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Research article

Cupping alleviates lung injury through the adenosine/A_{2B}AR pathway

Yifan Ren [a,b], Lei Qi [c], Lin Zhang [a,d], Jinkai Xu [b], Jiancan Ma [b], Yi Lv [a,d], Yuanyuan Zhang [e], Rongqian Wu [a,*]

[a] National Local Joint Engineering Research Center for Precision Surgery & Regenerative Medicine, Shaanxi Provincial Center for Regenerative Medicine and Surgical Engineering, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China
[b] Department of General Surgery, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China
[c] Department of Neurosurgery, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China
[d] Department of Hepatobiliary Surgery, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China
[e] Department of Pediatrics, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China

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ABSTRACT

Background: Acute lung injury (ALI) is a serious condition. Inflammation plays a crucial role in the pathogenesis of ALI. Cupping, as a part of traditional Chinese medicine, is still a popular complementary and alternative therapy for a variety of ailments including respiratory diseases. However, reliable scientific data about cupping therapy are scarce. Adenosine, a purine nucleoside produced under metabolic stress by the action of extracellular ecto-nucleotidases (i.e. CD39 and CD73), can attenuate ALI through the A_{2B}AR receptor. The aim of this study was to investigate the protective effect of cupping in a rat model of ALI and the role of adenosine in it.

Methods: Male adult rats were subjected to ALI by intratracheal LPS instillation (0.3 mg/kg). Immediately after intratracheal LPS instillation, vacuum pressure was applied to a sanitized plastic bell cup on the back of the rat by suction for 10 min. Pulmonary injury and inflammation were assessed at 4 h after LPS challenge. The role of adenosine and A_{2B}AR in cupping's protection after LPS instillation were evaluated.

Results: Cupping alleviated LPS-induced lung injury, reduced inflammation and inhibited NF-kB activation in rats. Cupping upregulated CD39 and CD73 mRNA expression of the skin tissue at the cupping site and increased circulating levels of adenosine. Administration of PSB1115, a specific adenosine A_{2B}AR receptor antagonist, abolished cupping's beneficial effects in LPS-induced ALI.

Conclusions: Cupping attenuates lung inflammation and injury through the adenosine/A_{2B}AR pathway. The current study provides evidence-based information about cupping therapy in ALI.

1. Introduction

Acute lung injury (ALI) is a common and devastating illness [1, 2], and the mortality rate of acute respiratory distress syndrome (ARDS) caused by ALI exceeds 40% [3]. Despite the increased understanding of the complex pathophysiology of ALI, there are few effective treatments for this devastating illness [4]. Cupping, a traditional treatment that has been used in several cultures for thousands of years, involves the application of a vacuum to a localized area of the skin [5]. Today, this practice, as a part of the broad spectrum of traditional Chinese medicine, is still a popular complementary and alternative therapy for a variety of ailments, including acute and chronic respiratory diseases, in East Asia [6, 7, 8, 9]. Remarkably, cupping is becoming increasingly popular in Western countries. Internet websites advertise and sell the necessary equipment, and online health forums provide details on the therapeutic properties of cupping. Despite the rich history of its medical applications, reliable scientific data about cupping therapy are scarce. Apparently, there is an urgent need to more clearly understand whether and how cupping adds value to existing conventional medical strategies and to identify the biological mechanisms through which cupping exerts beneficial effects.

Adenosine is a purine nucleoside that is produced by the body under metabolic stress, such as hypoxic conditions and acute or chronic inflammatory tissue insults. The main source of extracellular adenosine stems from a coordinated two-step enzymatic conversion of precursor nucleotides via ecto-apyrase (CD39) and ecto-5′-nucleotidase (CD73) [10]. Once released into the extracellular space, adenosine activates four individual G-protein-coupled adenosine receptors (A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR). Several studies have suggested an anti-inflammatory and tissue protective

* Corresponding author.
E-mail address: rwu001@mail.xjtu.edu.cn (R. Wu).

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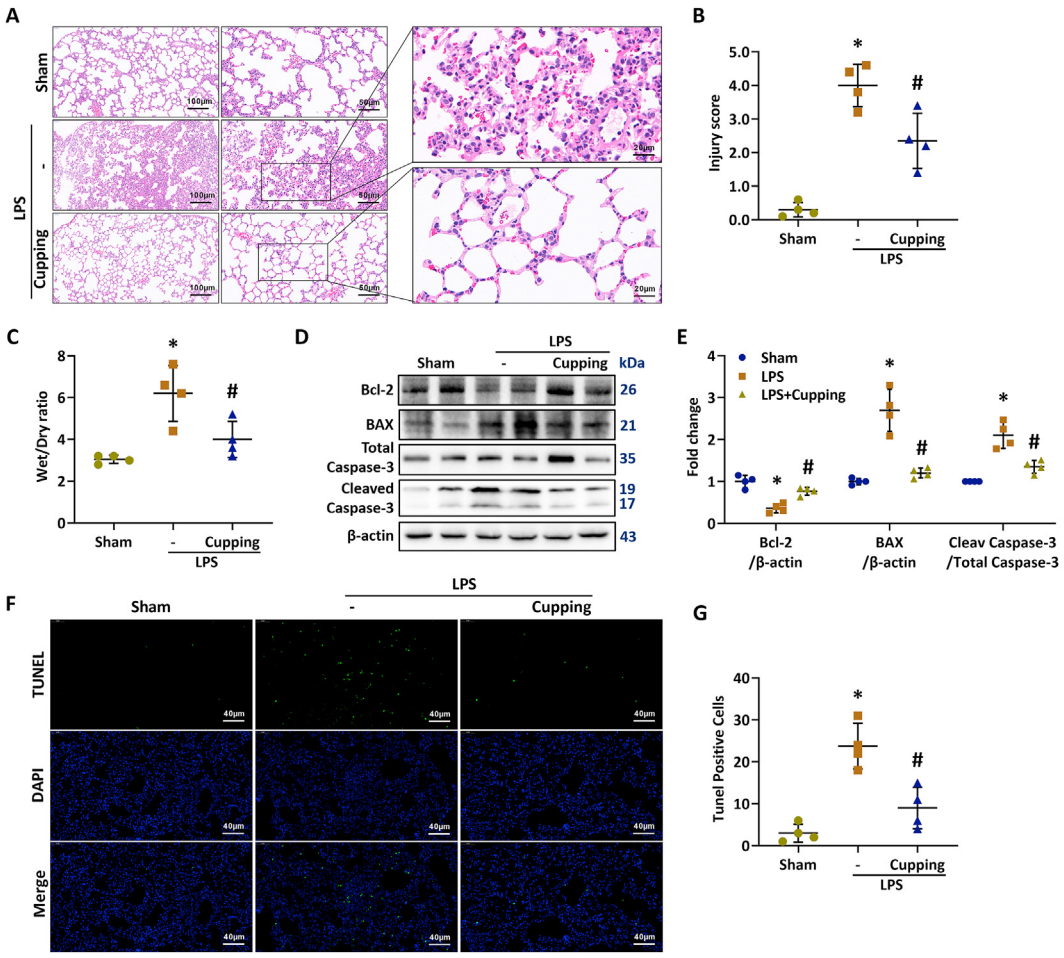

Figure 1. Cupping alleviates lung injury and apoptosis in experimental ALI. Rat ALI was induced by injecting 0.2 ml of LPS (0.3 mg/kg in PBS) into the trachea. Immediately after intratracheal LPS instillation, vacuum pressure was applied to a sanitized plastic bell cup on the back of the rat by suction (cupping group). Control animals were intratracheally challenged with an equal volume of PBS without LPS. The animals were sacrificed at 4 h after LPS instillation. (A) Representative images of haematoxylin and eosin (H–E) staining; (B) Pulmonary injury scores; (C) Lung dry-wet weight ratio; (D) Western blot analysis of the expression of Bcl-2, BAX, Total Caspase-3 and Cleaved Caspase-3 in the lungs; (E) Quantitative analysis of Bcl-2, BAX and Cleaved Caspase-3 expression; (F) Representative images of TUNEL staining (green) and corresponding nuclear counterstaining (blue) in the lungs; (G) Quantitative analysis of TUNEL-positive cells. n = 4–6; mean ± SD; *p < 0.05 versus Sham group; #p < 0.05 versus LPS alone group. LPS, lipopolysaccharide; W/D, dry-wet weight ratio; kDa, kilodalton; Bcl-2; B-cell lymphoma-2; TUNEL, TdT-mediated dUTP Nick-End Labelling; DAPI, 4′,6-diamidino-2-phenylindole.

role of extracellular adenosine under various conditions, including lung injury [11, 12, 13, 14]. The dominant adenosine receptor in the lungs is A_{2B}AR [15, 16]. We therefore hypothesize that cupping attenuates lung inflammation and injury through the adenosine/A_{2B}AR pathway. In the current study, we investigated the protective effect of cupping in a rat model of ALI and focused on the roles of adenosine and A_{2B}AR.

2. Methods

Rat Model of Acute Lung Injury: Male adult Sprague Dawley rats (weighing 250–300 g) from the Animal Experiment Centre of Xi'an Jiaotong University Health Science Centre were housed in a temperature-controlled room on a 12-h light/dark cycle and fed a standard Purina rat chow diet. Prior to the induction of lung injury, the rats were fasted overnight but allowed water *ad libitum*. The rats were anaesthetized with isoflurane inhalation, and the ventral neck and back were shaved and washed with 10% povidone iodine. A 0.5 cm midline incision was made above the sternum. The trachea was then exposed by blunt dissection. A 28-gauge needle was inserted into the trachea above the carina, and 0.2 ml of LPS (0.3 mg/kg in PBS, Sigma) was instilled. The dose of LPS was chosen based on previous publications [17, 18]. The skin incision was then closed with silk sutures. Control animals were intratracheally challenged with an equal volume of PBS without LPS. Blood and tissue samples were collected. At the end of the experiment, all animals were anesthetized and sacrificed by inhalation of isoflurane (Concentration 4%, Yuyan scientific instrument co., LTD, Shanghai, China). All animal experiments were performed in accordance with the guidelines of the China Council on Animal Care and Use and approved by the Institutional Animal Care and Use Committee of the Ethics Committee of Xi'an Jiaotong University Health Science Center, China.

Method for Cupping Therapy: Immediately after intratracheal LPS instillation, vacuum pressure was applied to a sanitized plastic bell cup (Beijing Kangda World Medical Appliance Centre, Beijing, China) on the back of the rat by suction. The diameter of the plastic cup was 1.8 cm. To standardize the cupping procedure, the same vacuum pressure [−0.06 mPa, determined by a vacuum gauge (Thermo Scientific)] and the same area of the back were used to place the cups for all animals. The cups were left in place for 10 min and then removed by gently pressing the adjacent skin to release the negative pressure. To determine the role of A_{2B}AR in beneficial effects of cupping after LPS instillation, PSB1115 (5 mg/kg BW, P0373-10MG, Sigma, USA), a specific A_{2B}AR antagonist [19, 20], was administered i.p. immediately before cupping. At 4 h after LPS challenge, blood, dorsal skin tissue to which the plastic cup and vacuum had been applied and lung tissue samples were collected for various measurements.

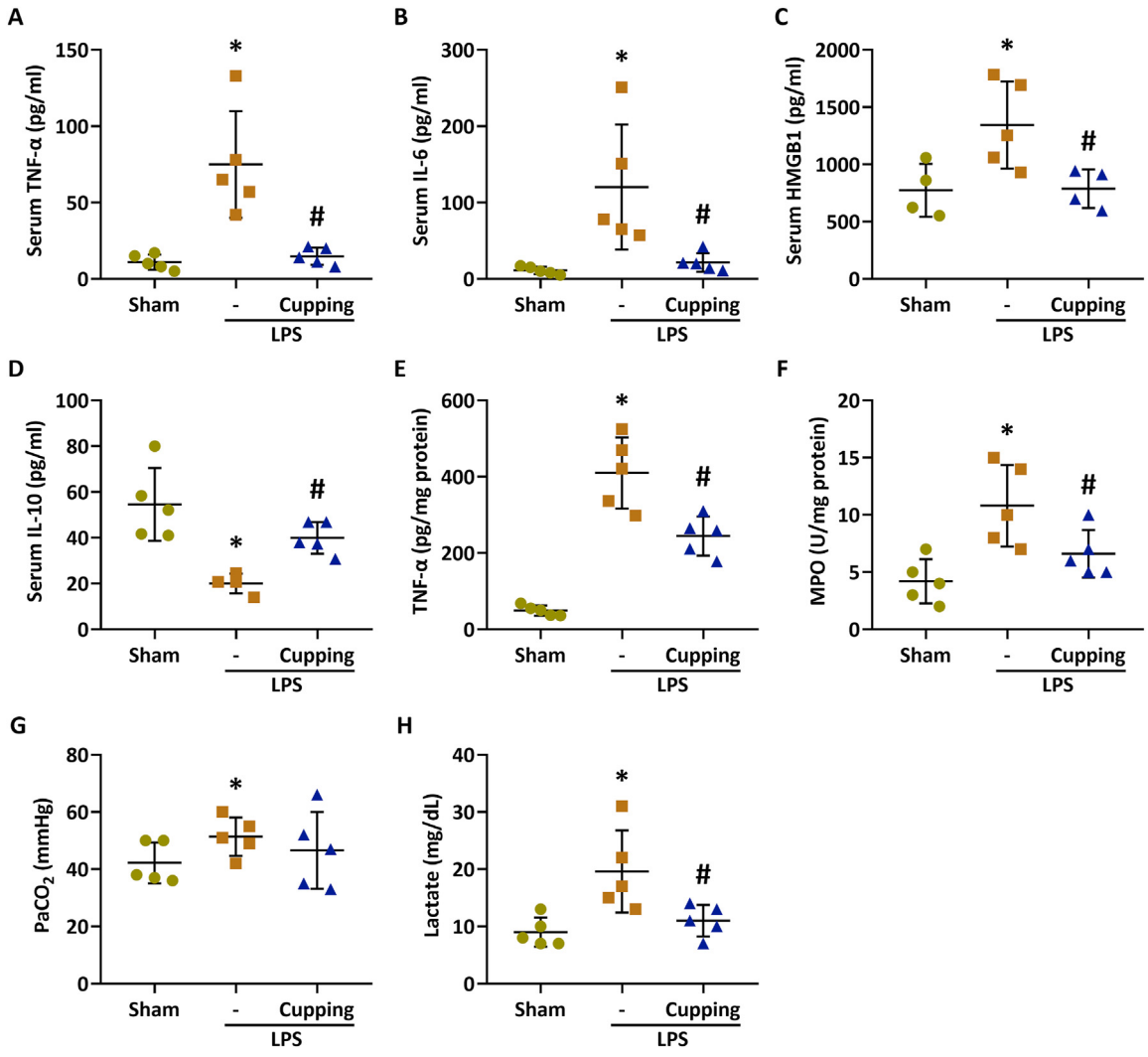

Figure 2. Cupping reduces inflammatory cell infiltration and systemic inflammation in experimental ALI. Rat ALI was induced by injecting 0.2 ml of LPS (0.3 mg/kg in PBS) into the trachea. Immediately after intratracheal LPS instillation, vacuum pressure was applied to a sanitized plastic bell cup on the back of the rat by suction (cupping group). Control animals were intratracheally challenged with an equal volume of PBS without LPS. The animals were sacrificed at 4 h after LPS instillation. (A) Serum TNF-α levels; (B) Serum IL-6 levels; (C) Serum HMGB1 levels; (D) Serum IL-10 levels; (E) TNF-α levels in the lungs; (F) MPO levels in the lungs; (G) $PaCO_2$ levels in the lungs; (H) Lactate levels in the lungs. n = 4–6; mean ± SD; *p < 0.05 versus Sham group; #p < 0.05 versus LPS alone group. LPS, lipopolysaccharide; TNF-α, tumour necrosis factor-α; IL-6, interleukin 6; HMGB1, high mobility group box 1 protein; IL-10, interleukin 10; MPO, myeloperoxidase; $PaCO_2$, arterial carbon dioxide tension.

Morphological examination: The morphological alterations in the lungs were examined. The samples were fixed in buffered formalin and processed for haematoxylin and eosin (H&E) staining and histologic analysis. Rao's method was used for semi-quantitative scoring of pulmonary edema, alveolitis, interstitial lung inflammation, atelectasis, and pulmonary haemorrhage [21] Details of histopathological scoring are detailed in the supplementary materials.

Water content determination: Lung oedema was estimated by comparing tissue water content. Briefly, pulmonary tissues were dried in a 70 °C oven for 48 h. The lung water content was calculated as the wet weight/dry weight ratio.

TUNEL staining: We used a TUNEL assay (11684795910, Roche, Switzerland) to identify apoptosis in the lung as reported previously [22]. After sectioning and fluorescence staining, the sections were observed by a fluorescence microscope at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Fluorescence staining was quantitatively analysed using Image-Pro Plus 6.0 software.

Assay of TNF-α, IL-6, IL-10 and High Mobility Group Box-1 (HMGB1): Serum or lung homogenate levels of TNF-α, IL-6, IL-10 and HMGB1 were measured using enzyme-linked immunosorbent assay (ELISA) kits (Cloud-Clone Corp, China) according to the manufacturer's instructions.

Granulocyte myeloperoxidase (MPO) assessment: Neutrophil accumulation within the pulmonary tissue was estimated using an MPO activity assay (SEA601Ra, Cloud-Clone Corp, China) according to the manufacturer's instructions.

Measurement of blood gas values: Arterial blood samples (0.4ml in a heparinized glass syringe) were collected for blood gas measurement at 4h after intratracheal LPS or PBS instillation. Blood gas values were analyzed with a ABL9 blood gas analyzer (radiomeyer copenhagen, DK-2700, Brønshøj). The blood gas values were adjusted to standard atmospheric pressure and temperature.

Determination of Circulating Levels of Lactate: Serum levels of lactate were determined by using specific assay kit according to the manufacturer's instructions (Pointe Scientific, Lincoln Park, MI).

Western Blot Analysis of NF-κB Nuclear Translocation: Lung tissues were freshly harvested. Nuclear extracts were prepared with a nuclear extract kit (P0027, Beyotime Biotechnology, China) according to

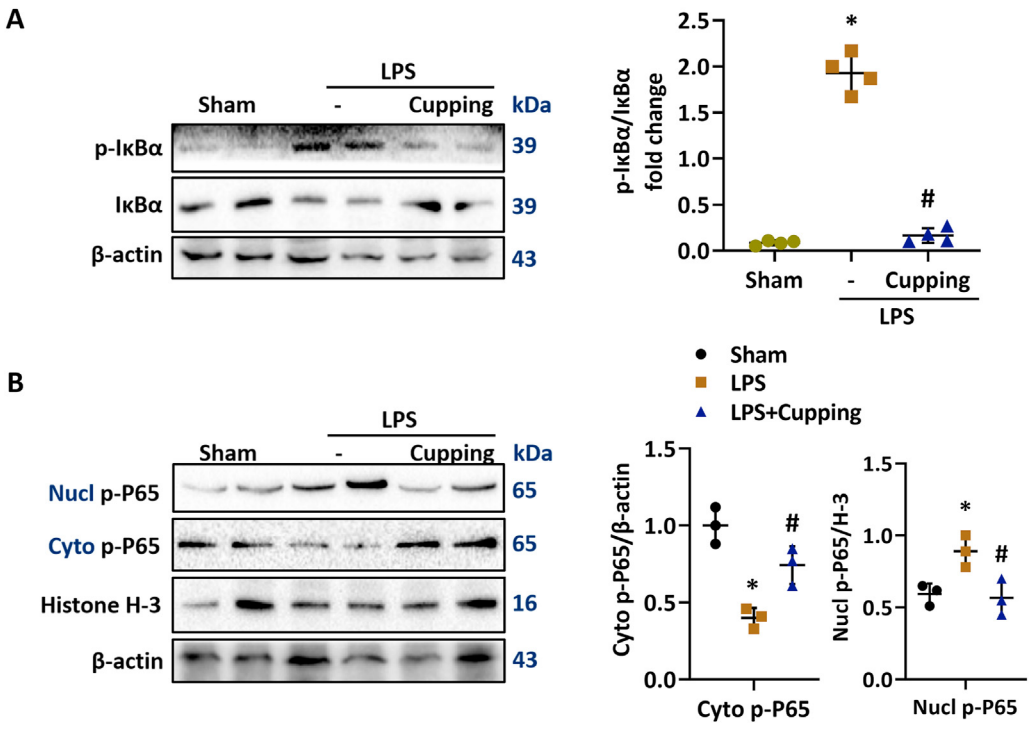

Figure 3. Cupping relieves inflammation through the NF-κB signalling pathway. Rat ALI was induced by injecting 0.2 ml of LPS (0.3 mg/kg in PBS) into the trachea. Immediately after intratracheal LPS instillation, vacuum pressure was applied to a sanitized plastic bell cup on the back of the rat by suction (cupping group). Control animals were intratracheally challenged with an equal volume of PBS without LPS. The animals were sacrificed at 4 h after LPS instillation. (A) Western blot analysis and quantitative analysis of the expression of p-IκBα and IκBα in the lungs; (B) Western blot analysis and quantitative analysis of the expression of Nucl p-P65 and Cyto p-P65 in the lungs. n = 4–6; mean ± SD; *p < 0.05 versus Sham group; #p < 0.05 versus LPS alone group. LPS, lipopolysaccharide; kDa, kilodalton; p-IκBα, phosphorylated inhibitor of NF-κB; IκBα, inhibitor of NF-κB; Nucl, cell nucleus; Cyto, cytoplasm; p-P65, phosphorylated P65; H-3, histone-3.

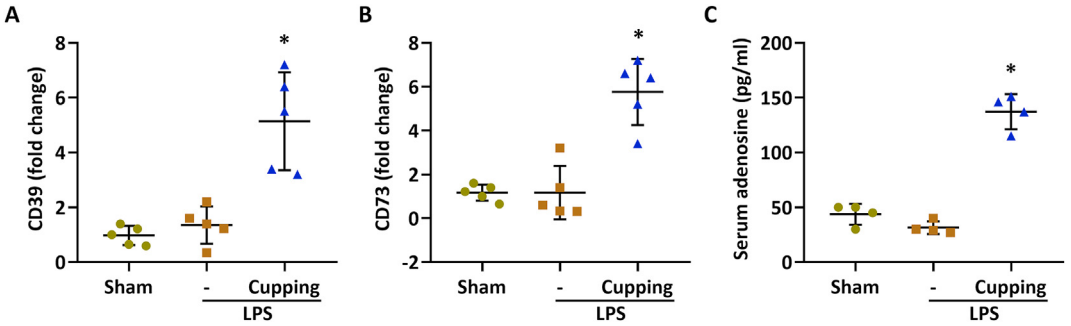

Figure 4. Cupping increased CD39, CD73 and adenosine levels in ALI rats. Rat ALI was induced by injecting 0.2 ml of LPS (0.3 mg/kg in PBS) into the trachea. Immediately after intratracheal LPS instillation, vacuum pressure was applied to a sanitized plastic bell cup on the back of the rat by suction (cupping group). Control animals were intratracheally challenged with an equal volume of PBS without LPS. The animals were sacrificed at 4 h after LPS instillation. (A) Levels of CD39 mRNA expression in the dorsal skin; (B) Levels of CD39 mRNA expression in the dorsal skin (C) Serum adenosine levels. n = 4–6; mean ± SD; *p < 0.05 versus Sham group; #p < 0.05 versus LPS alone group. LPS, Lipopolysaccharide.

the manufacturer's instructions. Equal amounts of nuclear or cytoplasmic protein (20 μg/lane) were resolved on a 4–12% Bis-Tris gel and transferred to a 0.2 μm nitrocellulose membrane. The nitrocellulose blots were blocked by incubation in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% BSA for 1 h. The blots were incubated with rabbit phosphorylated NF-κB P65 and IκBα polyclonal antibodies (1:1000, Cell Signaling Technology, Beverly, MA) overnight at 4 °C. The blots were then washed in TBST 5 times for 10 min, incubated with horseradish peroxidase-linked anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) for 1 h at room temperature, and then washed 4 times in TBST for 10 min. The bands were developed using a digital gel image analysis system (Bio-Rad, California, USA), and the protein expression levels were calculated by using ImageJ 2x software as the relative intensity normalized to β-actin or Histone-3.

Western Blotting Analysis of Apoptosis-related proteins: Lung tissues were freshly harvested. Western blot analysis was performed as previously described [23]. The blots were incubated with rabbit phosphorylated Bcl-2, BAX, Total Caspase-3 and Cleaved Caspase-3 polyclonal antibodies (1:1000, Cell Signaling Technology, Beverly, MA) overnight at 4 °C. The blots were then washed in TBST 5 times for 10 min. The blots were incubated with horseradish peroxidase-linked anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) for 1 h at room temperature and then washed 4 times in TBST for 10 min. The bands were developed using a digital gel image analysis system (Bio-Rad, California, USA), and the protein expression levels were calculated by using ImageJ 2x software as the relative intensity normalised to β-actin.

Assessment of CD39 and CD73 mRNA: CD39 and CD73 mRNA expression in the skin and underlying tissues to which the plastic cup and vacuum were applied were measured by qPCR. The following rat primers were used: G3PDH (XM_579386): 5′-ATG ACT CTA CCC ACG GCA AG-3’ (forward), 5′-CTG GAA GAT GGT GAT GGG TT-3’ (reverse); rat CD39 (NM_022587): 5′-GGA CTG ACC CAC AAC AAA CCA-3’ (forward), 5′-CGG CAT CCA GCA CAA TCC-3’ (reverse); and rat CD73 (NM_021576.1): 5′-CAC AGG AAA TCC ACC TTC CAA-3’ (forward), 5′-ATC GTC AGA GGT GAC TAT GAA TGG-3’ (reverse).

Assay of Serum Adenosine: A rat adenosine ELISA kit (JL27987-48T, JiangLai Biotechnology, Shanghai, China) was used for the detection of the serum levels of adenosine according to the manufacturer's instructions.

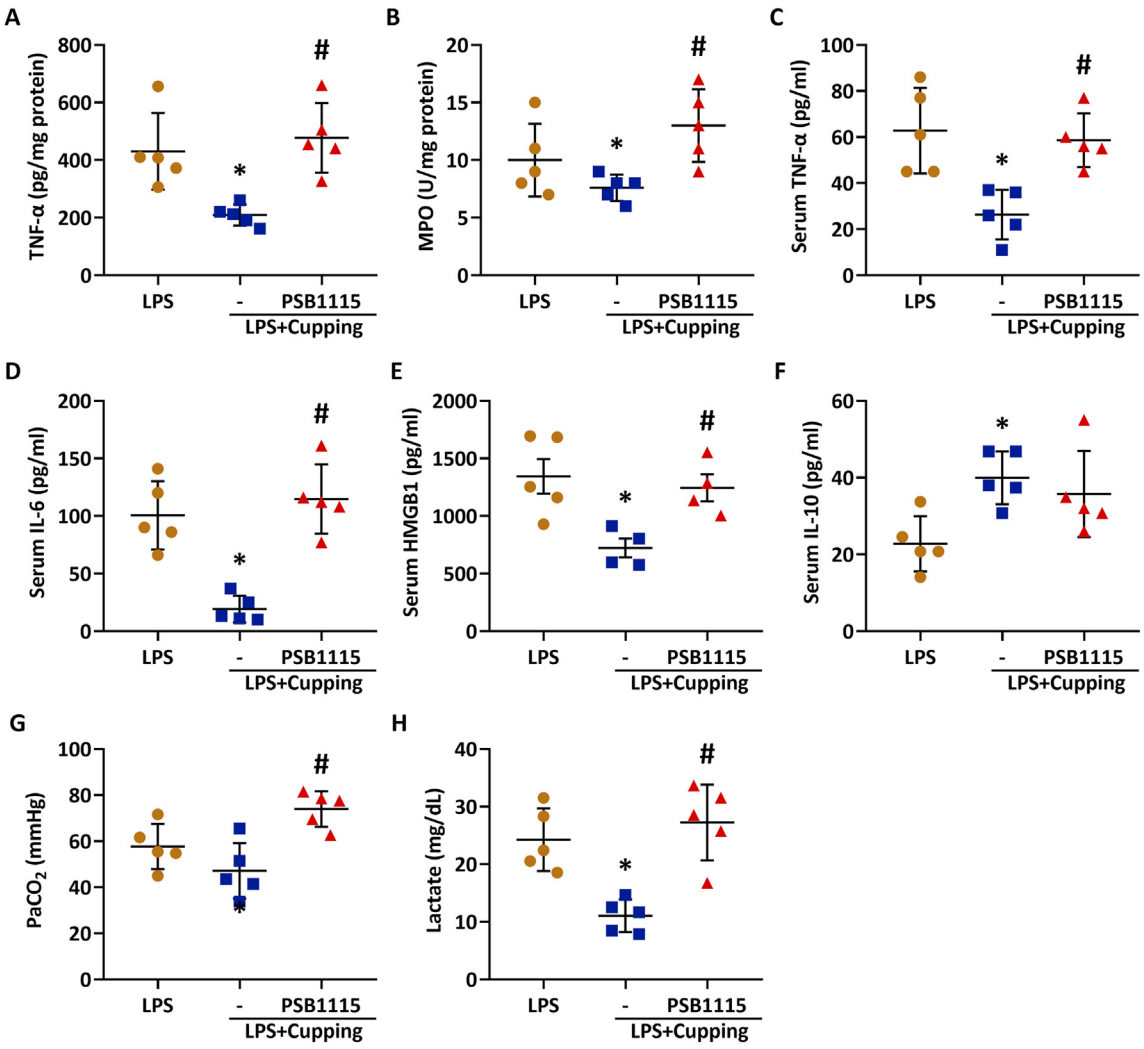

Figure 5. Cupping alleviates inflammation through the adenosine/A_{2B}AR pathway in experimental ALI. Rat ALI was induced by injecting 0.2 ml of LPS (0.3 mg/kg in PBS) into the trachea. Immediately after intratracheal LPS instillation, vacuum pressure was applied to a sanitized plastic bell cup on the back of the rat by suction (cupping group). To determine the role of A_{2B}AR in the beneficial effects of cupping after LPS instillation, PSB1115 (5 mg/kg BW), a specific A_{2B}AR antagonist, was administered i.p. immediately before cupping. The animals were sacrificed at 4 h after LPS instillation. (A) TNF-α levels in the lungs; (B) MPO levels in the lungs; (C) Serum TNF-α levels; (D) Serum IL-6 levels; (E) Serum HMGB1 levels; (F) Serum IL-10 levels; (G) $PaCO_2$ levels in the lungs; (H) Lactate levels in the lungs. n = 4–6; mean ± SD; *p < 0.05 versus LPS alone group; #p < 0.05 versus LPS + Cupping group. LPS, lipopolysaccharide; kDa, kilodalton; TNF-α, tumour necrosis factor-α; HMGB1, high mobility group box 1 protein; IL-10, interleukin 10; MPO, myeloperoxidase; IL-6, interleukin 6.

Statistical Analysis: The data are normally distributed, and all measurement data are expressed as the mean ± standard deviation (SD). The overall significance of the means of multiple groups was determined by one-way analysis of variance (ANOVA). Differences between individual means were assessed by the SNK test. Statistical analyses were interpreted at a 5% significance level.

3. Results

3.1. Cupping alleviates lung injury in experimental ALI

The successful establishment of ALI was confirmed by H&E staining of the lung. To evaluate the protective effect of cupping on LPS-induced ALI, pathological staining and quantification of injury were performed. As shown in Figure 1A&B, bleeding, inflammatory cell infiltration, and patchy necrosis were present in LPS-induced ALI animals. The lung injury score was increased by 8-fold. Administration of cupping improved lung architecture and significantly reduced the lung injury score at 4 h after LPS instillation ($P < 0.05$).

The lung W/D weight ratio is an index of water accumulation in the lung, which was measured to estimate the degree of pulmonary oedema [24]. As shown in Figure 1C, the lung water content of LPS-instilled rats was two times that of the control rats, suggesting that LPS induces acute pulmonary oedema in rats. Cupping effectively reduced the degree of pulmonary oedema at 4 h after LPS instillation ($P < 0.05$).

3.2. Cupping alleviates lung apoptosis in experimental ALI

BAX and Bcl-2 belong to the *bcl-2* gene family. Bcl-2 is an inhibitor of apoptosis. BAX not only antagonizes Bcl-2-mediated inhibition of apoptosis but also promotes apoptosis [25]. As shown in Figures 1D-E, Bcl-2 was decreased and BAX was increased in LPS-instilled rats compared with those of the PBS-instilled animals (i.e., Sham control, $P < 0.05$). For lung apoptosis, cleaved caspase-3 (i.e. active caspase-3) is another important apoptosis marker [26]. Therefore, we further detected the level of caspase-3 expression in lung tissue. Results showed that cleaved caspase-3/total caspase-3 ratio was significantly elevated in LPS-instilled rats (Figures 1D-E). Moreover, TUNEL staining showed that

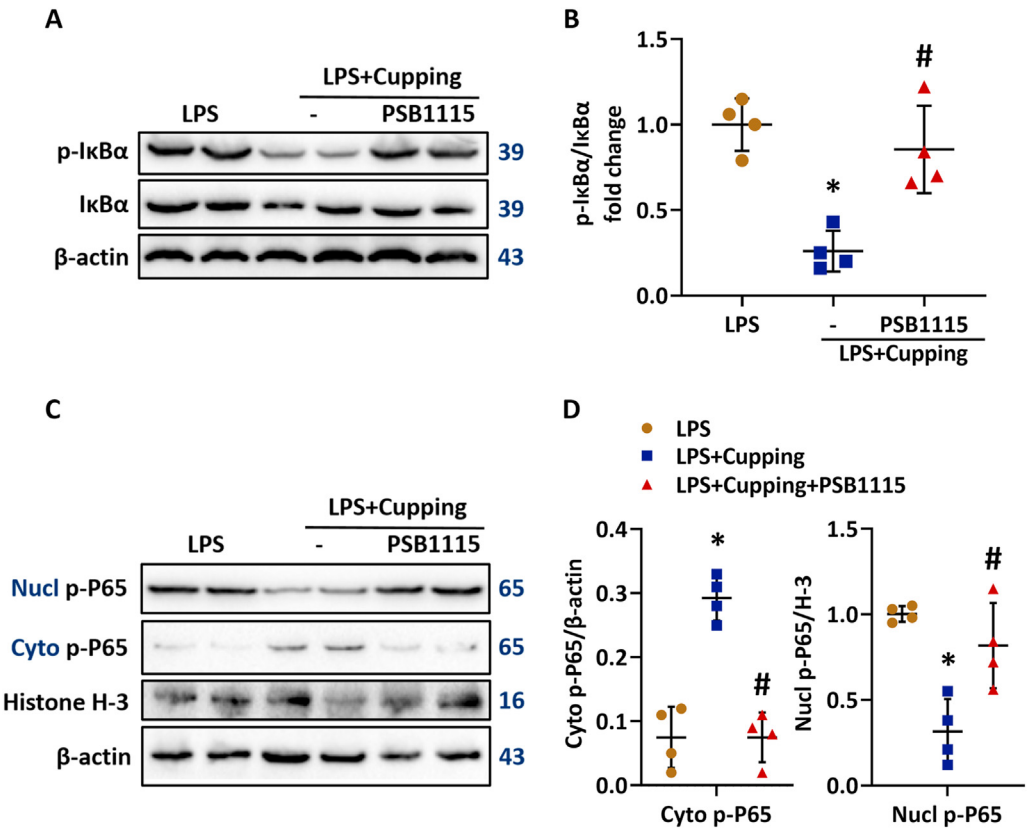

Figure 6. Cupping inhibits NF-κB activation through the adenosine/A_{2B}AR pathway in experimental ALI. Rat ALI was induced by injecting 0.2 ml of LPS (0.3 mg/kg in PBS) into the trachea. Immediately after intratracheal LPS instillation, vacuum pressure was applied to a sanitized plastic bell cup on the back of the rat by suction (cupping group). To determine the role of A_{2B}AR in the beneficial effects of cupping after LPS instillation, PSB1115 (5 mg/kg BW), a specific A_{2B}AR antagonist, was administered i.p. immediately before cupping. The animals were sacrificed at 4 h after LPS instillation. (A&B) Western blot analysis and quantitative analysis of the expression of p-IκBα and IκBα in the lungs; (C&D) Western blot analysis and quantitative analysis of the expression of Nucl p-P65 and Cyto p-P65 in the lungs. n = 4; mean ± SD; *p < 0.05 versus LPS alone group; #p < 0.05 versus LPS + Cupping group. LPS, lipopolysaccharide; kDa, kilodalton; p-IκBα, phosphorylated inhibitor of NF-κB; IκBα, inhibitor of NF-κB; Nucl, cell nucleus; Cyto, cytoplasm; p-P65, phosphorylated P65; H-3, histone-3.

the number of TUNEL-positive cells in LPS-instilled rats was increased (Figure 1F-G, P < 0.05). These results suggest that LPS treatment increases the apoptosis of lung cells in rats. Cupping therapy for 10 min increased the expression of Bcl-2, decreased the expression of BAX, cleaved caspase-3 and reduced the number of TUNEL-positive cells in LPS-instilled rat lungs (Figure 1D-G, P < 0.05).

3.3. Cupping reduces inflammatory cell infiltration and systemic inflammation in experimental ALI

Activation of the inflammatory system and infiltration of inflammatory cells into the lungs are the most important aetiologies of ALI [27]. As shown in Figures 2A-C, serum levels of TNF-α, IL-6 and HMGB1 in LPS-instilled rats increased by 14-, 16- and 2-fold, respectively, compared with those of the PBS-instilled animals (i.e., Sham control, P < 0.05). Cupping therapy for 10 min reduced serum levels of TNF-α, IL-6 and HMGB1 to levels similar to those of the sham controls. IL-10 is a protective inflammatory mediator, and serum levels of IL-10 in LPS-instilled rats decreased by 3-fold compared with those of the PBS-instilled animals. Cupping increased IL-10 by 2-fold compared with that of the LPS-instilled rats (Figure 2D, P < 0.05).

Similarly, markedly increased TNF-α levels in the lungs after LPS instillation were also significantly decreased by cupping (Figure 2E). The level of myeloperoxidase (MPO) activity is an indicator of neutrophil infiltration. As shown in Figure 2F, pulmonary levels of MPO activity increased significantly at 4 h after LPS instillation. Cupping prevented the LPS-induced increase in pulmonary levels of MPO activity (P < 0.05). Moreover, the arterial carbon dioxide tension ($PaCO_2$) increased from 43.3 ± 0.95mmHg in PBS-instilled rats to 53.8 ± 1.44mmHg in LPS-instilled rats (Figure 2G, P < 0.05). Cupping therapy resulted in a 13% decrease in $PaCO_2$ values after intratracheal LPS instillation (P < 0.1). Similarly, increased serum levels of lactate after LPS instillation were also markedly decreased by cupping (Figure 2H, P < 0.05).

3.4. Cupping relieves inflammation through the NF-κB signalling pathway

Activation of IKK leads to the phosphorylation and isolation of inhibitor of NF-κB-α (IκBα), which is bound to nuclear factor kappa-B p65 (NF-κB p65). The dissociated NF-κB p65 is transferred from the cytoplasm to the nucleus and binds with the corresponding inflammation-related genes to initiate the transcription of inflammatory cytokines and induce inflammation [28].

As shown in Figure 3A, phosphorylated IκBα was significantly increased in LPS-instilled rat lungs (P < 0.05). Accordingly, phosphorylated NF-κB p65 in the cytoplasm decreased, while phosphorylated NF-κB p65 in the nucleus increased, suggesting that NF-κB p65 dissociated and translocated into the nucleus from the cytoplasm in LPS-instilled rats (Figure 3B, P < 0.05). Cupping significantly reduced the dissociation of phosphorylated IκBα and the translocation of phosphorylated NF-κB p65 from the cytoplasm into the nucleus (Figure 3A&B, P < 0.05).

3.5. Cupping alleviates inflammation through the adenosine/A_{2B}AR pathway in experimental ALI

To determine the role of adenosine in the beneficial effects of cupping after LPS instillation, CD39 and CD73 mRNA expression in the skin tissue at the cupping site was measured by real-time quantitative PCR (qPCR). As shown in Figure 4A&B, a slight increase in skin CD39 and CD73 mRNA expression was observed at 4 h after intratracheal LPS instillation without cupping. However, the increase was not statistically significant. Cupping for 10 min, on the other hand, increased skin CD39 and CD73 mRNA expression dramatically (P < 0.05). In response, serum adenosine levels also increased after cupping (Figure 4C, P < 0.05).

Recent studies have shown that the dominant adenosine receptor in the lungs is A_{2B}AR [29, 30, 31, 32]. This receptor is predominantly expressed on pulmonary epithelia and inflammatory cells [30, 31, 33,

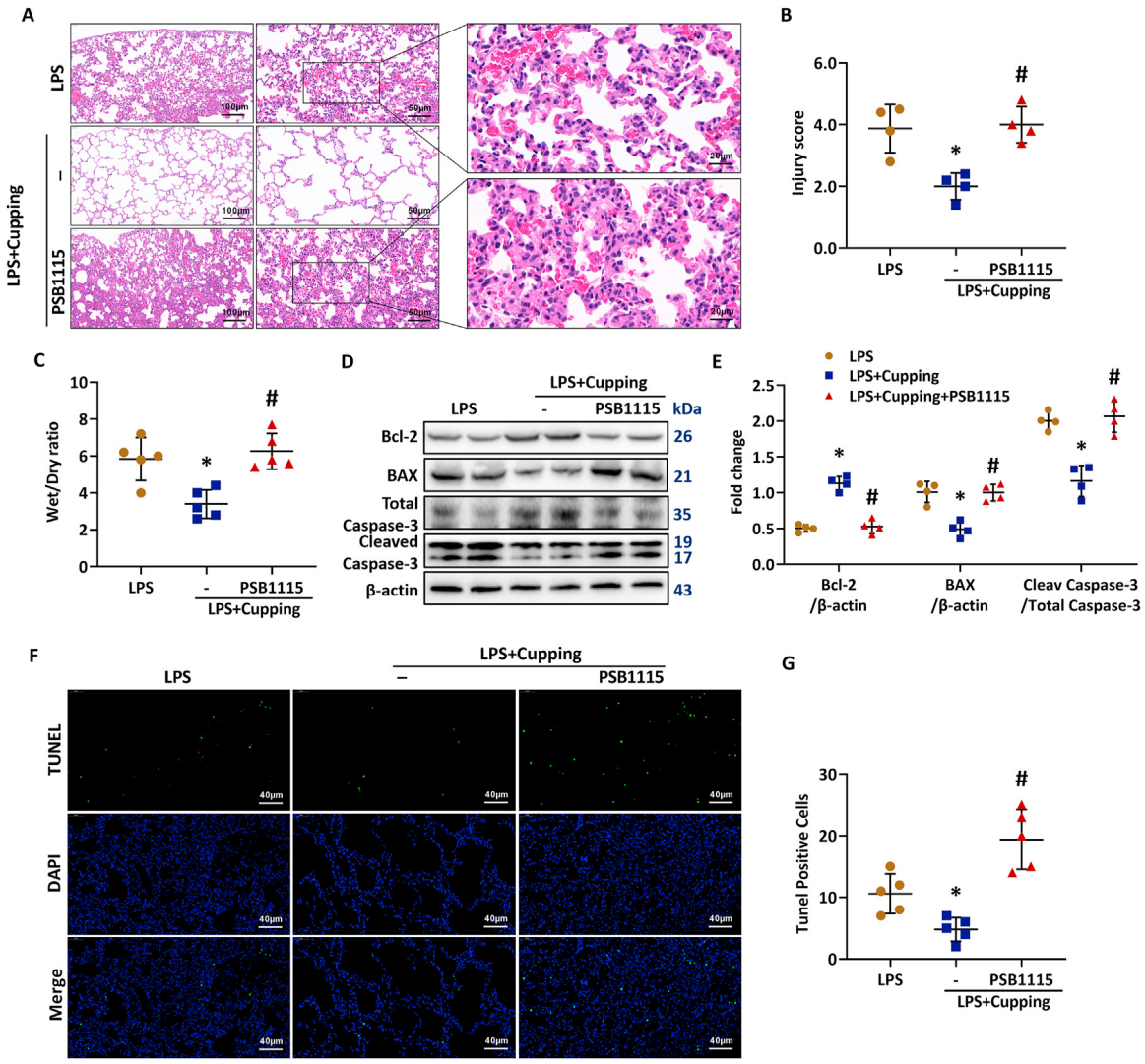

Figure 7. Cupping alleviates lung injury through the adenosine/A_{2B}AR pathway in experimental ALI. Rat ALI was induced by injecting 0.2 ml of LPS (0.3 mg/kg in PBS) into the trachea. Immediately after intratracheal LPS instillation, vacuum pressure was applied to a sanitized plastic bell cup on the back of the rat by suction (cupping group). To determine the role of A_{2B}AR in the beneficial effects of cupping after LPS instillation, PSB1115 (5 mg/kg BW), a specific A_{2B}AR antagonist, was administered i.p. immediately before cupping. The animals were sacrificed at 4 h after LPS instillation. (A) Representative images of haematoxylin and eosin (H–E) staining; (B) Pulmonary injury scores; (C) Lung dry-wet weight ratio; (D) Western blot analysis of the expression of Bcl-2, BAX, Total Caspase-3 and Cleaved Caspase-3 in the lungs; (E) Quantitative analysis of Bcl-2, BAX and Cleaved Caspase-3 expression; (F) Representative images of TUNEL staining (green) and corresponding nuclear counterstaining (blue) in the lungs; (G) Quantitative analysis of TUNEL-positive cells. n = 4–6; mean ± SD; *p < 0.05 versus LPS alone group; #p < 0.05 versus LPS + Cupping group. LPS, lipopolysaccharide; W/D, dry-wet weight ratio; kDa, kilodalton; Bcl-2; B-cell lymphoma-2; TUNEL, TdT-mediated dUTP Nick-End Labelling; DAPI, 4′,6-diamidino-2-phenylindole.

34]. To determine the role of A_{2B}AR in the beneficial effects of cupping after LPS instillation, PSB1115, a specific A_{2B}AR antagonist [32], was administered i.p. immediately before cupping. PSB1115 is a widely used, time-tested, specific adenosine A_{2B}AR receptor antagonist *in vivo*. It not only inhibits adenosine A_{2B}AR receptor in lung tissue [20, 29, 32, 34], but also widely used in a variety of other organs to antagonize the function of adenosine A_{2B}AR [35, 36, 37, 38].

As shown in Figure 5A&B, PSB1115 treatment completely eliminated the effects of cupping on pulmonary levels of TNF-α and MPO activity at 4 h after LPS instillation. PSB1115 exacerbated not only pulmonary inflammation but also systemic inflammation. Serum HMGB1, TNF-α, and IL-6 in the PSB1115 treatment group was increased and IL-10 in the PSB1115 treatment group was decreased compared with those of the cupping group (Figure 5C-F, $P < 0.05$). As expected, the recovery effect of cupping on PaCO2 and Lactate disappeared after PSB1115 was added (Figure 5G&H, $P < 0.05$). PSB1115 also inhibited the inhibitory effect of cupping on the NF-κB signalling pathway in LPS-instilled rats (Figures 6A-D, $P < 0.05$).

3.6. *Cupping alleviates lung injury through the adenosine/A_{2B}AR pathway in experimental ALI*

To determine the role of adenosine in the beneficial effects of cupping after LPS instillation, we again examined lung injury in rats. As shown in Figures 7A-B, PSB1115 treatment completely eliminated the pathological improvement due to cupping in the lung at 4 h after LPS instillation ($P < 0.05$). Similarly, the effects of cupping on pulmonary oedema were also eliminated by PSB1115 treatment (Figure 7C, $P < 0.05$). The expression levels of the apoptosis-related proteins Bcl-2, BAX and cleaved caspase-3 as well as the fluorescence TUNEL staining showed that PSB1115 effectively antagonized the inhibitory effect of cupping on lung apoptosis in LPS-instilled rats (Figure 7D-G, $P < 0.05$).

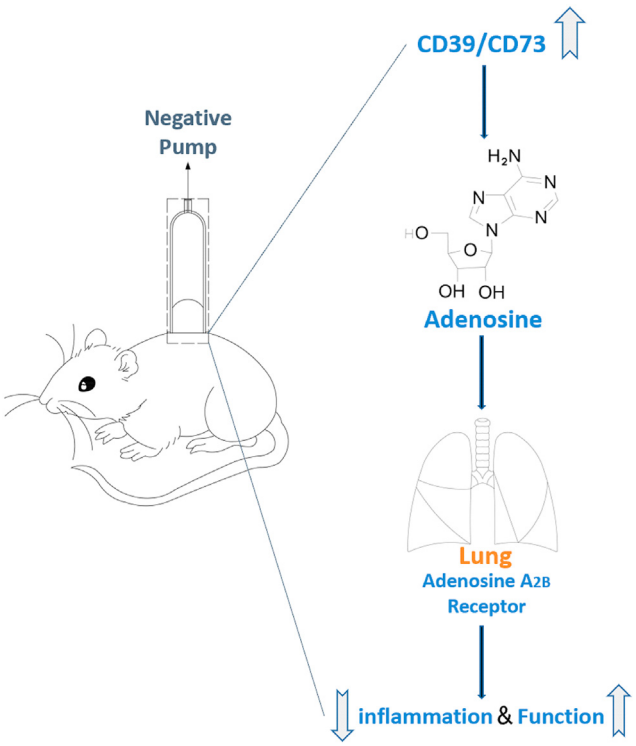

Figure 8. The graphical abstract. Cupping alleviates lung inflammation and injury through the adenosine/A2BAR pathway.

4. Discussion

In the current study, we show for the first time that cupping alleviates LPS-induced ALI in rats, inhibits inflammatory cell infiltration and alleviates the systemic inflammatory response through the NF-κB pathway. The beneficial effects of cupping in ALI appear to be associated with elevated adenosine and actions on the A_{2B}AR receptor (Figure 8).

Cupping has been claimed to work via a range of "mechanisms" from "unblocking the flow of QI" (i.e., the vital energy or life force) to "detoxification"; however, these explanations do not fit into modern medical theories. Using a rat model of intratracheal LPS instillation-induced ALI, we have clearly shown that cupping attenuates lung inflammation and injury. Adenosine, a purine nucleoside, is produced under metabolic stress, such as hypoxic conditions and acute or chronic inflammatory tissue insults [39]. The role of adenosine as an extracellular signalling molecule was first established by Drury and Szent-Gyorgyi in 1929 [40]. Subsequently, evidence has been obtained for the protective effects of extracellular adenosine in the lungs, heart, brain, kidneys, skeletal muscle and adipose tissues [11, 41, 42, 43]. Based on these observations, a unifying hypothesis for adenosine action was formulated by Newby [44], and the term 'retaliatory metabolite' was coined to describe the protective function of adenosine.

The physiological effects of adenosine are mediated through its interactions with cell-surface receptors. Four adenosine receptors have been identified by pharmacological and molecular studies. All of them are members of the G-protein-coupled family of receptors [45]. The genes for these receptors have been analysed in detail and are designated A_1, A_{2A}, A_{2B} and A_3. Recent studies have shown that adenosine attenuates ALI through the A_{2B} adenosine receptor (A_{2B}AR) [29, 46]. The synthesis of adenosine involves the catabolism of adenine nucleotides by the action of extracellular ectonucleotidases (CD39 and CD73). Our current study showed that CD39 and CD73 gene expression in the skin tissue at the cupping site increased markedly at 4 h after cupping therapy. Administration of PSB1115, a specific A_{2B}AR antagonist, before cupping eliminated the beneficial effects of cupping on LPS-induced lung inflammation and injury. This result suggests that cupping attenuates lung inflammation and injury through the adenosine/A_{2B}AR pathway. Due to the nature of cupping treatment, we were not able to perform *in vitro* experiment to support the signalling pathway, which is a limitation of this study.

The anti-inflammatory pathway involved in cupping was investigated by examining NF-κB signalling molecules in the lung. It has been well established that various signalling pathways are involved in inflammatory responses in ALI, including the NF-κB-, extracellular signal-regulated kinase (ERK)-, c-Jun N-terminal kinase (JNK), and p38-pathways [47, 48, 49]. Since signalling pathways involved in the anti-inflammatory activities of cupping have not been investigated, we conducted this study to determine whether cupping influences NF-κB p65 signalling molecules. When the signalling pathway is activated, the receptor protein first activates κB kinase (IKK) after stimulation. IKK phosphorylates a serine residue at the IκB sub-fundamental node of the NF-κB-IκB complex in cells, leading to ubiquitylation of the IκB subunit, which is then degraded by proteases, thereby releasing the NF-κB dimer. Free NF-κB enters the nucleus, binds to genes that have NF-κB binding sites, and initiates transcription. NF-κB also activates the expression of the IκBα gene, and the newly formed IκBα inhibits the activity of NF-κB again, thus forming a spontaneous negative feedback loop. Such a design principle forms the phenomenon of NF-κB oscillation [50, 51, 52]. Briefly, male adult rats were subjected to ALI and subsequent cupping therapy as described above. The lungs were harvested 4 h after LPS instillation to assess the NF-κB signalling molecules. To determine whether cupping had any effects on the proposed signalling molecules at this time point, pulmonary levels of inflammatory cytokines (i.e., TNF-α, IL-6 and IL-10) were measured by ELISA. The results showed that cupping inhibited activation of the NF-κB signalling pathway and reduced inflammation in rats with ALI.

5. Conclusion

In summary, dry cupping attenuated pulmonary injury and inflammation in LPS instillation-induced ALI. The protective effect of cupping in ALI appears to be related to an increase in adenosine levels. Using PSB1115, an inhibitor of the adenosine receptor A_{2B}AR, blocked the protective effect of cupping in ALI rats. Taken together, our results suggest that cupping attenuates lung inflammation and injury, maybe in part, through the adenosine/A2BAR pathway. We believe that the current study will increase the knowledge of these effects, efficacy, and potential of cupping therapy in ALI.

Declarations

Author contribution statement

Performed the experiments; analyzed and interpreted the data and wrote the paper. Yifan Ren; Lei Qi and Lin Zhang.

Analyzed and interpreted the data and contributed reagents, materials, analysis tools or data: Jinkai Xu; Jiancan Ma and Yi Lv.

Conceived and designed the experiments and wrote the paper: Yuanyuan Zhang and Rongqian Wu.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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