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C.S. Kurokawa
http://orcid.org/0000-0003-1380-7527
J.P. Araújo Júnior
https://orcid.org/0000-0002-9153-1485
R.B. Pires
http://orcid.org/0000-0002-3961-5534
M.F. Carpi
http://orcid.org/0000-0002-1482-564x
M.A. Moraes
http://orcid.org/0000-0003-0007-1506
J.R. Fioretto
http://orcid.org/0000-0003-4034-5962

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HMGB1 and inflammatory cytokines in experimental acute lung injury induced in rabbits

[*HMGB1 e citocinas inflamatórias em lesão pulmonar aguda experimental induzida em coelhos*]

C.S. *Kurokawa*[1], J.P. *Araújo Júnior*[2], R.B. *Pires*[1], M.F. *Carpi*[1],
M.A. *Moraes*[1], L.T.L. *Medeiros*[1], J.R. *Fioretto*[1]

[1]Unesp - Faculdade de Medicina - Botucatu, SP
[2]Unesp - Instituto de Biociências - Botucatu, SP

ABSTRACT

The aim of this work was to measure HMGB1, TNF-alpha, and IL-8 in bronchoalveolar lavage (BAL), serum and TLR2 and TLR4mRNA expression in lung tissue of rabbits with two grades of acute lung injury (ALI). The animals were randomly assigned to groups with severe (S) and mild/moderate (MM) ALI, induced with warm saline, and a control group. HMGB1, TNF-alpha, IL-8, TLR2mRNA and TLR4mRNA were measured after ALI induction. The results showed increased levels of IL-8, TNF-alpha, HMGB1 and TLR4mRNA in the ALI groups. HMGB1, IL-8 and TNF-alpha concentrations in BAL were higher in S compared MM. Increased TLR4mRNA was observed in S and MM versus control. The results suggest an early participation of HMGB1 in ALI together with IL-8 and TNF-alpha and association with severity. TLR4 has early expression and role in ALI pathophysiology but is not associated with severity.

Keywords: rabbit, acute lung lesion (ALI), lung lesion, HMGB-1, cytokines

RESUMO

O objetivo deste trabalho é determinar os níveis de HMGB1, TNF-alfa e IL-8 no lavado broncoalveolar (BAL), bem como quantificar a expressão sérica de TLR2 e TLR4 mRNA em tecido pulmonar de coelhos com dois graus de lesão pulmonar aguda (LPA). Os animais foram distribuídos aleatoriamente em grupos com LPA grave (S) e leve / moderada (MM), induzidas com solução salina morna, e um grupo controle. HMGB1, TNF-alfa, IL-8, TLR2mRNA e TLR4mRNA foram medidos após a indução de LPA e quatro horas de ventilação mecânica. Os resultados mostraram níveis aumentados de IL-8, TNF-alfa, HMGB1 e TLR4mRNA nos grupos com LPA. As concentrações de HMGB1, IL-8 e TNF-alfa no LBA foram maiores no S comparado ao MM. Aumento de TLR4mRNA foi observado em S e MM versus *controle. Os resultados sugerem uma participação precoce da HMGB1 na LPA em conjunto com IL-8 e TNF-alfa e associação com a gravidade da LPA. O TLR4 foi expresso na ALI e possivelmente possui papel precoce na fisiopatologia da LPA, mas sem associação com a gravidade.*

Palavras-chave: coelho, lesão pulmonar aguda (LPA), lesão pulmonar, HMGB-1, citocina

INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are conditions characterized by inflammation, disruption of the alveolocapillary barrier, interstitial and alveolar edema, decreased lung compliance, lung hypertension, ventilation/perfusion imbalance, and refractory hypoxemia (Redding, 2001). Despite progress in the understanding of the pathophysiology of ALI and technological advances in monitoring and treatment, mortality due to ARDS remains high (McNicholas *et al.*, 2018).

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E-mail: cilmery.kurokawa@unesp.br

The biological aggression in ALI is due to stimulation of inflammatory mediators, activation of the complement system, coagulation cascade and inflammatory cytokine production, and possible stimulation of damage-associated molecular patterns (DAMPs) (Takeuchi and Akira, 2010; Levitt *et al*., 2009; Chiumello *et al*., 1999, Kotani *et al*., 2004) 3, 4, 5). It has been proposed that DAMPs are involved in the mechanism of lung injury and can be a trigger and perpetuator of the uncontrolled inflammatory response and production of inflammatory cytokines (Takeuchi and Akira, 2010).

HMGB1 is a DAMP prototype and a cytokine produced by macrophages associated with severe systemic inflammatory response syndrome (SIRS), sepsis, and ALI (Scaffidi *et al*., 2002; Cohen, 2002). It is abundant in the cell nucleus, cytoplasm and cell membrane and is expressed in large amounts in lung tissues (Cohen, 2002). The receptor for HMGB1, called RAGE (receptor for advanced glycation end products), is usually expressed on the basolateral surface of type I pneumocytes. This receptor is involved in lung development and in the injury described in ALI (Uchida *et al*., 2006). Expression of RAGE is higher in the lung than in other organs, a fact rendering the lung more susceptible to the effects of HMGB1 and injury by inflammatory cytokines (Uchida *et al*., 2006; Calfee *et al*., 2008; Buckley and Ehrhardt, 2010).

Studies have shown the involvement of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), in lung injury induced by biological insults such as infection and DAMPs (Xiang & Fan, 2009). PRRs are evolutionarily conserved receptors whose ligands are pathogen-associated molecular patterns (PAMPs) and DAMPs. DAMPs are proteins or metabolites released during cell death or tissue damage (Xiang and Fan, 2009) that can amplify and perpetuate the inflammatory response in ALI, as described in sepsis and SIRS, suggesting a similar mechanism involved in ALI and its degrees of severity. Both DAMPs and PAMPs can be the initial trigger of the inflammatory response in ALI and can sustain the process (Xiang & Fan, 2009).

The presence of HMGB1 in ALI is due to epithelial cell death after injury or subsequent release as an inflammatory cytokine after macrophage stimulation (Calfee and Matthay *et al*., 2010; Ogawa *et al*., 2006). After its release into the microenvironment, HMGB1 binds to RAGE, TLR2, and TLR4 receptors on alveolar macrophages, neutrophils and lung epithelial or endothelial cells, inducing the production of TNF-alpha, IL-1beta, IL-6, IL-8, and MMP-8 (Calfee and Matthay *et al*., 2010; Ogawa *et al*., 2006). TLRs 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, and 13 have been shown to play a role in ALI and ARDS. However, TLR2 and TLR4 have been more extensively studied because of their capacity to bind bacterial components and their agonist role in the stimulation induced by HMGB1 and RAGE (Gattinoni *et al*., 2003; Li *et al*., 2009). TLR2 and TLR4 are found on macrophages, dendritic cells, neutrophils, and epithelial and endothelial cells (Xiang & Fan, 2009).

The latest theory about the development of post-traumatic SIRS proposes activation of the innate immune response mediated by the binding of PAMPs and DAMPs to PRRs of monocytes, neutrophils and endothelial cell and the same mechanism may be suggested for ALI and ARDS (Calfee and Matthay, 2010). We used an experimental model with two different degrees of severity of ALI to better understand the role of HMGB1, TLR2 and TLR4 in this condition and their association with the severity of ALI. Protective mechanical ventilation was used to minimize the influence of mechanical ventilation on the genesis of ALI. Inflammatory cytokines and perpetuation of the local or systemic inflammatory response have received attention due to their role in the induction of ALI (Levitt *et al*., 2009). The model of warm saline infusion is suitable to evaluate the importance of cytokines and inflammatory mediators in ALI, although the reason for perpetuation of the inflammatory response and its modulation is unclear. The hypothesis that the severity of ALI is associated with the production of HMGB1, IL-8, TNF-alpha, and mRNA expression of TLR2 and TLR4 was tested. Therefore, this study evaluated the concentrations of HMGB1, IL-8, TNF-alpha in bronchoalveolar lavage (BAL) and mRNA expression of TLR2 and TLR4 in lung tissue of rabbits with two different degrees of ALI severity.

MATERIAL AND METHODS

The study was approved by the Animal Care and Use Committee (approval number CEEA-892 2011) of São Paulo State University (UNESP). This study can be consulted in the Medical School of Botucatu, UNESP. Forty-five male New Zealand white rabbits, weighing 2.0-3.0kg, were anesthetized by intramuscular injection of ketamine (50mg/kg) and xylazine (4mg/kg). Anesthesia was maintained with continuous intravenous infusion of ketamine (10mg/kg/h) and xylazine (4mg/kg/h). Muscle paralysis was induced by the intravenous administration of pancuronium bromide (0.2mg/kg) and maintained with 0.1mg/kg doses. A tracheotomy was performed by inserting a tracheal tube (3.0–3.5mm inner diameter; Portex, Hythe, UK). Immediately after tracheotomy, ventilation was initiated with an Inter 7 Plus ventilator (Intermed, São Paulo, Brazil) in the pressure-regulated volume control mode using the following initial parameters: FiO_2 1.0; tidal volume 6mL/kg; PEEP 5cmH_2O; respiratory rate 40-50 breaths/min.

These settings were maintained for 15min for stabilization. Once the tracheotomy was performed, a vascular catheter was inserted into the common carotid artery (22 Gauge Jelco, Introcan W Safety™, B. Braun, Melsungen, Germany) and a double lumen catheter (5Fr; Arrow International, Inc., Reading, PA, USA) was advanced into the superior vena cava through the jugular vein. The arterial catheter was used to assess blood gases and arterial blood pressure with a monitoring system (LogiCalw, Medex, Dublin, OH, USA) connected to a conventional physiological monitor of the brand Dixtal model DX 2010, Manaus, Brazil. The double lumen catheter was used for continuous infusion of sedatives, maintenance fluids and vasoactive drugs to maintain hemodynamic stability.

Acute lung injury was induced by removal of the surfactant through successive washes with warm saline infused into the lung as described in other studies (Viana *et al*., 2004). The volume of saline used was 30mL/kg and infusions were performed at a maximum pressure of 30cmH_2O. The procedure was repeated every 3-5 minutes until the criterion for mild/moderate ($PaO_2/FiO_2 \leq$ 300mmHg) or severe ALI ($PaO_2/FiO_2 \leq$ 100mmHg) was achieved.

Mechanical ventilation after ALI was performed with an Inter 7 mechanical ventilator (Intermed São Paulo, Brazil) in the pressure-regulated volume control mode, with the pressure set to 30cmH_2O, PEEP to 10cmH_2O, and tidal volume to 6mL/kg. The initial respiratory rate was 40 cycles per minute and FiO_2 was 1.0. The animals were divided into three groups: a control group consisting of 15 rabbits without ALI submitted to sham procedures in the neck region and receiving protective mechanical ventilation (PEEP of 5cmH_2O and tidal volume of 6mL/kg); severe ALI group consisting of 15 rabbits with severe ALI ($PaO_2 \leq$ 100mmHg) receiving protective mechanical ventilation (PEEP of 10cmH_2O and tidal volume of 6mL/kg); mild/moderate ALI group consisting of 15 rabbits with moderate ALI (100mmHg > PaO_2 < 300mmHg) receiving protective mechanical ventilation (PEEP of 10cmH_2O and tidal volume of 6mL/kg).

For the confirmation of ALI severity and oxygen monitoring, arterial partial pressure of oxygen was measured in all animals at the beginning of the procedure, after tracheotomy, after ALI confirmation according to the experimental protocol, and every 30 minutes during the 4 hours of ventilation. After the 4 hours of ventilation, IL-8, TNF-alpha and HMGB1 levels were measured in BAL and plasma. Lung tissue was obtained for the measurement of TLR2 and TLR4 mRNA expression and for histopathological analysis of lung injury.

The pulmonary parameters obtained in mechanical ventilation were used for the confirmation of ALI and its severity. We used PaO_2 and compliance to confirm ALI and its different degrees of severity. HMGB1 was measured in BAL obtained from the left lung by the infusion of saline (15mL/kg) using the Rabbit HMGB1 ELISA kit (IBL International, Hamburg, Germany). The intra- and inter assay coefficients provided by the manufacturer were 10%.

The concentrations of IL-8 and TNF-alpha were determined in tissue, BAL and plasma by ELISA using kits from USCN Life Science Incorporation (Wuhan, China), specific for rabbit

cytokines. The intra- and inter assay coefficients of variation were less than 10 and 12%, respectively. The animals were euthanized with intravenous pentobarbital (100mg/kg). The lung was removed, filled with oxygen, occluded, and the presence of pneumothorax was observed. The position of the vascular catheters and endotracheal tube was confirmed. Lung fragments (50mg) were collected from the middle lobe for the quantification of TLR2 and TLR4 mRNA expression.

The left lung was used for histopathological analysis and collection of BAL. The lung was fixed in 10% formalin and the slides were stained with hematoxylin-eosin. Lung injury was evaluated in five randomly chosen areas by attributing a score to the following seven parameters: alveolar inflammation, interstitial inflammation, alveolar hemorrhage, interstitial hemorrhage, edema, atelectasis, and necrosis. Each item analyzed had 4 grades of severity or 4 points, for a maximum score of 28 points or crosses (Rotta *et al*., 1999).

Fragments from the middle lobe of the right lung were stored in RNA later™ until the time of total RNA extraction. Total RNA was extracted using the SV Total RNA Isolation System from Promega®. The lung fragments were disrupted in a Tekmar Tissumizer with 1mL lysis buffer of the SV Total RNA kit. For elimination of genomic DNA after extraction, the total RNA samples were again treated with DNAse (Sigma) according to the protocol of the manufacturer. The elimination of genomic DNA was confirmed by real-time PCR using the extracted RNA treated with DNAse in the real-time PCR assay. All samples were quantified in a NanoDrop 2000/2000c spectrophotometer (Thermo Scientific) and purity was verified by determining the 260/280 ratio. Samples with a ratio of 1.9 to 2.0 were used for cDNA synthesis and real-time PCR. Other samples were discarded.

cDNA and real-time PCR: The total RNA extracted was submitted to reverse transcription for the synthesis of cDNA using the Improm enzyme (Promega™) according to manufacturer instructions. The cDNA was stored at -70ºC for quantification by real-time PCR. TLR2 and TLR4 (Kajikawa *et al*., 2005) (19) were quantified by reverse transcription of mRNA to cDNA and real-time PCR. Real-time PCR was performed in the Applied Biosystems 7300 Real-Time PCR System using the GoTaq® qPCR Master Mix (Promega, Madison, WI, USA). Both genes were normalized to the GAPDH gene. The sample volume was 4L of cDNA in a reaction volume of 20µL containing enzymes, nucleotides, and the primers specific for each gene. The number of cycles was 40, with an initial activation at 95ºC for 1 minute, denaturation at 95ºC for 15 seconds, and annealing and extension at 60ºC for 1 minute. The melting curve was analyzed to eliminate the presence of nonspecific amplification and primer-dimer formation.

Parametric data were analyzed by ANOVA and the Tukey test and by ANOVA for repeated measures. The Kruskal-Wallis and Friedman tests were used for nonparametric data.

RESULTS

The weight of the animals ranged from 2.3 to 2.6kg, with no difference between groups. The median number of infusions for ALI induction was 14 in the severe ALI group and 5 in the mild/moderate group. This difference was due to the intensity of the injury proposed. Nevertheless, the percentage of recovered saline volume was similar in the groups. Blood oxygen values were similar in all groups at the beginning of the study. After infusion of warm saline, some animals with PaO_2 below 100mmHg were excluded from the severe group to ensure homogeneity of the groups. All animals were ventilated for 4 hours to collect ventilation parameters and biological material. Oxygen concentration was 219.81±81.31mmHg over the 4 hours, reinforcing the presence of ALI (Figure 1A).

Baseline PaO_2 was 230mmHg in the group with mild/moderate ALI, which differed from the severe and control groups. PaO_2 values improved over the period of evaluation in this group, reaching values similar to those of the control group at T120. The same trend was not observed in the severe group. There was little variation in PaO_2 values over the period of evaluation in the control group and the values after 4 hours of ventilation were similar to baseline (Figure 1A).

Pulmonary compliance was evaluated to confirm the presence and severity of lung injury. Compliance was low in animals with severe and mild/moderate ALI when compared to the control group without lung injury (Figure 1B). Pulmonary compliance decreased after the induction of ALI and remained stable until the end of the experiment in the severe and mild/moderate groups, confirming the presence of ALI. Differences in compliance were observed between the severe and mild/moderate groups after the induction of ALI (Figure 1B).

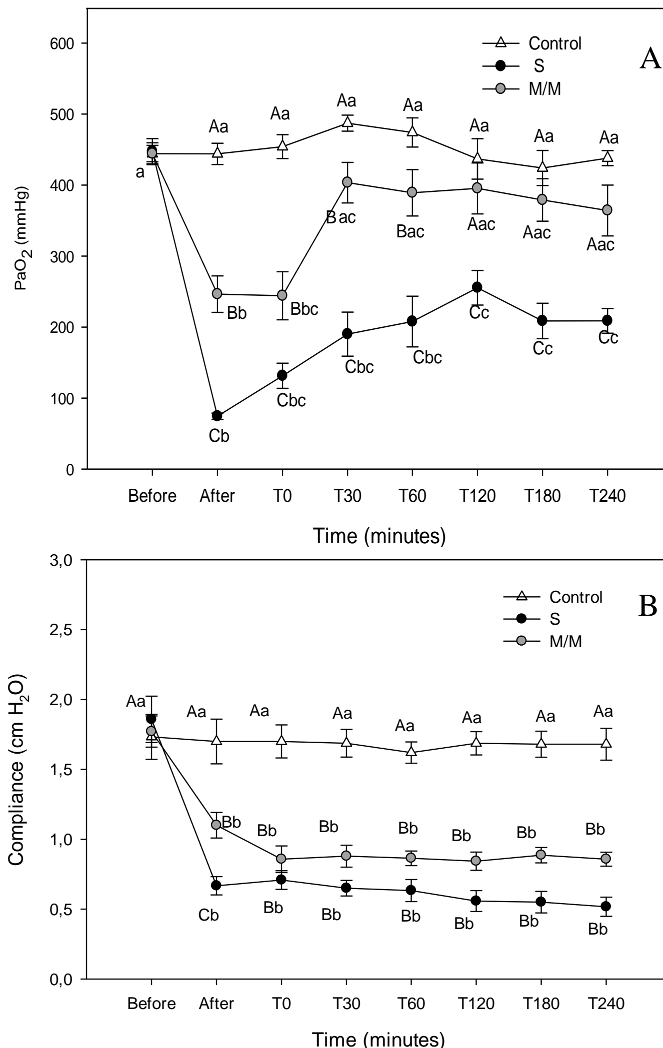

Figure 1. PaO_2 (A) and compliance (B) in the control group and the groups with severe (S) and mild/moderate (MM) acute lung injury in rabbit. Capital letters: comparison between groups at the same time point. Lower case letters: comparison between time points in the same group. Values with different letters differ significantly ($P \leq 0.05$, Kruskal-Wallis test for groups and Friedman between times in the same group).

HMGB1 was measured in BAL and plasma. The results showed higher HMGB1 levels in BAL in the severe group compared to the moderate/mild group, while plasma levels were similar (Table 1). The concentration of IL-8 in BAL and plasma differed between the groups with ALI, while no difference was observed when tissue homogenates were analyzed (Table 1). The levels of TNF-alpha in BAL and plasma differed between the two intensities of lung injury, although plasma levels of this cytokine were similar in the mild/moderate and control groups.

Evaluation of TNF-alpha levels in lung tissue homogenates revealed a significant difference only between the severe and control groups.

Table 1. HMGB1, IL-8 and TNF-alpha in bronchoalveolar lavage, lung tissue homogenate and plasma of rabbits after induction of acute lung injury and mRNA expression of TLR-2 and TLR-4 in lung tissue

Variable median (25th; 75th percentile).	Control (n=15)	Severe (n=15)	Mild/Moderate (n=15)	p value
HMGB1 (ng/mL)				
BAL	16 (14 - 26) a	62 (20 - 79) b	34 (9 - 45) a	0.035
Plasma	2 (0 - 4) a	1.3 (0 - 3) a	2.4 (0.3 - 5.5) a	0.954
IL-8 (pg/mL)				
BAL	128 (14 - 180)a	592 (347 - 704)b	350 (140 - 672) c	0.02
Lung tissue	686 (529 - 1751)a	1315 (610 - 2863)a	1317 (674 - 2267)a	0.80
Plasma	241 (77 - 540) a	344 (69 - 934) b	75 (39 - 199) a	0.014
TNF-alpha (pg/mL)				
BAL	45 (25 - 86) a	1078 (382 - 1224) b	167 (121 - 750) c	≤ 0.001
Lung tissue	1122 (960 - 1329) a	1526 (1324 - 1693) b	1477 (1172 - 1647) ab	0.035
Plasma	78 (40 - 80) a	143 (64 - 227) b	17 (6,.5 - 30.6) a	0.016
Histology Score	0.80 (0.6 - 1.0)a	2.1(1.9 - 2.3)b	1.0 (0.4 - 1.2)a	0.02
TLR2/GAPDH	0.690 (0.35 - 0.87)a	0.490 (0.30 - 1.84)a	0.316 (0.19 - 0.38)a	> 0.05
TLR4/GAPDH	0.153 (0.93 - 0.22)a	0.248 (0.21 - 0.30)b	0.261 (0.24 - 0.31)b	< 0.05

n = number of animals, BAL = bronchoalveolar lavage. Values with lowercase letters do not differ significantly (P≤0.05, Kruskal-Wallis).

Histopathological analysis of lung tissue from control animals demonstrated alveolar integrity and a small number of neutrophils in the alveolar space. There was no increase in the inflammatory infiltrate in the mild/moderate group compared to control, but increased inflammation was observed in animals with severe ALI (Table 1). The highest histological scores were observed in the severe ALI group (Table 1), followed by the mild/moderate and control groups. Hematoxylin-eosin staining demonstrated a difference in scores between the groups with severe and mild/moderate ALI, but the latter did not differ from the control group.

The expression of TLR2 mRNA in the severe and mild/moderate ALI groups was similar to that observed in the control group (Table 1). On the other hand, increased TLR4 mRNA expression was found in animals with ALI.

DISCUSSION

The experimental model, LPA induced with warm saline in rabbit, can be used for the identification of new biomarkers. In this model, the intensity of the lesion can be monitored by PaO_2 and it was used to confirm the presence of lung injury and allowed the classification of animals with different degrees and intensity of ALI, but compliance only showed the ALI presence without difference in intensity of lesions. Rabbits with mild/moderate ALI had PaO_2 values in the first hour that were situated between the severe and control groups. Thereafter, the PaO_2 concentration was similar to that of the control group, but IL-8 and TNF-alpha were higher in BAL, differing from the control group and confirming the presence of mild/moderate ALI. In this respect, mild/moderate ALI is characterized by PaO_2 of 200mmHg in the first hour, with 200mmHg > PaO_2 < 300mmHg after the first hour, and severe ALI is characterized by PaO_2 below 200mmHg over the first 4 hours. Differences in the histological score were also observed between the groups with ALI, confirming the presence of mild/moderate and severe injury.

Pulmonary compliance confirmed the presence of lung injury after the measurement of PaO_2. The group with mild/moderate exhibited ventilatory parameters similar to those observed in the group with severe injury over the 4 hours of mechanical ventilation. The PaO_2 values in the mild/moderate group (300 to 200mmHg) indicated recovery of oxygenation after 4 hours of ventilation. PaO_2 can be used to confirm the

degree of injury in rabbits and compliance confirmed the presence of injury in both forms of ALI.

The period of observation was longer than one hour, this choice permitted better evaluation of the role of inflammatory cytokines and receptors, as well as the detection of differences between experimental groups and of associations with the intensity of injury. The gentle ventilation was crucial for this experiment to avoid lung injury induced by high volumes and pressures as frequently used for this purpose. This experimental model can show the role of HMGB1, TNF-alfa, IL-8, TLR-2, and TLR-4 in ALI and their association with its severity. Rabbits with severe ALI have increasedHMGB1 , TNF-alfa and IL-8 levels.

HMGB1 concentration differed between the groups with ALI, suggesting that this protein could be associated with the injury and recruitment of neutrophils and have a role in the modulation of macrophages (Entezari *et al*., 2014) (20). TNF-alpha and IL-8 were also able to distinguish mild/moderate from severe injury and could therefore be used as an auxiliary tool in lung injury.

We observed that HMGB1 could induce the early expression of IL-8 and TNF-alpha, suggesting its participation in the deterioration of lung function (Imai *et al*., 1999; Mukaida, 2003; Kao *et al*., 2014) (21, 22, 23). Although cytokines and associated receptors have roles in the lung injury process, in this study, TLR2 expression in the two groups with ALI did not differ from that of the control group, ruling out its involvement during the first 4 hours of ALI. TLR2 may participate in later stages of ALI or after 4 hours of evaluation. In contrast to TLR2, higher expression of TLR4 was observed in the groups with ALI, demonstrating the early participation of this receptor in the induction of lung injury in this animal model.
Other studies have demonstrated the participation of TLR2 and TLR4 as receptors of HMGB1 (Kao *et al*., 2014) (23) and in the recruitment of STAT3, resulting in inflammatory responses and lung injury (Wolfson *et al*., 2014) (24). TLR2, TLR4 and STAT3 are recruited in cyclic stretch using high-amplitude ventilation in human lung micro vessel endothelial cells. This cyclic stretch increases HMGB1 expression. TLR2, TLR4 and STATs 1 and 3 are recruited for HMGB1 expression and inflammatory cytokine production (Wolfson *et al*., 2014) (24). TLR2 and TLR4 interact with HMGB1 and induce NF-kB-dependent transcriptional activation through TLR2 and TLR4; MyD88 and Mal/TIR pathways could be activated for cytokine production and amplify the lung injury (Huang *et al*., 2010) (25).

The detection of HMGB1 in BAL and serum over the period of 4 hours demonstrates the involvement of this cytokine in the early stage of the inflammatory response. Ogawa *et al*. (2006) described HMGB1 in late stage of ALI lesion and maintenance of inflammation (14). The intense stimulus present at the onset of aggression may induce a decrease in some receptors associated with inflammation. After this initial period, these receptors could increase their expression and influence lung deterioration over time (Wolfson *et al*., 2014) (24). HMGB1 can induce an intense inflammatory response and stimulate the production of other inflammatory cytokines (Lotze and Tracey, 2005; Anderson and Tracey, 2011) (26, 27).

Our findings suggest that HMGB1 together with TLR4 stimulates inflammation, sustaining and self-perpetuating the inflammatory response observed in ALI over the first hours of injury (Deng *et al*., 2013) (28). The HMGB1-specific receptor RAGE probably participates in the physiopathology of ALI. This receptor is expressed in large amounts in the lung during aggression and, together with TLR4 and TLR2, may induce cytokine production (Buckley & Ehrhardt, 2010; Sparvero *et al*., 2009; Achouiyi *et al*., 2013) (11, 29, 30). TLR4, TNF-alpha, IL-8, and HMGB1 are induced during ALI and we may suggest that they are produced early and participate in the onset and maintenance of the inflammatory response observed in ALI.

The absence of a correlation between HMGB1 measured in BAL and in plasma may be due to the experimental model used, which does not include aggressive ventilation, LPS or other stimuli described in primary ALI models. Therefore, the time interval between the release of cytokines in the lung and their detection in plasma needs to be longer, in agreement with the idea of early involvement of HMGB1 in the onset of primary ALI.

The association of TNF-alpha, IL-8 and HMGB1 levels with the intensity of injury may be used as a complementary tool in the determination of ALI. However, the best site for measurement seems to be BAL, a fact limiting studies in patients. The measurement of HMGB1 may be feasible and could be used as an auxiliary tool together with other biomarkers of aggression in the onset of ALI if obtained in tracheal aspirate rather than BAL, which is an invasive and dangerous procedure.

The maintenance of inflammation in ALI seems to involve HMGB1 and TLR receptors, although we found no increase in the expression of TLR2. HMGB1 binds to TLR2 and TLR4, as well as to RAGE, but the model used here was only able to verify the expression of TLR4 in the early stage of ALI (Jiang *et al*., 2006) (31). The expression of TLR2 in ALI was similar to that of the control group, suggesting its participation after 4 hours of injury or in situations of concomitant bacterial infections with capability to link TLR2. TLR2 was similar to the control group in our experiment but is likely to participate in ALI, especially in the presence of Gram-positive bacteria since this receptor binds to cell wall components of these bacteria for the stimulation of inflammatory cytokines. Both TLR2 and TLR4 are receptors of HMGB1 and are likely to participate in the physiopathology of ALI. However, blockade of these receptors, which could provide more accurate information about the role of TLR4 and HMGB1 in TNF-alpha and IL-8 production (Deng *et al*., 2013; Ueno *et al*., 2004; Park *et al*., 2004) (28, 32, 33), was not performed in the present study because of the lack of specific antibodies against TLRs for rabbits.

The source of early production of HMGB1 may be necrosis of pulmonary or capillary epithelial cells or production by lung-resident macrophages. Neutrophils and macrophages can produce HMGB1 as demonstrated in an experimental model of aggressive ventilation with a tidal volume of 30mL/kg and ventilator-induced lung injury. The levels of HMGB1 in BAL obtained by these authors ranged from 50 to 600ng/mL and were higher than those observed in the present study (Ogawa *et al*., 2006) (14). These differences were due to the mode of ventilation used.

In contrast to previous studies (Matsusawa *et al*., 2010; Ding *et al*., 2013) (34,35), we used gentle or nonaggressive ventilation, which protects the lung tissue against injury. In another study, BAL levels of HMGB1 were 130ng/mL in LPS-instilled mice compared to 33ng/mL in the control group without LPS (Ueno *et al*., 2004) (32). The control group level was similar to the values found in our study. Despite this difference, we detected HMGB1 in BAL and its early production.

HMGB1 has been suggested to be produced late in systemic inflammatory processes or lung injuries. However, our study demonstrated the expression of HMGB1 in early stages of noninfectious ALI, even in the protective ventilation mode and in severe and mild/moderate injury. Furthermore, we observed the early involvement of TLR4 in ALI, one of the receptors to which HMGB1 binds. Binding of HMGB1 to TLR4 may be a mechanism of cell stimulation and activation of nuclear factors to induce the expression of TNF-alpha and IL-8. HMGB1 has been shown to require TLR2 and TLR4 to induce an increase in NFkB. Our findings support this hypothesis, except that HMGB1 plays an early and not a late role as suggested.

Rabbits are an experimental model that permits several measures of blood oxygen, evaluation of ventilatory therapy and lung lesion induced by mechanical ventilation. This model could be used in test drugs or surfactants usually employed to human ALI treatment, SDRA or lung disease. ALI in rabbits induced with warm saline enables the control of the lesion intensity and exclusion of lesion induced by bacterial antigen. In conclusion, HMGB1 is involved in the early stages of ALI and the inflammatory response, as well as in the maintenance of inflammation in the late stages of this condition. This participation of HMGB1 in the early and late stages of ALI and the stimulation of TLR2 and TLR4 may induce the production of inflammatory cytokines, with consequent deterioration of lung function.

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