton *et al.* (2007), utilizing mice with specific deletion of the HSP72 genes proved that GLN's mediated protection against lung injury is dependent on HSP72 expression. Another study done by Morrison *et al.* (2006) found that in heat shock factor-1 knockout cells, GLN's ability to generate an HSP response is lost and the concomitant protection of GLN's is also abolished. In agreement with much prior data, we deem that GLN mediated protection is highly correlated with HSP72 enhancement.

It is known that GLN is translocated out of lung and skeletal muscle in response to many forms of injury and stress (Tjader *et al.* 2007). Studies have indicated that in sepsis or lung injury, the down regulation of HSP72 expression may be due to a deficiency of lung tissue GLN. This relative decrease of HSP72 expression may lead to a worsening of lung tissue metabolism, subsequent lung injury, and organ failure (Bongers *et al.* 2007, Singleton *et al.* 2005, Zhang *et al.* 2009, Jing *et al.* 2007). In the present study, we also observed that HSP72 expression appears to be down-regulated at 8-h after LPS inhalation (Fig.2) and the down-regulation of HSP72 in turn aggravated lung injury; whereas prophylactic GLN inhalation not only prevented the occurrence of HSP72 down-regulation (Fig.2), it also attenuated the LPS-induced lung injury. These observations suggest that the LPS associated HSP72 down-regulation could be

abolished by GLN pretreatment, and in addition the protection effect of GLN is dependent on the level of HSP72 expression.

The limitation of this study is the lack of data investigating the effect of therapeutic GLN given at the time of LPS insult or thereafter, which would be of practical utility in a clinical setting. The present study does imply a positive outcome from prophylactic GLN inhalation, and an investigation of therapeutic applications of GLN for the treatment of acute lung injury is planned as part of our future studies.

Aerosol therapy is commonly used to administer certain drugs, and it provides a means to treat various pulmonary diseases. The advantage of aerosol therapy is the minimal systemic adverse effects with the use of smaller doses, besides giving a rapid response. In recent times, more and more new drugs, including bronchodilators, mucolytics, antibiotic, anti-inflammatory agents and insulin are becoming available for use as aerosol therapy (Rubin 2010). The increasing evidence, including the present study implicating GLN as a nontoxic, clinically relevant enhancer of HSP72 expression, supports the development of GLN for use as an inhalation agent providing a novel means of therapeutic intervention in a clinical setting.

In conclusion, the present results imply, for the first time that a single dose of prophylactic glutamine inhalation can enhance tissue HSP72 synthesis; these results

also indicate that enhanced HSP72 may have functional significance as GLN-pretreated animals developed less lung damage following LPS inhalation. GLN inhalation appears to be a possible strategy to induce HSP72 to manage inflammatory airway diseases. Apart from these findings, future research could focus on the prophylactic function of inhaled GLN against occupational pneumoconiosis and other chronic lung diseases.

Conflict of Interest

The author declares no conflicts of interest.

Acknowledgments

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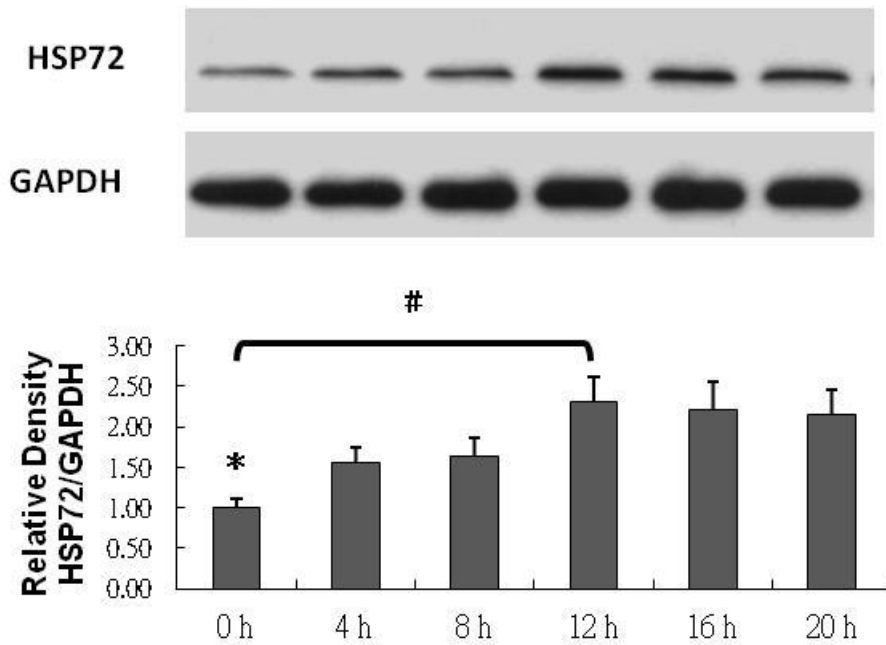


Fig.1. Time course effect of inhaled GLN on HSP72 expression in lung tissue of rats.

HSP72 expression was analyzed by Western blotting analysis. Relative density refers to the ratio of HSP72 to GAPDH. Image represents results found in triplicate. Relative densitometric results are expressed as mean \pm SD of levels relative to that of the control group (0 h), * $P < 0.05$ vs. all other groups. Notable findings include a peaking at 12 h ($^{\#}P < 0.0001$ vs. 0 h) and a non-significant decay after 16 h and 20 h (compared with 12 h, $P > 0.5$, $P > 0.3$).

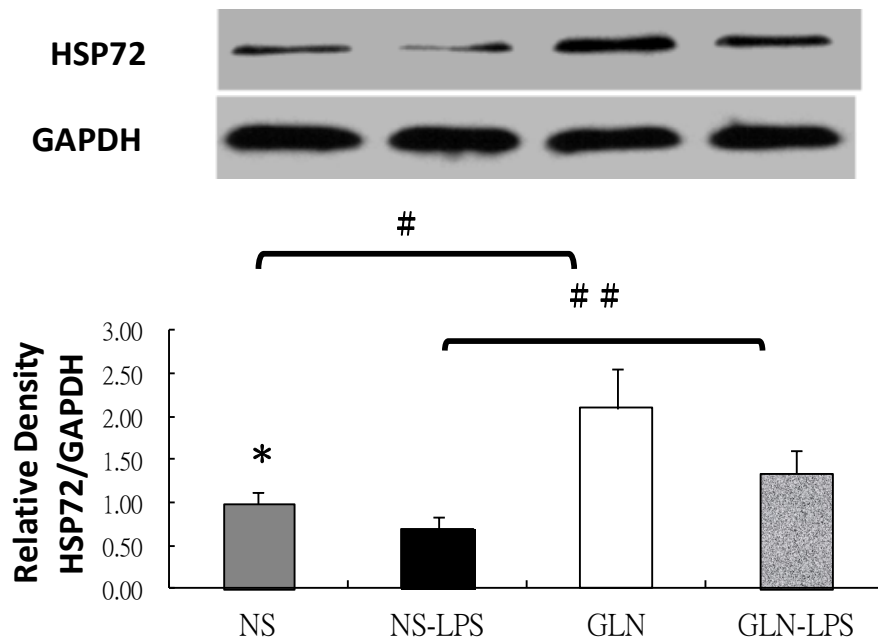


Fig.2. Western blot analysis of HSP 72 in lung tissue from different groups of rats.

HSP72 expression was analyzed by Western blot analysis. Relative density refers to the ratio of HSP72 to GAPDH. Image represents results found in triplicate.

Relative densitometric results are expressed as mean \pm SD of levels relative to that of the control group (NS), * $P < 0.05$ vs. all other groups. LPS inhalation down-regulates HSP72 expression. Inhaled GLN up-regulates lung HSP72 expression both in non LPS-treated rats ($^{\#}P < 0.0001$ NS vs. GLN) and LPS-treated rats ($^{\#\#}P < 0.001$ NS-LPS vs. GLN-LPS).

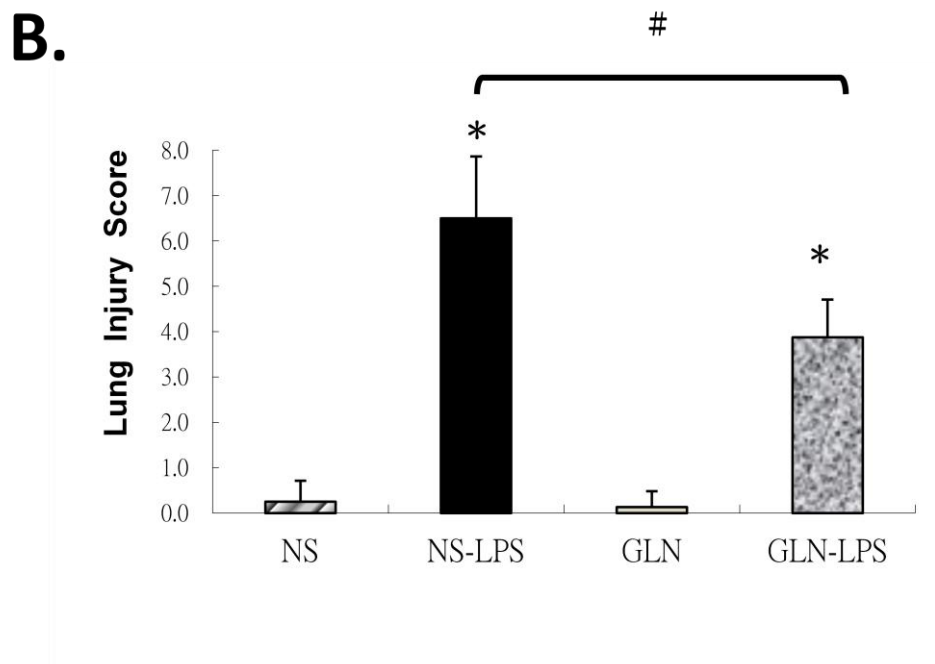
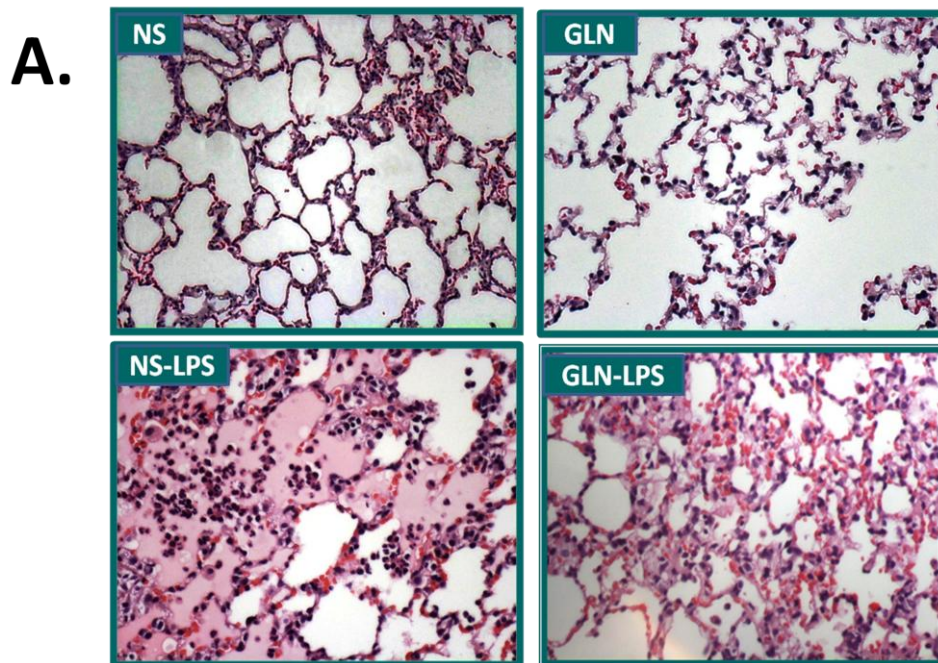


Fig.3. **A.** Illustrative microphotographs showing the lung histology of lung tissue obtained in four experimental groups. Note the severe pulmonary edema and cellular infiltration in the NS-LPS group and the attenuation of lung pathology with GLN pretreatment (GLN-LPS). Hematoxylin and eosin stain, original

magnification $\times 20$. **B.** The data were obtained from scoring the lung pathological changes (edema formation and inflammatory cells infiltration) in rat lungs. * $P < 0.0001$ vs. NS, # $P < 0.0001$ NS-LPS vs. GLN-LPS.

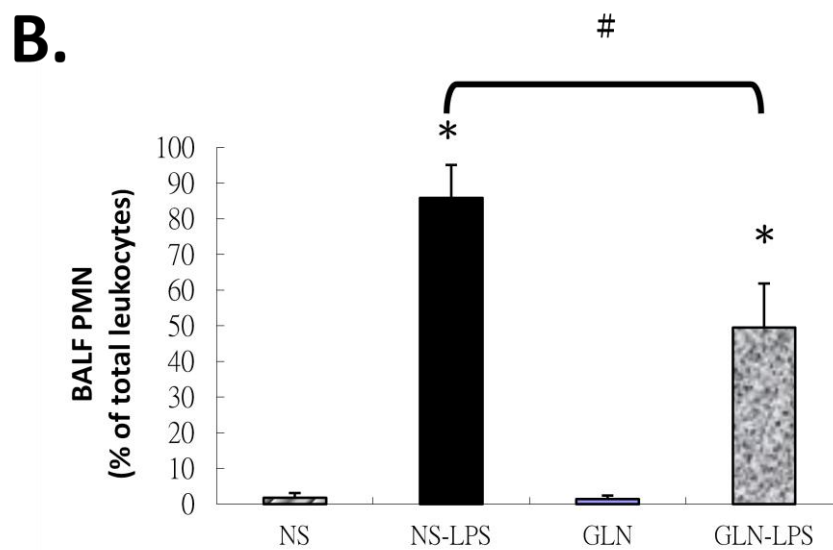
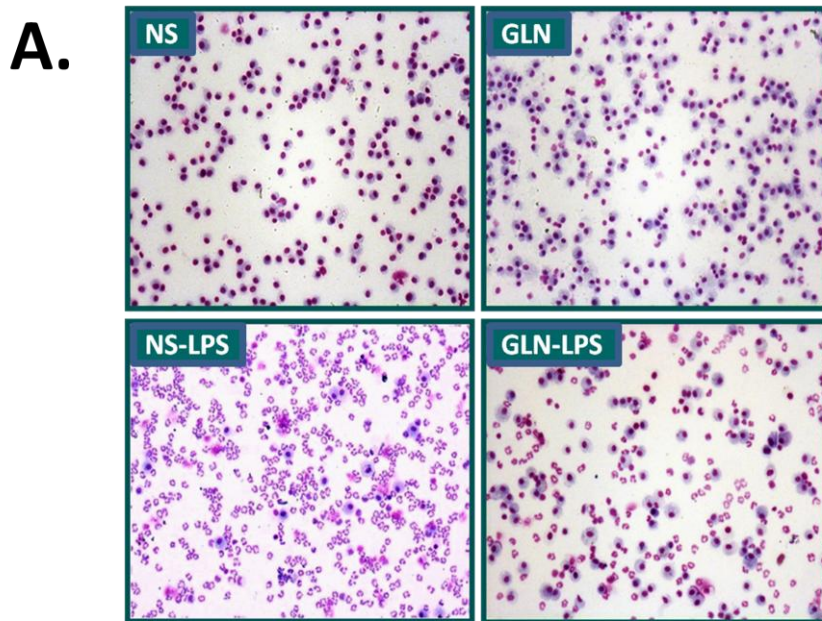


Fig.4. **A.** Differential cell counts and images of BALF cells. PMNs were evaluated for air space migration by morphological analysis (original magnification, $\times 10$) The cells were stained with Giemsa and counted double blind under a microscope. **B.** PMN in BALF is presented as percent of total cells collected.

LPS caused marked increases in these parameters. Pretreatment with GLN significantly attenuated the LPS effects. *P<0.0001 vs. NS, # P<0.0001 NS-LPS vs. GLN-LPS.

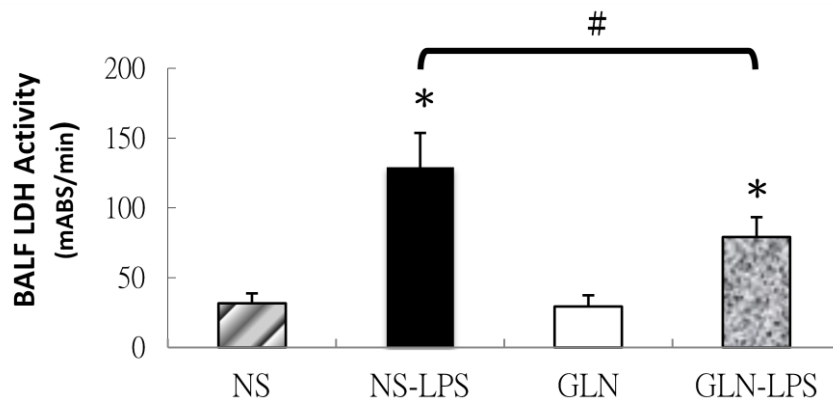


Fig.5. Measurement of LDH activity in BALF. LPS caused marked increases in these parameters. Pretreatment with GLN significantly attenuated the LPS effects.

* $P < 0.0001$ vs. NS , [#] $P < 0.0001$ NS-LPS vs. GLN-LPS.