Prophylactic effects of short-term acupuncture on Zusanli (ST36) in Wistar rats with lipopolysaccharide-induced acute lung injury

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Objective: To evaluate the prophylactic effects of short-term manual acupuncture stimulation on Zusanli (ST36) on acute lung injury (ALI) caused by lipopolysaccharide instillation in Wistar rats.

Methods: Thirty-two Wistar rats were randomized into 4 groups (n=8) classified by the absence or presence of lipopolysaccharide instillation [negative (NG) and positive control (PC), respectively], and performance of sham or real needle stimulation [sham (SA) or real (RA) acupuncture, respectively]. Manual acupuncture was performed daily for 5 minutes over four consecutive days (days 1 to 4). Lipopolysaccharide instillation was performed on day 4 after the last acupuncture stimulation. Systemic blood samples, bronchoalveolar lavage, and bone marrow blood sample were collected on day 5 to measure the count of leukocytes.

Results: RA reduced the inflammatory response on ALI as indicated by blood cell count and bronchoalveolar lavage cell count. SA presented a minor efficacy to reduce inflammatory response in rats with experimental ALI. Bronchoalveolar lavage showed increased cell counts of rats in the PC group as compared with the NG group, and less but no significant differences in cell counts in the PC-RA group as compared with the PC-SA group.

Conclusion: Prophylactic manual needling stimulation of ST36 can mitigate ALI caused by lipopolysaccharide instillation in Wistar rats. Further studies should address the mechanisms of immune system response induced by needle stimulation of ST36 acupoint.

Keywords: acupuncture; lipopolysaccharides; acute lung injury; rats

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短期针刺足三里对脂多糖诱导的大鼠急性肺损伤的预防作用

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目的: 研究短期手法针刺足三里治疗对脂多糖诱导的实验性大鼠急性肺损伤的预防作用。

方法: 根据是否进行脂多糖刺激及采用的针刺方法的不同，将 32 只 Wistar 大鼠随机分为 4 组，即正常对照组、模型组、假针刺组和针刺组，每组 8 只。假针刺组和针刺组大鼠行手法针刺治疗，连续治疗 4 d，每次

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5 min. The 4th day needle was used to stimulate the rectum, liver, and spleen. The 5th day, collect blood, and use the spleen to promote the immune response to stimulate the lung.

**Results:**
- Blood cell counts and immune response were monitored. Needles were used to stimulate the rectum and liver, and the spleen was used to promote the immune response.
- Acupuncture was effective in reducing the symptoms of ALI, with the elderly showing a better response.

**Conclusion:**
- Acupuncture is effective in reducing the symptoms of ALI, with the elderly showing a better response.
- Further research is needed to understand the mechanism of action.

**Keywords:**
- Acupuncture
- Liver
- Spleen
- Immune response
- Elderly

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The primary function of the respiratory system is to allow gas exchange and to protect the organism from diseases caused by microorganisms and other agents. Microorganisms move in and out of the airways during breathing. Acute lung injury (ALI) is the leading cause of acute illnesses worldwide and contributes to the high mortality related to respiratory diseases both in children and adults. High-risk populations for developing fatal ALI include the very young, the elderly, and the immunocompromised. Defense mechanisms include airway reflexes and angulations, epiglottis, and mucociliary function. However, as these barriers are transposed, the immune system must prevent ALI by cellular and humoral regulation with an appropriate inflammatory response. Although vaccines may be used for prevention of ALI, available effective vaccines are limited in supply and are expensive, putting them out of reach of most vulnerable populations in developing countries. Several other vaccines face technical challenges or obstacles to the development-related intellectual property rights. Thus, research on methods for prevention of ALI are still needed for these populations.

Traditional Chinese medicine (TCM) has a long history in disease prevention and treatment. Cases description, explanation, and prescriptions for infectious diseases (traditionally called shanghai – “catch cold”) can be found on books written almost two millennia ago and are still applied to clinical practice. Theory of TCM on physiology attributes to the lungs (Fei) the breathing process and the promotion of the defensive function against external factors, actually interpreted mainly as infectious agents. Such resistance is properly developed with the assistance of the spleen (Pi) and kidneys (Shen) by promotion of blood (Xue). If the defensive function is preserved and the external agent is relatively weak, the infection does not develop. However, when the lung function is weak, the defensive process becomes compromised. In this case, stimulation of acupuncture points may be used to increase the strength of both lung and defensive functions.

Scientific evidence recognized by the World Health Organization (WHO) suggested the use of acupuncture stimulation for the treatment of several diseases including those affecting the respiratory tract. However, the preventive aspect is not emphasized by the WHO documents. Traditionally, Zusani (ST36) acupoint has been related to immune functions and is often used in clinical practice for disorders of the immune system. Scientific literature seems to corroborate this widespread traditional information. Evidence found in animal experiments includes, increasing intestinal electrical activity, hypotension effect and prophylaxis of cardiomycyte hypertrophy on spontaneous hypertension, reduction of renovascular hypertension by increasing activity of nitric oxide synthase (NOS), improvement of glucose tolerance, and anxiolytic effect on restrained-induced stress. The wide range of scientific evidence reinforces the traditional consideration of ST36 as one of the most important acupoint related to health promotion in TCM.

Lipopolysaccharide (LPS) is a constituent of cell walls for Gram-negative microorganisms that contributes to the local inflammation and systemic toxicity of infections. In vivo administration of bacterial LPS triggers a network of inflammatory responses. LPS mediates many of its effects by ligand-receptor interaction with a number of immune cells, which is followed by a release of a vast array of pro-inflammatory mediators that orchestrate the acute inflammatory response. The therapeutic effects of electro-acupuncture stimulation of ST36 on LPS-induced ALI had been demonstrated. Effects of 30-min needle electric stimulation at ST36 one hour prior to LPS injection reduced lung sepsis by down-regulation of inducible nitric oxide synthase (iNOS). In humans, repeated acupuncture treatments at ST36, Quchi (LI11), Xuehai (SP10), and Dazhui (GV14) induced a significant reduction in leukocytes and lymphocytes circulating values, suggesting a modulation of the immune response system via needle stimulation. Although electro-acupuncture is currently available, manual stimu-
lation still comprises the most used technique on private practice in developing countries. Moreover, none of those studies assessed the prophylactic effects of short-term acupuncture on ALI.

The aim of this paper was to evaluate the prophylactic effects of a short-term manual acupuncture on ST36 on the immune system response to ALI caused by LPS instillation in Wistar rats. It was hypothesized that acupuncture stimulation may improve the immune system response to ALI.

1 Materials and methods

1.1 Experimental protocol Thirty-two Wistar rats (male, 200 – 250 g) were randomized into 4 groups (n=8) classified by the absence or presence of LPS instillation [negative (NC) and positive control (PC), respectively] and performance of sham or real manual needle stimulation [sham acupuncture (SA) or real acupuncture (RA), respectively]. The resulting groups were NC, PC, PC-SA, and PC-RA groups. The protocol in this study was approved before its execution by the Animal Care Committee of Augusto Motta University Center (certificate number 06/08).

1.1.1 Needle stimulation The timeline of the experimental protocol was presented in Figure 1. Both SA and RA stimulations were performed for a four-consecutive-day experimental procedure (day 1 to 4). Following needle insertion a manual acupuncture was performed continuously for 5 minutes with needle bilateral rotation. RA was performed with stainless-steel needles (0.25 mm diameter) inserted at depth of approximately 1.5 mm into the right-sided ST36 acupoint. ST36 was identified as the belly of the tibialis cranialis muscle below the cranial crest of the tibia.[6,16] SA used the same needle type but was performed by puncturing a point approximately 5 mm laterally to ST36 original location. All rats in the PC groups were immobilized during acupuncture stimulation by using hands to minimize stress. Rats in the NC group were also immobilized for 5 minutes without acupuncture stimulation to induce the same stress level due to manipulation.

1.1.2 LPS instillation Rats in the PC group received LPS instillation [Escherichia coli] serotype O55:B5 (M63 ID C482199), 10 μg in 0.05 mL of saline per rat intranasal] on day 4 after acupuncture stimulation. A micropipette was used for intranasal instillation after anesthesia with 1 minimum alveolar concentration (0.950 02%) of vaporized halothane (Flu-o-Pen, Narcosul, Porto Alegre, Brazil). Rats were sacrificed on day 5 with a high-dose thiopental (intraperitoneal injection).

1.2 Inflammatory cells in blood and lung Peripheral blood samples obtained from the tail vein were collected from rats on days 1 and 4, both after acupuncture stimulation. On day 5, no further acupuncture stimulation was performed and all rats were sacrificed. In sequence, the bronchoalveolar lavage (BAL) was performed with 15 mL (3 washes of 5 mL each) of PBS containing 10 mmol/L ethylenediamine tetraacetic acid (EDTA). The recovery rate of BAL was 12 mL. BAL fluid was centrifuged at 4°C for 10 min at 400 x g and the cell pellets were resuspended in 0.25 mL PBS for further leukocytes enumeration. Total counts of cells recovered in the fluid and peripheral blood were made in Neubauer chamber under bright-field and the differential cell counts were performed as discriminated below.

1.3 Leukocytes enumeration in effluents from bone marrow The right femur was isolated and the femoral head and condyles were removed. Displaceable cells were removed by flushing out the lumen of the femur shaft with 2 mL of RPMI 1640 medium containing 40 U/mL heparin. The bone marrow suspension was passed through a 19-gauge needle to better dissociate the cells. Total cell count recovered in the bone marrow was made in Neubauer chamber under bright-field and the differential cell counts were performed on May-Grunwald-Giemsa stained cytospin preparations under oil immersion objective to determine the percentages of neutrophils and other cells (eosinophils and basophils).

1.4 Statistical analysis Comparison of total leukocytes in day 1 among all studied groups was performed with the Kruskall-Wallis test. Comparison of total leukocytes count between days 1 and 5 was performed for each group by Wilcoxon Signed Rank test. Also, comparisons of NC vs PC group and PC-SA vs PC-RA were performed on the same day (1 or 5, accordingly) with the Mann-Whitney U test. Blood cells, BAL, and marrow cells obtained on day 5 (total leukocytes, monocytes, neutrophils, lymphocytes, and others cells) were compared between the NC group and the PC group, and between the PC-SA group and the PC-RA group by using the Mann-Whitney U test. Data in Tables represent median [minimum (Min), maximum (Max)]. All analysis was performed with SPSS 16.0 for Windows. Statistical significance was considered with P<0.05.

2 Results

2.1 Total leukocytes count All rats in the PC groups survived from LPS instillation. Counts of total leukocytes in all groups on days 1 and 5 were presented in Table 1. There were no significant
2.2 Differential count of blood cells  Results of blood cells analysis obtained on day 5 were summarized in Table 2. After intervention, there were significant differences in counts for total leukocytes (P = 0.0003), neutrophils (P = 0.01), and lymphocytes (P = 0.0003) in the PC-Ra group as compared with the PC-SA group, but not for monocytes counts (P = 0.61). No significant differences were found on NC vs PC groups comparisons except the count of neutrophils (P = 0.02).

2.3 Differential count of BAL  Table 3 summarized the results of differential counts in BAL obtained on day 5. Higher counts of leukocytes (P = 0.002), neutrophils (P = 0.004), and monocytes (P = 0.002) were obtained from BAL in the PC group than those in the NC group. Reduced neutrophils counts were observed in the PC-Ra group as compared with the PC-SA group (P = 0.01). Less but not significant differences were found on all cell counts in the PC-Ra group as compared with the PC-SA group (leukocytes, P = 0.54; monocytes, P = 0.19).

2.4 Counts of bone marrow cells  Comparison of bone marrow cells obtained on day 5 was presented in Table 4 for all groups. RA reduced less count of other cells (P = 0.01) as compared with SA. No significant differences were found on counts of leukocytes or neutrophils among all other experimental groups.
Table 3  BAL cells analysis obtained on day 5

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Count of total leukocytes</th>
<th>Count of neutrophils</th>
<th>Count of monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>8</td>
<td>3.9 [3.3, 5.5]</td>
<td>0.0 [0.0, 0.0]</td>
<td>3.9 [3.3, 5.5]</td>
</tr>
<tr>
<td>PC</td>
<td>8</td>
<td>38.2 [17.5, 47.8]</td>
<td>9.6 [8.4, 18.3]</td>
<td>14.0 [7.0, 23.4]</td>
</tr>
<tr>
<td>P value (NC vs PC)</td>
<td>0.002</td>
<td>0.004</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>PC-SA</td>
<td>8</td>
<td>25.5 [10.0, 62.2]</td>
<td>19.9 [7.9, 27.0]</td>
<td>16.1 [5.8, 32.4]</td>
</tr>
<tr>
<td>PC-RA</td>
<td>8</td>
<td>13.0 [9.5, 47.5]</td>
<td>4.1 [1.5, 6.2]</td>
<td>7.8 [6.2, 12.5]</td>
</tr>
<tr>
<td>P value (SA vs RA)</td>
<td>0.54</td>
<td>0.01</td>
<td>0.19</td>
<td></td>
</tr>
</tbody>
</table>

Table 4  Bone marrow cells analysis obtained on day 5

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Count of total leukocytes</th>
<th>Count of neutrophils</th>
<th>Count of other cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>8</td>
<td>41.0 [36.5, 44.0]</td>
<td>8.6 [5.5, 15.0]</td>
<td>26.5 [24.5, 31.6]</td>
</tr>
<tr>
<td>PC</td>
<td>8</td>
<td>40.8 [36.0, 48.6]</td>
<td>12.6 [9.9, 16.9]</td>
<td>26.2 [16.6, 29.3]</td>
</tr>
<tr>
<td>P value (NC vs PC)</td>
<td>0.94</td>
<td>0.18</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>PC-SA</td>
<td>8</td>
<td>31.8 [25.5, 37.5]</td>
<td>4.7 [2.6, 14.6]</td>
<td>14.9 [14.0, 18.2]</td>
</tr>
<tr>
<td>PC-RA</td>
<td>8</td>
<td>38.2 [30.2, 42.0]</td>
<td>6.7 [4.7, 9.2]</td>
<td>22.6 [20.5, 28.4]</td>
</tr>
<tr>
<td>P value (SA vs RA)</td>
<td>0.08</td>
<td>0.42</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

3 Discussion

This study evaluated the prophylactic effects of short-term acupuncture on ST36 on the immune system response to ALI caused by LPS instillation. The main finding of this work was that short-term stimulation for 4 consecutive days blunted the inflammatory reaction to LPS by immune system response.

A model of LPS-induced ALI was applied to mimic morphological and functional changes observed in clinical situations resulting from circulating LPS. It is well documented that LPS administration triggers a network of inflammatory responses mediated by a number of immune cells, which is followed by the release of a vast array of pro-inflammatory mediators that orchestrate the acute inflammatory response\(^{[17,18]}\). Hence, it is a useful model to test the prophylactic effects of TCM interventions.

No significant differences were found in any groups between days 1 and 5 despite the increase in dispersion on all PC groups. Although there was no significant difference between the NC and PC groups on day 5, the high dispersion in total leukocytes count found in the PC group was not observed in the NC group, suggesting that the (small) sample size may influence that result. However, the occurrence of sepsis in the PC group cannot be completely ruled out as indicated by the high dispersion on cell count. This absence of significant difference may also be explained by stress of animal manipulation, which increases the production of adrenocortical hormone\(^{[19]}\). Animals were 'slightly' immobilized as this maneuver should significantly minimize the stress and subsequently decrease the adrenocortical hormone production induced by immobilization and needle insertion. As all groups were kept immobilized during the same time, it is believed that such effect would influence all groups in the same manner, if any. Finally, SA did not blunt the immune system reaction as demonstrated by a significant increase in total leukocytes count as compared with RA, which gives evidence on the prophylactic effect of the latter.

Blood analysis performed on day 5 showed that SA on the ALI rats induced by LPS stimulation could not significantly change monocytes, neutrophils, or lymphocytes counts. As stated before, the low median value of total leukocytes found in the PC group could be influenced by the small sample size. However, RA could significantly decrease neutrophils and lymphocytes counts in the PC-RA group. The high value of total leukocytes found in PC-SA reinforces simultaneously that sepsis occurred and suggests an effect of point location. Altogether, those data suggested that the correct point location may stimulate the immune system, probably acting by increasing adrenocortical hormone\(^{[19]}\). Thus we believe that SA induced a minor effect on ALI while RA was more effective in the prophylaxis of ALI.

The BAL analysis corroborates the blood cells results and clearly confirms the installation of sepsis in all PC groups. RA promoted reduction in leukocytes, monocytes, and neutrophils counts as compared with SA. Cell count in the PC-SA group
was higher than that in the PC-RA group, although without significant difference. Again, the high dispersion of data on PC-SA group may interfered in this result.

The lack of statistical significance of PC vs NC group comparison of bone marrow cell count may be a consequence of leukocyte trafficking from marrow to blood stream in their way to the lungs[30]. The obtained results sustain this hypothesis by the simultaneous consideration of blood cell analysis and BAL (Tables 2 and 3, respectively). PC-SA group presented increased cell counts in both blood and BAL analysis (as compared with the NC and PC-RA groups) for almost all studied immune cell types. This suggests that leukocytes migration occurred after SA but not RA, what reinforces that RA promoted the prophylaxis of ALI.

The results of this study are in agreement with other works on acupuncture. For instance, Huang et al. showed that 30-minute manual acupuncture stimulation of ST36 1 h before LPS instillation mitigated the ALI in rats. The anti-inflammatory capacity of ST36 observed in our study was confirmed by other animal studies. Tian et al. showed that electro-acupuncture on ST36 could decrease TNF-α transcription on experimental model of ulcerative colitis in rats. The same decreased production of interleukin-2 (IL-2) in Wistar rats with sepsis was observed. Hence, our data clearly indicated the anti-inflammatory capacity of ST36 by needle stimulation.

The respiratory tract is an important site for interface of the immune system with the environment. Such reasoning is in agreement with Chinese physiology which states that the lungs are responsible for the host defense against external febrile diseases. This work reinforces the need of research on integrative medicine to pose scientific-based hypothesis for the natural phenomena observed during its development.

Differences between species should be considered before further data interpretation. Although data from this study indicated the therapeutic potential of acupuncture stimulation of ST36 as a prophylaxis measure against lung infection, there was no evidence to support the empirical use of needle stimulation as the unique treatment for lung infection. More studies are needed before further clinical application on prophylaxis can be considered.

REFERENCES

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