

Glutamine attenuates acute lung injury by inhibition of high mobility group box protein-1 expression during sepsis

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Heat shock protein 70 (HSP70) is reported as the main factor responsible for the beneficial effects of glutamine (GLN) and as a negative regulator of high mobility group box protein-1 (HMGB-1) expression. Our aim was to determine whether GLN attenuates acute lung injury (ALI) by the inhibition of HMGB-1 expression during sepsis. Male Sprague–Dawley rats were subjected to caecal ligation and puncture (CLP) to induce sepsis. GLN or saline was administered through tail vein 1 h after CLP. Then, quercetin (Q), an inhibitor of HSP70, was utilised to assess the role of the enhanced HSP70. We observed the survival of the subjects. At 24 h post-CLP, we measured lung HSP70, phosphorylated heat shock factor-1 (HSF-1-p) and HMGB-1 expressions, NF- κ B DNA-binding activity and ALI occurrence. We also measured serum HSP70, IL-6 and HMGB-1 concentrations. GLN improved survival during sepsis. In GLN-treated rats, lung HSP70 and HSF-1-p expressions were enhanced, lung HMGB-1 expression and NF- κ B DNA-binding activity were suppressed, and ALI was attenuated. Furthermore, in GLN-administered rats, serum HSP70 concentration was higher, and serum IL-6 and HMGB-1 concentrations were lower than those in non-treated rats. Q inhibited the enhancement of HSP70 and HSF-1-p expressions and abrogated the GLN-mediated benefits. In conclusion, GLN attenuated ALI and improved survival by the inhibition of HMGB-1 expression during sepsis in rats. These benefits were associated with the enhancement of HSP70 expression by GLN.

Glutamine: High mobility group box protein-1: Lung injury: Sepsis

Sepsis is defined as a complex clinical syndrome caused by a harmful or damaging host response to infection and may progress to the development of one or multiple organ dysfunctions and death⁽¹⁾. The lung is the most often affected organ in multiorgan dysfunction syndrome after sepsis^(2,3).

High mobility group box protein-1 (HMGB-1) is a nuclear DNA-binding protein, which regulates gene expression and transcription under normal conditions⁽⁴⁾. It can be regarded as an important DNA chaperone involved in the regulation of nuclear transactions⁽⁵⁾. In pathologic conditions, HMGB-1 is redistributed from the nucleus into the cytoplasm⁽⁶⁾. Then, inflammatory cells such as neutrophils, monocytes and macrophages release HMGB-1 into the extracellular milieu, where it functions as a proinflammatory cytokine⁽⁷⁾. HMGB-1 is known as a sufficient and necessary mediator of the lethal multi-organ failure during sepsis⁽⁸⁾. The elevated serum level of HMGB-1 is associated with the mortality in patients with sepsis^(6,9,10). The direct contribution of HMGB-1 to acute lung injury (ALI) was demonstrated by previous studies^(6,9,10). They showed that HMGB-1 itself caused an acute pulmonary inflammatory response manifested by neutrophil infiltration, alveolar congestion, pulmonary haemorrhage, destruction of pulmonary parenchyma cells and increased production of

proinflammatory cytokines in lungs. In addition, anti-HMGB-1 antibodies attenuated endotoxin-induced lung injury but not the early release of TNF- α and IL-1 β , indicating that HMGB-1 is a late mediator of endotoxin-induced ALI⁽⁹⁾. Unlike other proinflammatory cytokines such as TNF- α or IL-1 β , HMGB-1 is a late appearing inflammatory mediator and provides a wider time frame for clinical intervention against the progressive inflammatory cascade of sepsis⁽¹¹⁾.

Glutamine (GLN) is known as a safe enhancer of heat shock protein 70 (HSP)-70 and potentially down-regulates NF- κ B pathway activation and proinflammatory cytokines release⁽¹²⁾. In many clinical trials, GLN improved infectious morbidity and mortality in critically ill patients, particularly when administered in doses greater than 0.3–0.5 g/kg per d⁽¹³⁾. In experimental studies, a single 0.75 g/kg dose of intravenous GLN attenuated ALI and improved survival by the inhibition of anti-inflammatory responses during sepsis⁽¹⁴⁾. The role and mechanisms of anti-inflammatory and protective effects of GLN on critically ill patients have not been clearly determined. However, recent experimental data demonstrated that HSP70 enhancement is the main factor responsible for the beneficial effects of GLN on ALI during sepsis⁽¹⁵⁾. HSP is a family of proteins, which mediates a self-protective

Abbreviations: ALI, acute lung injury; CLP, caecal ligation and puncture; GLN, glutamine; HMGB-1, high mobility group box protein-1; HSF, heat shock factor; HSF-1-p, phosphorylated HSF-1; HSP, heat shock protein; Q, quercetin.

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phenomenon, heat shock response⁽¹⁶⁾. During heat shock response, heat shock factor-1 (HSF-1) monomer is released and converted to trimer in the cytoplasm. Then, HSF-1 is phosphorylated and migrates to nucleus. In nucleus, the phosphorylated HSF-1 (HSF-1-p) binds to the promoters of HSP genes and induces HSP transcription⁽¹⁷⁾. Synthesised HSP confers protective role against environmental stresses^(18,19). Of the various HSP, HSP70 plays a vital role in cellular protection during sepsis, endotoxaemia and adult respiratory distress syndrome^(14,20). HSP70 overexpression reduced both mortality and organ dysfunction during sepsis^(16,21–23). As a molecular chaperone, HSP70 acts as a quencher of various denatured proteins and prevents the activation of inflammatory cells⁽²⁴⁾. Recently, *in vitro* studies reported a role for HSP70 as a negative regulator of HMGB-1 cytoplasmic translocation and release induced by cellular stresses^(24,25). However, no *in vivo* study has examined the effect of GLN, a safe enhancer of HSP70, on the release of HMGB-1 during sepsis.

Therefore, we hypothesised that GLN would down-regulate HMGB-1 expression and that the inhibition of HMGB-1 expression would be associated with the enhancement of HSP70 expression by GLN during sepsis. The aim of the present study was to determine whether a single dose of GLN attenuates ALI by the inhibition of HMGB-1 expression in a rat caecal ligation and puncture (CLP) model.

Materials and methods

Animal preparation

Experiments were performed on male Sprague–Dawley rats (body weight, 300–350 g) purchased from the Orient Bio Inc. (Seongnam, Korea). Animals were maintained *ad libitum* on a laboratory chow (Lab diet) and water and housed in a specific pathogen-free room at constant temperature (20–22°C) with 10 and 14 h of light and dark exposure, respectively. Animals underwent an acclimatisation period of 14 d before being used in experiments. All experiments conducted were approved by the Animal Experiment Committee of the Clinical Research Institute of Seoul National University Hospital in accordance with current Korean Animal Protection Laws.

Experimental procedures

Sepsis was induced by CLP performed as previously described⁽¹⁴⁾. Briefly, after inducing anaesthesia with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg), a midline incision was made in abdominal wall and caecum was extruded. Approximately, 25% of the caecum was ligated below ileocaecal valve. The distal caecum was punctured twice using a sterile eighteen-gauge needle to allow a small amount of faecal material to extrude into the peritoneal cavity. Abdominal muscle and skin layers were then closed. All animals received 20 ml/kg of normal saline fluid after the procedures. No antibiotics were administered. Animals were returned to their cages and allowed *ad libitum* food and water. It is of note that our model consistently yielded 100% mortality within 72 h in control animals. Rats were divided into five groups: the SHAM group, animals of which underwent a sham operation; the CLP group, CLP alone; the CLP + GLN group, CLP plus GLN (0.75 g/kg

once via a tail vein) administration; the CLP + quercetin (Q) group, CLP plus Q (400 mg/kg intraperitoneal) administration; the CLP + GLN + Q group, CLP plus GLN (0.75 g/kg) administration plus Q (400 mg/kg) administration. The CLP + GLN and CLP + GLN + Q groups were administered GLN solution, and the SHAM, CLP and CLP + Q groups were administered saline vehicle through a tail vein 1 h after CLP, respectively⁽¹⁴⁾. GLN was administered as alanyl-GLN dipeptide (Sigma-Aldrich, St Louis, MO, USA) prepared as a 37.5% solution dissolved in saline⁽¹⁴⁾. The CLP + Q and CLP + GLN + Q groups received Q (Sigma-Aldrich), an inhibitor of HSP70, and the SHAM, CLP and CLP + GLN groups received vehicle at 6 h before CLP to assess the role of the enhanced HSP70 on the HMGB-1 expression during sepsis, respectively⁽¹⁴⁾.

For survival studies, animals were allocated to the SHAM group (*n* 6) and the other four treatments groups (*n* 15/group). Then, the animals were closely observed for occurrence of mortality. Moribund animals (defined as severely lethargic and unresponsive to painful stimulation) were killed with a lethal dose of ketamine and xylazine. To analyse the effect of GLN on ALI and the expression of HSP70 and HMGB-1, a separated set of animals were allocated based on the data of survival studies: the SHAM group (*n* 6); the CLP group (*n* 18); the CLP + GLN group (*n* 10); the CLP + Q group (*n* 18); the CLP + GLN + Q group (*n* 18). Then, to measure HSP70 and HMGB-1 expression simultaneously, six animals in each group were sacrificed at 24 h after CLP. Blood samples were obtained by cardiac puncture and lung tissues were harvested. Blood was centrifuged at 3000 rpm for 10 min at 4°C, and separated serum was stored at –70°C for subsequent assays. The right upper lobe of lung of each animal was separated and immediately fixed with 4% formaldehyde in 0.1 M phosphate buffer for histological examinations. Remaining lung tissues were thoroughly washed in PBS, immediately frozen in liquid nitrogen, and stored at –70°C until required.

Western blot analysis

Western blotting was performed as previously described^(14,24). Briefly, lung tissues were homogenised in 1 ml of ice-cold tissue protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) containing 1% protease inhibitor cocktail (Roche, Basel, Switzerland). Homogenates were centrifuged at 12 000 rpm for 20 min, and the supernatants were stored at –70°C. Total protein concentrations in supernatants were determined using the bicinchoninic acid protein assay kits (Pierce Biotechnology). Protein extracts (45 µg per lane) were run on 8% SDS-PAGE gels and then transferred to polyvinylidene fluoride membranes (Schleicher & Schuell, Dassel, Germany). The following primary antibodies were used for immunoblotting: mouse monoclonal anti-rat-HSP70; polyclonal anti-rat-HSF-1 antibodies (diluted 1:1000; Stressgen, Victoria, BC, Canada); mouse monoclonal anti-rat-HMGB-1 antibody (diluted 1:1000; Stressgen). Secondary antibodies, anti-mouse-IgG (Amersham International, Amersham, Buckinghamshire, UK), coupled with peroxidase were diluted 1:5000 in Tris (2-amino-2-(hydroxymethyl)propane-1,3-diol)-buffered saline-Tween (TBS-T). Protein bands were detected using the ECL™ enhanced chemiluminescence system

(Amersham International). Optical densities were quantified by a computer-assisted densitometric analysis of the exposed films (Lap Work Software; Seoulin Bioscience, Seoul, South Korea). All blots were normalised against β -actin to control for protein loading. For β -actin measurements, the above-mentioned Western blot technique was applied utilising a specific mouse monoclonal anti- β actin antibody (Sigma-Aldrich, St Louis, MO, USA).

Histological analysis of lung tissues

The separated right upper lung lobes were embedded in paraffin and sectioned at 4 μ m. For histological examination, the sections were deparaffinised, stained with haematoxylin and eosin and reviewed by pathologists blinded to the treatments groups. ALI score was calculated as previously described^(26,27). In brief, lung injury was assessed by scoring four items: alveolar congestion; haemorrhage; infiltration or aggregation of neutrophils in air spaces or vessel walls; thickness of the alveolar wall/hyaline membrane formation. The severity for each item was rated on a five-point scale graded from 0 (minimal) to 4 (maximal), giving a range of total scores from 0 to 16 (most severe).

Immunohistochemical analysis for lung high mobility group box protein-1 expression

Immunohistochemical analysis was carried out as previously described⁽²⁸⁾. Briefly, the sections of lung tissues were deparaffinised and incubated with mouse polyclonal anti-rat-HMGB-1 antibody (diluted 1:1000; Stressgen) overnight at 4°C. The sections were washed with PBS and incubated with anti-mouse-IgG (Amersham International), coupled with peroxidase and diluted 1:1000. The slides were stained using the LSAB2 kit (Dako, Glostrup, Denmark) including the biotin-avidin-peroxidase complex system. Then, the slides were counterstained with haematoxylin and reviewed by pathologists blinded to the treatments groups.

Assessment of lung apoptosis

With the sections of lung tissues, the terminal deoxynucleotidyl transferase dUTP nick end labelling assay was performed using the ApopTag plus peroxidase *in situ* apoptosis detection kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions⁽²⁹⁾.

Lung NF- κ B DNA-binding activity

Preparation of nuclear extracts from lung tissues was performed using the Nuclear Extract kit (Active Motif, Carlsbad, CA, USA). Then, 2.5 μ g nuclear extract was submitted to the TransAM NF- κ B p65 kit (Active Motif) for determination of the DNA-binding activity of NF- κ B p65⁽³⁰⁾. The Jurkat nuclear extract was provided as a positive control for NF- κ B activation.

ELISA assays

Serum HSP70 concentration was measured using an ELISA kit (Assay designs, Ann Arbor, MI, USA), and serum HMGB-1 concentration was determined using an ELISA kit

(SHINO-TEST Corp., Tokyo, Japan) in duplicate, according to the manufacturer's instructions. Serum IL-6 concentration was measured with the DuoSet ELISA kits (R&D System, Minneapolis, MN, USA). ELISA plates were measured on a Versa Max microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA) at 450 nm, and concentrations of the respective proteins in serum were calculated according to calibration curves.

Statistical analysis

Fisher's exact test was used to compare survival data in the five study groups. Lung HSP70, HSF-1-p and HMGB-1 expressions, ALI scores, NF- κ B DNA-binding activity and serum HSP70, HMGB-1 and IL-6 concentrations in the groups were compared using the one way ANOVA test followed by Duncan grouping as a *post hoc* test⁽¹⁴⁾. *P* values of <0.05 were considered statistically significant, and the significance levels quoted are two-sided. All analyses were conducted using SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Survival at 72 h after caecal ligation and puncture

No mortality occurred in the SHAM group. In the CLP + GLN group, seven out of fifteen rats (46.7%) survived at 72 h post-CLP. However, in the CLP and CLP + Q groups, all animals expired. In the CLP + GLN + Q group, only one

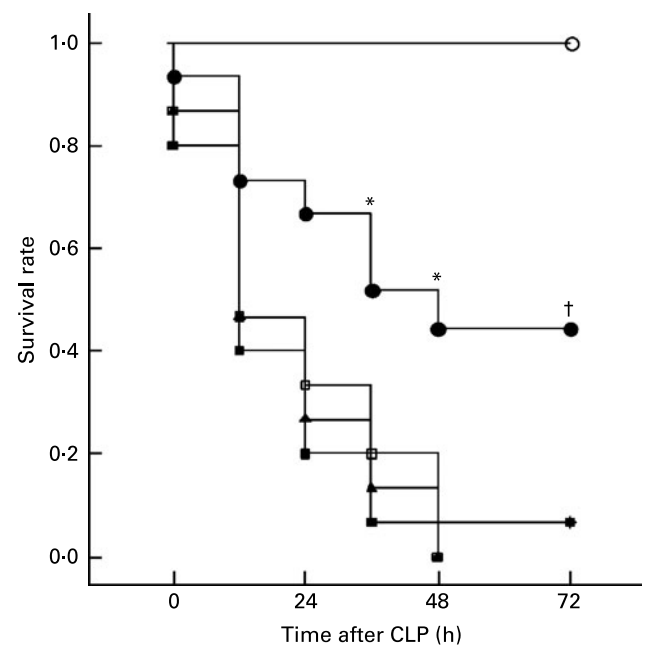


Fig. 1. Survival at 72 h after caecal ligation and puncture (CLP). The Kaplan-Meier survival curve shows that glutamine (GLN) administration reduced mortality due to sepsis caused by CLP. **P*<0.05 *v.* the CLP + GLN + quercetin (Q) group; †*P*<0.05 *v.* the CLP, CLP + Q and CLP + GLN + Q groups. SHAM, sham operation; CLP + GLN (0.75 g/kg) injection; CLP + Q administration (400 mg/kg). ○, SHAM; ▲, CLP; ●, CLP + GLN; □, CLP + Q; ■, CLP + GLN + Q.

of fifteen (6.7%) animals survived. The 72-h mortality in the CLP + GLN group was lower than that in the CLP, CLP + Q and CLP + GLN + Q groups (P values were 0.006, 0.006 and 0.035, respectively; Fig. 1).

Heat shock protein 70, phosphorylated heat shock factor-1 and high mobility group box protein-1

In the CLP + GLN group, lung HSP70 and HSF-1-p expressions were significantly higher than those in the CLP, CLP + Q and CLP + GLN + Q groups (Fig. 2(a) and (b)). Q inhibited the enhancements of HSP70 and HSF-1-p by GLN in the CLP + GLN + Q group. Lung HMGB-1 expression was significantly lower in the CLP + GLN group than that in the CLP, CLP + Q and CLP + GLN + Q groups (Fig. 3(a)). Immunohistochemical analysis showed the increased redistribution of HMGB-1 from nucleus to cytoplasm and extracellular areas (Fig. 3(b)). The serum concentrations of HSP70 and HMGB-1 paralleled their lung expressions (Table 1).

Histological examination of lung tissues

The representative photomicrographs of lung parenchyma showed ALI including alveolar congestion, haemorrhage, infiltration and aggregation of neutrophils in air spaces

or vessel walls and thickening of alveolar wall/hyaline membrane formations (Fig. 4). ALI score was found to be lower in the CLP + GLN group than that in the CLP, CLP + Q and CLP + GLN + Q groups (Table 1).

Lung apoptosis

In the CLP, CLP + Q and CLP + GLN + Q groups, marked appearances of TUNEL-positive cells were observed, but no or minimal TUNEL-positive cells were observed in the SHAM and CLP + GLN group (Fig. 5). The appearance of TUNEL-positive cells was more prominent in the CLP + Q group than that in the CLP group.

Lung NF- κ B DNA-binding activity and serum IL-6 concentration

In the CLP + GLN group, lung NF- κ B DNA-binding activity and serum IL-6 concentration were significantly lower than those in the CLP, CLP + Q and CLP + GLN + Q groups (Table 1). Q induced minimal decrease of lung NF- κ B DNA-binding activity and serum IL-6 concentration in the CLP + Q and CLP + GLN + Q groups, when compared to the CLP group. However, there were no statistical significances.

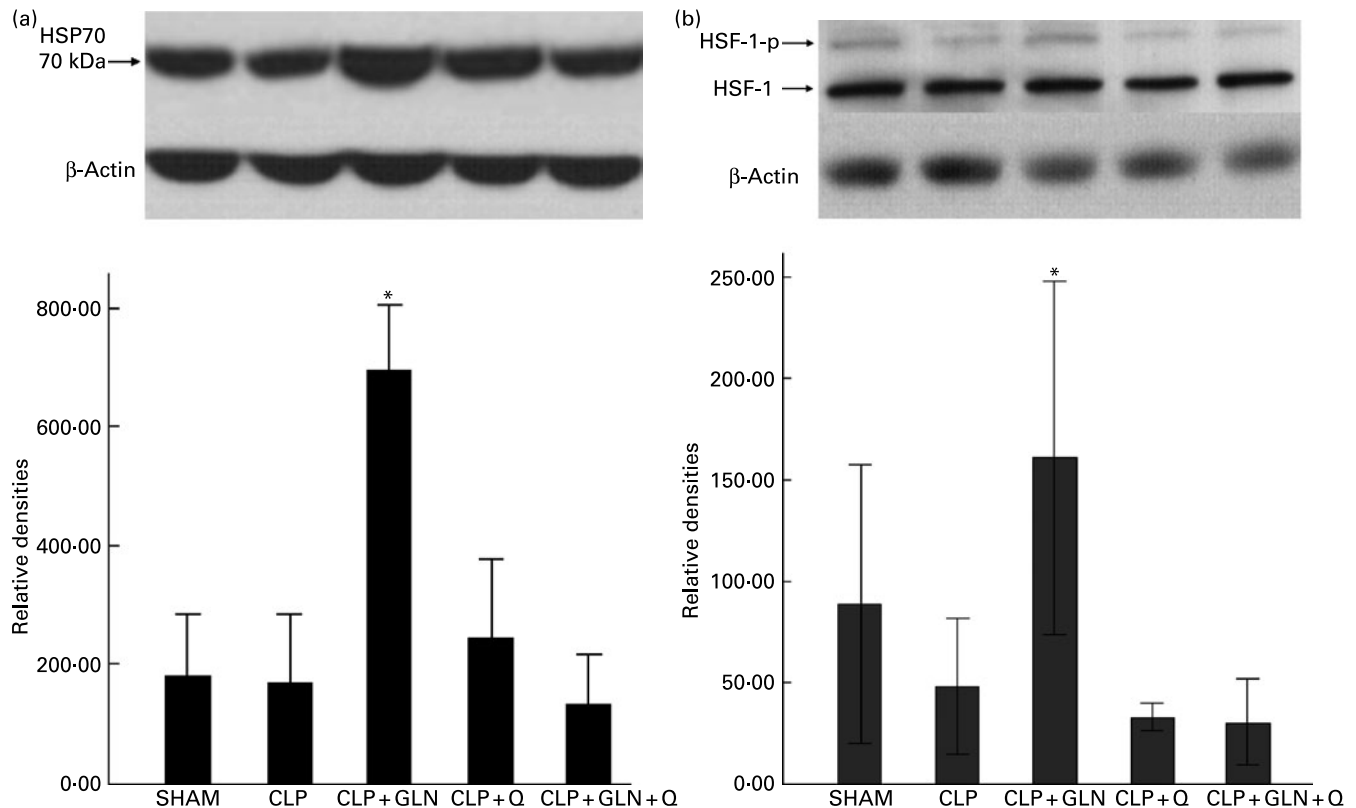


Fig. 2. Expression of heat shock protein 70 (HSP70) and phosphorylated heat shock factor-1 (HSF-1-p) in lung tissues. The expressions of HSP70 (a) and HSF-1-p (b) in lung tissue 24 h after caecal ligation and puncture (CLP) were detected by Western blotting. Blots are representative of six animals within each group. In the CLP + glutamine (GLN) group, lung HSP70 and HSF-1-p expressions were significantly higher than those in the CLP, CLP + quercetin (Q) and CLP + GLN + Q groups. Values are means with standard deviations represented by vertical bars. * Mean value was significantly different from that of the CLP, CLP + Q and CLP + GLN + Q groups ($P < 0.05$).

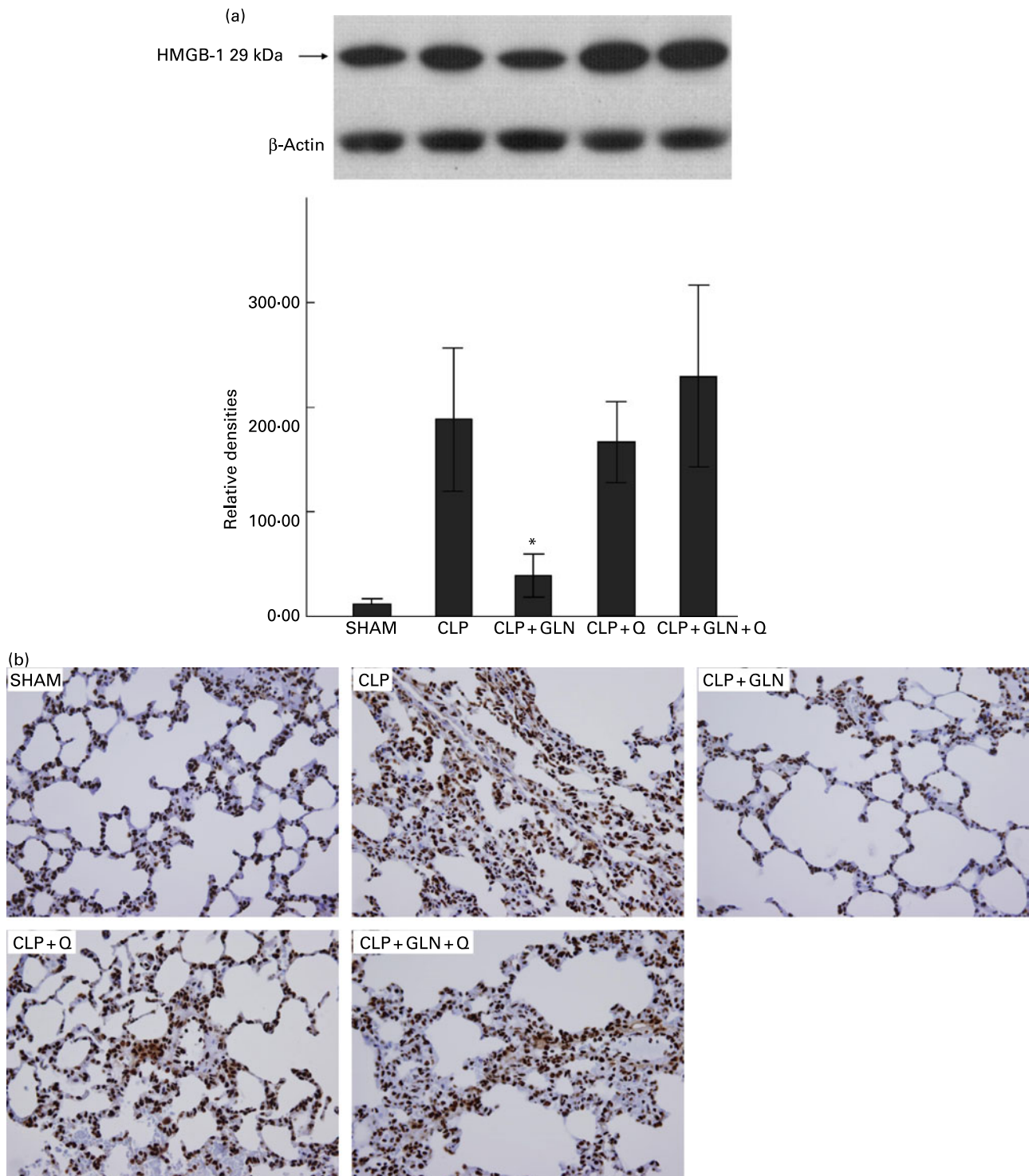


Fig. 3. Expression of high mobility group box protein-1 (HMGB-1) in lung tissues. The expression of HMGB-1 in lung tissues detected by Western blotting (a) and represented by immunohistochemical analysis (b). In the caecal ligation and puncture (CLP) + glutamine (GLN) group, lung HMGB-1 expression was significantly lower than that in the CLP, CLP + quercetin (Q) and CLP + GLN + Q groups. Representative photomicrographs of the lung immunohistochemistry ($\times 400$) showed the increased redistribution of HMGB-1 from nucleus to cytoplasm and extracellular areas. Values are means with standard deviations represented by vertical bars. * Mean value was significantly different from that of the CLP, CLP + Q and CLP + GLN + Q groups ($P < 0.05$).

Discussion

Our experimental data demonstrated that an administration of GLN enhanced HSP70 and HSF-1-p expressions but inhibited HMGB-1 expression in both lung and serum, attenuated ALI

and improved survival. These benefits were abrogated by the administration of Q, an inhibitor of HSP70. These findings mean that enhanced HSP70 in part mediated the inhibition of HMGB-1 expression resulting in ALI attenuation and

Table 1. Serum heat shock protein 70 (HSP70), high mobility group box protein-1 (HMGB-1), and IL-6 concentrations, lung NF- κ B DNA-binding activity, and acute lung injury (ALI) score

(Mean values with their standard deviation for six rats per group)

	SHAM		CLP		CLP + GLN		CLP + Q		CLP + GLN + Q	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Serum										
HSP70 (pg/ml)	453.83	339.80	299.67	169.55	2007.33†	931.50	225.17	120.33	233.50	113.67
HMGB-1 (pg/ml)		ND	12.97*	9.18	1.20†	0.94	13.51*	9.49	14.14*	13.54
IL-6 (pg/ml)	163.78	62.04	478.96*	181.44	165.17†	49.05	374.58*	140.55	368.66*	80.26
Lung										
NF- κ B DNA-binding activity (OD)‡	0.38	0.01	0.55*	0.09	0.39†	0.02	0.53*	0.07	0.52*	0.10
ALI score	7.00	1.10	9.50*	1.52	6.33†	1.63	8.83*	0.98	9.33*	1.21

SHAM, sham operation; CLP, caecal ligation and puncture, GLN, glutamine; Q, quercetin; OD, optical density; ND, not detectable.

CLP + GLN (0.75 g/kg) injection; CLP + Q, administration (400 mg/kg).

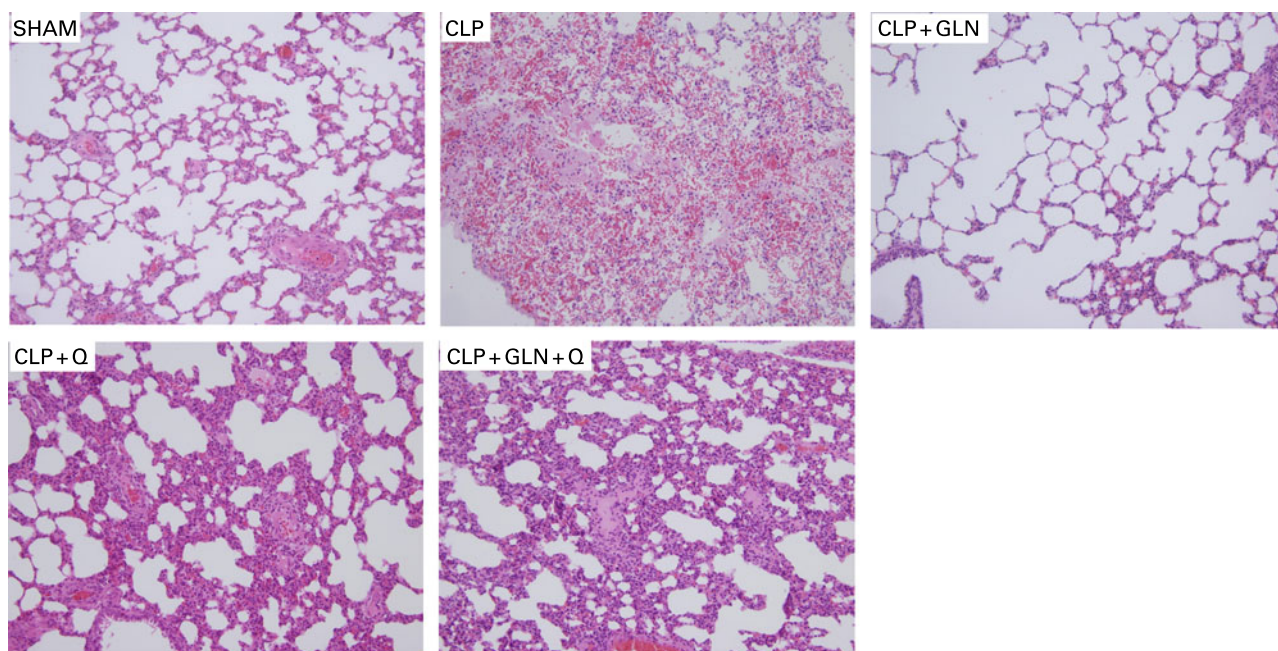
* Mean value was significantly different from that of the SHAM group ($P < 0.05$).† Mean value was significantly different from that of the CLP, CLP + Q, and CLP + GLN + Q groups ($P < 0.05$).

‡ Results were represented as OD normalised to the positive control with sd.

survival improvement during sepsis. Although the mechanisms of the interactions between HSP70 and HMGB-1 have not been clearly shown, we propose two possible mechanisms.

The first involves the intracellular interaction between HSP70 and HMGB-1, which inhibits the expression of HMGB-1. In 2007, an *in vitro* study suggested that HSP70 translocated into the nucleus directly or indirectly interacts with HMGB-1, and the intranuclear HSP70/HMGB-1 connection consequently prevents the cytoplasmic translocation and subsequent release of HMGB-1⁽²⁴⁾. Furthermore, another *in vitro* study suggested that the chromosome region maintenance 1-dependent nuclear export pathway is thought to be associated with this interaction between HSP70 and HMGB-1⁽²⁵⁾. Our data also showed that the enhancements of HSP70 and HSF-1-p, HSP70 transcriptional factor, were associated

with the decreased redistribution of HMGB-1 from nucleus to cytoplasm and extracellular area. The second proposal is that enhanced HSP70 and HSF-1-p inhibits NF- κ B pathway activation and early proinflammatory cytokine release, which suppresses the late expression of HMGB-1. It has been well known that proinflammatory cytokines damage cells and stimulate the secretion of intracellular HMGB-1 into the extracellular milieu⁽¹¹⁾. Many previous studies reported that HSF-1 overexpression induces competitive inhibition of nuclear NF- κ B-related signal transduction and suppresses the proinflammatory cytokine release^(16,21–23,31). In the present study, we also found that the lung HSP70, HSF-1-p expressions and serum HSP70 concentration showed negative correlation with lung NF- κ B DNA-binding activity and serum IL-6 concentration. These results of the present study suggested that both the

**Fig. 4.** Representative photomicrographs of the lung histology (haematoxylin and eosin, $\times 100$). Acute lung injury such as alveolar congestion, haemorrhage, infiltration and aggregation of neutrophils in air spaces or vessel walls and thickening of alveolar wall/hyaline membrane formations was found to be attenuated in the caecal ligation and puncture (CLP) + glutamine (GLN) group than in the CLP, CLP + quercetin (Q) and CLP + GLN + Q groups.

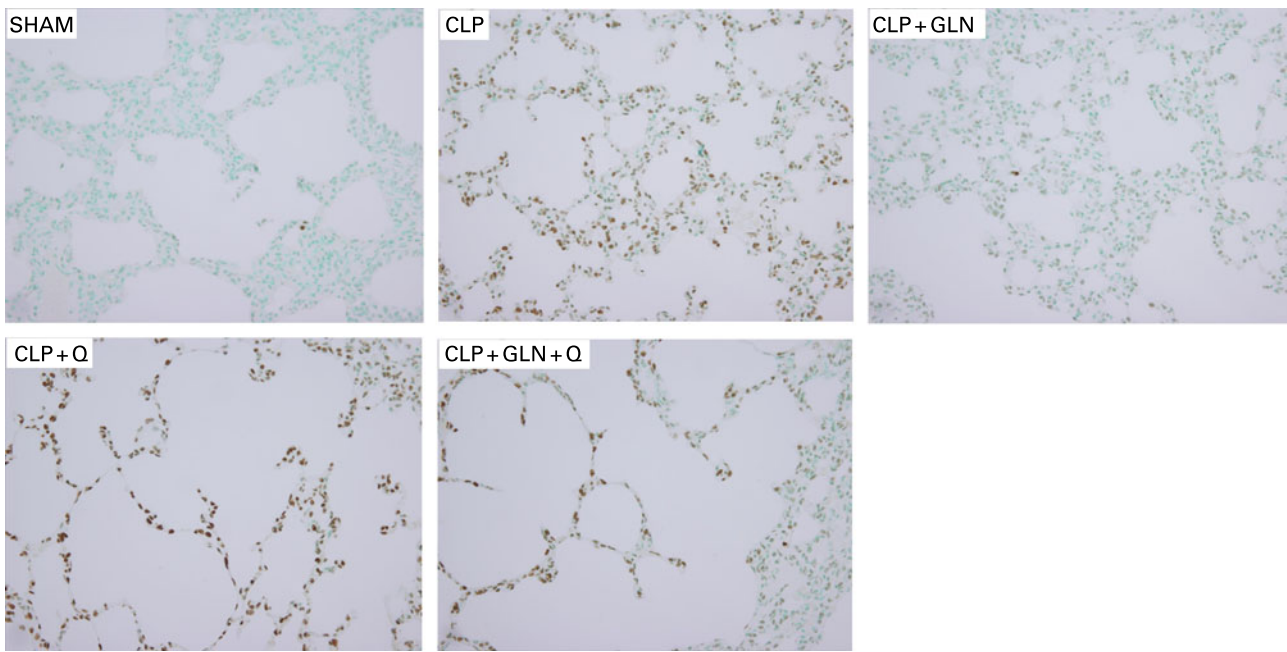


Fig. 5. Representative photomicrographs of the lung apoptosis (the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, $\times 400$). In the caecal ligation and puncture (CLP), CLP + quercetin (Q) and CLP + glutamine (GLN) + Q groups, marked appearances of TUNEL-positive cells were observed, but no or minimal TUNEL-positive cells were observed in the sham operation (SHAM) and CLP + GLN groups. The appearance of TUNEL-positive cells was more prominent in the CLP + Q group than that in the CLP group.

intranuclear HSP70/HMGB-1 connection and the competitive inhibition of nuclear NF- κ B-related signal transduction by HSF-1-p may contribute to the inhibition of HMGB-1 expression.

In addition, recent data have demonstrated that extracellular HSP70 suppresses proinflammatory cytokine release through NF- κ B pathway inhibition⁽³²⁾. We did not examine the role of extracellular HSP70, separately. However, the negative correlation between the serum HSP70 and IL-6 concentrations observed in our data suggested that extracellular HSP70 may contribute to the inhibition of proinflammatory cytokine release.

HSP70 is a protective protein synthesised in stressed conditions. However, no differences in HSP70 expression were observed between the SHAM and CLP groups in our data. Furthermore, lung HSF-1-p in the CLP group was slightly lower than that in the SHAM group. These results were consistent with the results in other previous experimental studies⁽³³⁾. The authors of the studies proposed that failed HSP70 expression contributes to lung injury during sepsis, and the correction of the deficient HSP70 expression improves prognosis of sepsis.

To determine the effect of GLN on HSP70 and HMGB-1 expression, we used a single 0.75 g/kg dose of intravenous GLN. Previous data reported that a single 0.75 g/kg dose of intravenous GLN to a rat resulted in a plasma GLN level of 3–7 mm/l and that maximal HSP70 expression occurred at this level⁽²⁰⁾. To examine the relationship between HSP70 and HMGB-1, we sacrificed animals at 24 h post-CLP. Since HSP70 is detected in serum from 6 to 24 h post-stimulation, and HMGB-1 is secreted into serum approximately 20 h after the induction of sepsis, we sacrificed the subjects at 24 h post-stimulation to assess the expressions of HSP70 and HMGB-1 simultaneously^(7,34).

Since we used Q for the inhibition of HSP70 overexpression, our data showed only the correlation between HSP70 and HMGB-1. To demonstrate the direct mechanistic link between HSP70 and HMGB-1, complimentary *in vivo* studies should be conducted with appropriate knockout animal models⁽¹⁵⁾. Q inhibits HSP70 overexpression by blocking HSF-1 and HSF-2 and reducing HSP70 mRNA accumulation⁽³⁵⁾. Therefore, Q has been used to down-regulate HSP70 synthesis in various studies^(14,35). Although Q is perhaps the best known HSP70 inhibitor and has been used as a pre-treatment to inhibit HSP70 expression in several previous studies, questions have been raised concerning its effect on tissue injury and apoptosis^(36,37). Many experimental and clinical data reported that Q induced apoptosis and inhibited NF- κ B activation and IL-6 expression during sepsis^(38,39). Our data showed prominent TUNEL-positive cells in the CLP + Q group, when compared to the CLP group. Minimal decreases of lung NF- κ B DNA-binding activity and serum IL-6 concentration were also observed in the CLP + Q and CLP + GLN + Q groups. However, there were no significant differences in NF- κ B DNA-binding activity and proinflammatory cytokine release between the Q-treated and non-treated groups. Furthermore, Q had no effect by itself on basal HSP70 and HSF-1-p expressions, HMGB-1 expression, ALI score and mortality, when compared between the CLP and CLP + Q groups. These results suggested that the main effect of Q on the present study is the inhibition of HSP70 overexpression in GLN-administered rats (the CLP + GLN + Q group). A recent experimental data also showed that the effect of Q on HSP70 inhibition was parallel to the effect of HSP70 knockout⁽¹⁵⁾.

The present study is the first experimental trial to observe the effect of GLN on HMGB-1 expression during sepsis

in vivo. Several preclinical and clinical trials are currently being undertaken with a view toward preventing the progression of multiorgan failure during sepsis by inhibition of HMGB-1 expression using different HMGB-1 inhibitors such as anti-HMGB-1 antibody, ethyl pyruvate, lysophosphatidylcholine, selective α -7 nicotinic acetylcholine receptor agonists and ghrelin^(11,40). The results of the present study indicate that GLN, a safe HSP70 enhancer, could be considered as a potential therapeutic agent for preventing HMGB-1 release during sepsis.

In conclusion, a single dose of GLN administration as a post-treatment after the initiation of sepsis was found to enhance the expression of HSP70 and to inhibit the expression of HMGB-1 in serum and lung tissues. GLN also attenuated ALI and improved 72-h survival by inhibition of the expression of HMGB-1 during sepsis in rats. These benefits were associated with the enhancement of HSP70 expression by GLN.

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