Evaluation of immunopathologic effects of aqueous extract of *Echinacea purpurea* in mice after experimental challenge with *Pasteurella multocida* serotype A

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Summary

In order to assess the immunopathological effects of aqueous *Echinacea purpurea* extract (EPE) on mice experimentally challenged with *Pasteurella multocida* serotype A, forty female BALB/c mice were randomly divided into four groups. The groups included a control group (received sterile distilled water 2 times/week for 2 weeks, intraperitoneally and then 100 µl sterile saline intranasally), a PMA group (received sterile distilled water as the control group and after 2 weeks, 5.6 × 10^7 CFU/ml of *P. multocida* serotype A, intranasally), an EPE+PMA group (received *E. purpurea* extract intraperitoneally 2 times/week for 2 weeks and then challenged as the PMA group) and an EPE group (received *E. purpurea* extract as EPE+PMA group and then 100 µl sterile saline intranasally). After 24 and 48 h post-challenge, half of the animals in each group were sacrificed and analyzed for bacterial counts in their lungs and livers, TNFα serum levels and histopathological changes. The results showed significant differences in lung bacterial counts between PMA and EPE+PMA groups. TNFα serum level was significantly higher in the PMA group. Histopathological examination revealed infiltration of neutrophils in alveolar septa and hyperemia in the PMA group. In addition, the criteria of bronchopneumonia were partially recovered in the EPE+PMA compared to the PMA group. According to the results, it seems that *E. purpurea* extract has an immunomodulatory effect and can be used to prevent or control of pneumonia caused by *Pasteurella*.

Key words: *Echinacea purpurea*, *Pasteurella multocida*, Mice, TNFα, Histopathology

Introduction

*Pasteurella multocida* is a gram negative bacterium causing widespread infections in various domestic animals; sniffles in rabbits, pneumonia and haemorrhagic septicaemia in cattle, sheep and goats and fowl cholera in chickens. Pasteurellosis, an infection with *Pasteurella* sp. which is found in humans and animals, is an important respiratory disease, especially in animals with high economic importance (Kuhnert and Christensen, 2008). Serotype *P. multocida* is one of the nasopharyngeal commensal pathogens associated mainly with respiratory diseases in animals (Vasfi Marandi and Mittal, 1997).

*Echinacea purpurea* (purple cone flower) has been used traditionally in North America for the treatment of various symptoms of “colds and flu”, as well as the treatment of respiratory diseases, candidiasis, and wound healing (Bauer, 1998; Barrett, 2003; Sharma et al., 2010). *Echinacea* extracts have been shown to have nonspecific immunomodulatory properties in *vitro* (Bauer and Wagner, 1991), including increased phagocytosis (Stotzem et al., 1992), increased cytokine production (Burger et al., 1997), increased natural killer cell activity (See et al., 1997) and increase immunoglobulin G levels in rats (Rehman et al., 1999).

Previous studies suggest the potential use of *Echinacea* for controlling bacterial infections (Sharma, 2010). Sharma et al. (2010) reported that respiratory bacteria such as *Streptococcus pyogenes*, *Hemophilus influenzae* and *Legionella pneumophila* were also readily inactivated by *Echinacea*, and their pro-inflammatory responses caused by different cytokines secreted by bronchial epithelial cell cultures in response to infection were reversed. According to their results, *Staphylococcus aureus* (methcillin-resistant and sensitive strains) and *Mycobacterium smegmatis* were less sensitive to the antibacterial effects of *Echinacea* but their pro-inflammatory responses were completely reversed. Therefore, they reported that *Echinacea* to have a dual action against respiratory bacteria: one being its anti-bacterial action, a bactericidal effect against some of the bacteria incriminated in upper respiratory infections, and the other an anti-inflammatory effect which could reverse inflammation caused by these bacteria.

Despite the *in vitro* immunomodulatory and bactericidal activity of *E. purpurea* extract on the immune system and on different bacteria, the principle role and *in vivo* activity of this plant on the immune system following experimental *P. multocida* infection have not been addressed. Therefore, an *in vivo* study was designed to investigate the immunopathologic effects of
Materials and Methods

Echinacea

Echinacea purpurea powder was obtained from aerial vegetative parts (Goldaru Co., Isfahan, Iran). Echinacea aqueous extract was prepared using distilled water incubated at 37°C in a water bath for 2 h. The suspension was filtered using sterile 0.45 µm PVDF syringe filter units. The filtered extract was stored at 4°C prior to use.

Bacteria

Pasteurella multocida serotype A was prepared from Razi Vaccine and Serum Research Institute (Iran, Karaj). The bacterium was cultured in blood agar medium containing 5% sheep blood. Bacteria identity was confirmed morphologically and biochemically. The bacteria were cultured overnight in 100 ml TSB medium at 37°C. The culture was centrifugated at 4000 rpm for 10 min and washed twice with sterile saline and resuspended in sterile PBS. The bacterial suspension OD 600 was adjusted to 0.1 and counted by surface plate method (Quinn et al., 2002).

Determination of LD 50 and ID 50

Female BALB/c mice with an age range of 6-8 weeks were obtained from Jundishapur Animal Laboratory Centre, Ahvaz, Iran. The storage condition of mice was standardized. Before determining LD 50, the pathogenicity of the P. multocida serotype A strain was tested in healthy mice by intraperitoneal inoculation of 0.5 ml PBS containing 3.5 × 10 5 CFU/ml of P. multocida serotype A. Subsequently, the determination of the LD 50 for P. multocida serotype A was calculated by Reed and Muench’s method (Reed and Muench, 1938). Briefly, appropriate bacterial volumes (100 µl) from serially tenfold dilutions of P. multocida serotype A prepared in sterile PBS were intranasally inoculated in six mice per group. The number of deaths was recorded. Animals which did not die from infection were killed by cervical dislocation according to animal ethics, and bacterial isolation was carried out on their lungs and livers. LD 50 and ID 50 of P. multocida serotype A were then calculated by the Reed and Muench method (Reed and Muench, 1938).

Experimental infection

Forty female BALB/c mice (25-27 g) were kept under controlled conditions. Food and water were allowed ad libitum. A commercial pelleted diet was used during the experiments. The animals were allowed to adapt to the laboratory conditions for 2 weeks before the beginning of the experiment. All animals were randomly assigned to four groups (10 mice per group). In the control group, mice received 0.5 ml sterile distilled water two times/week for two weeks followed by 100 µl sterile saline intranasally. In the second group (PMA), mice received sterile distilled water similar to the control group and after 2 weeks, 5.6 × 10 5 CFU/ml of P. multocida serotype A in a total volume of 100 µl, was administered intranasally. The mice were maintained in an upright position for 30 sec after inoculation. The third group (EPE+PMA) were injected with 0.5 ml of E. purpurea extract (40 mg/ml) intraperitoneally two times/week for two weeks and then challenged with P. multocida serotype A, similar to the PMA group. The fourth group (EPE) received 0.5 ml E. purpurea extract (40 mg/ml) intraperitoneally two times/week for two weeks and then 100 µl of sterile saline intranasally.

Blood sampling

After weighing and sacrificing mice at 24 and 48 h post-bacteria-challenge in the PMA and EPE+PMA groups, blood samples were collected from the animals by cardiocentesis (five mice at 24 and five mice at 48 h post infection). About 0.3 ml blood was collected in sterile microtubes without anticoagulant for serum separation. Serum samples were taken and stored frozen at -20°C for TNF-α assays.

Histopathology

After necropsy and weighing the lungs, parts of lungs, livers and spleens were fixed in 10% neutral buffered formalin for histopathological examination. Remaining lung and liver tissues were used for bacterial isolation. Specimens in formalin were processed routinely and embedded in paraffin wax. Tissue sections of 5 µm thickness were routinely stained with haematoxylin and eosin (H&E). The lesions were scored (-) as none; (+) as minimal; (++) as moderate and (+++) as severe.

Bacteriological examination

Approximately 0.1 g of each lung and liver was removed aseptically after necropsy at 24 and 48 h post challenge. The tissue was homogenized in 900 µl sterile normal saline and a serially tenfold dilution to 10 7 was prepared. Subsequently, 50 µl of each dilution was cultured on sheep blood agar plates and incubated at 37°C for 24 h. The number of colonies was counted and the number of bacteria was reported as tissue CFU g⁻¹.

Serum TNF-α analysis

A commercial ELISA kit (Boster Biological Technology, LTD) was used to determine TNF-α serum concentration by following the procedures recommended by the manufacturer.

Statistical analysis

Data were analyzed using SPSS (version 16, SPSS, Inc., Chicago, IL, USA). Lung index results together with bacterial counts and serum TNF-α profiles were compared between groups and subgroups (24 and 48 h) using analysis of variance and LSD tests. Semi-quantitative scoring of histopathological changes was analyzed by Kruskal-Wallis tests.
Results

Clinical observation
Clinical observations of mice in the PMA group included the inactivity of mice, depression, weakness, and tangled hair. Three mice died during 24 h, and two mice at 48 h post challenge. In the EPE+PMA group, one mouse died at 24 and 48 h post challenge. In the EPE and control groups the mice were healthy and appeared normal. No significant variation in lung indices of test groups were observed in comparison to the control group (P>0.05). Lung index data are shown in Table 1.

Lung and liver bacterial isolation and count
To assess the *in vivo* effects of *E. purpurea* extract on mice experimentally infected with *P. multocida*, bacterial counts of lung and liver homogenates of different groups were measured (Table 1). Mean bacterial counts for different groups are summarized in Table 1. In the PMA group, the average number of *P. multocida* at 24 and 48 h post challenging was $81.7 \times 10^9$ and $95.9 \times 10^9$ CFU/g in the lungs and $5.5 \times 10^9$ and $7.6 \times 10^9$ CFU/g in the livers. In the EPE+PMA group, the average number of *P. multocida* in the lungs at 24 h post challenging was $6.2 \times 10^9$ CFU/g and in 48 h, $2.6 \times 10^9$ CFU/g. In the livers, the average number of bacteria at 24 h post challenging was $0.7 \times 10^9$ CFU/g and $2.8 \times 10^9$ CFU/g at 48 h. No bacteria were isolated from lungs and livers in the control and EPE groups. Bacterial counts in the PMA group were higher at 48 h than 24 h. In the EPE+PMA group, bacterial count was higher in 24 h than 48 h. Significant differences were found between PMA and control and EPE groups. Bacterial counts in the PMA group were higher in 24 h than 48 h. In the EPE+PMA group, one mouse died at 24 and 48 h post challenge. In the EPE+PMA group, the average number of bacteria at 24 and 48 h were acute bronchopneumonia with diffuse and severe infiltration of polymorphonuclear cells within alveolar septa and alveolar lumen. Hyperemia was obvious in both subgroups (24 and 48 h), scored as moderate to severe. Seroproteinaceous exudates containing fibrin were detected within alveolar lumen, bronchioles and around vessels (Fig. 1A). In one mouse, bacterial colonies were seen with high magnification. Lungs of mice in the EPE+PMA group showed mild to moderate infiltration of neutrophils and moderate hyperemia (Fig. 1B). Significant differences were found in neutrophils infiltration between PMA and EPE+PMA (P<0.05). The lungs of control and EPE groups showed normal histological structure. Liver lesions in the PMA group were cell swelling and diffuse infiltration of neutrophils in sinusoidal spaces (Fig. 1C). Also, blood vessels were engorged with red and white blood cells. In EPE + PMA livers, the infiltration of inflammatory cells was decreased (Fig. 1D). Spleen histopathological changes in the PMA group were seen as the accumulation of neutrophils in the marginal zone of white pulp (Fig. 1E). The spleen of EPE + PMA mice showed less infiltration of neutrophils (Fig. 1F).

Pathological observation
The histopathologic study of PMA and EPE+PMA groups revealed different lesions in the lungs, livers and spleens. The predominant changes in the lungs of the PMA group after 24 and 48 h were acute bronchopneumonia with diffuse and severe infiltration of polymorphonuclear cells within alveolar septa and alveolar lumen. Hyperemia was obvious in both subgroups (24 and 48 h), scored as moderate to severe. Seroproteinaceous exudates containing fibrin were detected within alveolar lumen, bronchioles and around vessels (Fig. 1A). In one mouse, bacterial colonies were seen with high magnification. Lungs of mice in the EPE+PMA group showed mild to moderate infiltration of neutrophils and moderate hyperemia (Fig. 1B). Significant differences were found in neutrophils infiltration between PMA and EPE+PMA (P<0.05). The lungs of control and EPE groups showed normal histological structure. Liver lesions in the PMA group were cell swelling and diffuse infiltration of neutrophils in sinusoidal spaces (Fig. 1C). Also, blood vessels were engorged with red and white blood cells. In EPE + PMA livers, the infiltration of inflammatory cells was decreased (Fig. 1D). Spleen histopathological changes in the PMA group were seen as the accumulation of neutrophils in the marginal zone of white pulp (Fig. 1E). The spleen of EPE + PMA mice showed less infiltration of neutrophils (Fig. 1F).

Serum TNF α profile
To determine whether EPE had immunomodulator effect on inflammatory cytokine, TNF α serum TNF α was measured in all groups. Serum TNF α concentrations are presented in Table 2. Serum TNF α concentration was found to be higher in the PMA and EPE+PMA groups. However, the concentration of TNF α in the EPE+PMA group was lower than that of the PMA group. The results of the data analysis showed that the differences between serum TNF α concentrations were significant for the PMA and EPE+PMA groups (P<0.05), the PMA and EPE groups and the PMA and the control groups (P<0.01). The differences between the EPE and EPE+PMA groups, the EPE and the control groups and the EPE+PMA and the control groups was not significant (P>0.05).

### Table 1: Summary of lung index and bacteriological findings following intranasal infection of *Pasteurella multocida* in mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time of sacrificing after infection (h)</th>
<th>The average index of lung</th>
<th>The average number of <em>P. multocida</em> in lung (CFU/g)</th>
<th>The average number of <em>P. multocida</em> in liver (CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>0.96 ± 0.18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.89 ± 0.23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PMA</td>
<td>24</td>
<td>1.33 ± 0.36</td>
<td>81.7 × 10⁹</td>
<td>5.5 × 10⁹</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.25 ± 0.57</td>
<td>95.9 × 10⁹</td>
<td>7.6 × 10⁹</td>
</tr>
<tr>
<td>EPE+PMA</td>
<td>24</td>
<td>1.09 ± 0.36</td>
<td>6.2 × 10⁹</td>
<td>0.7 × 10⁹</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.14 ± 0.54</td>
<td>2.6 × 10⁹</td>
<td>2.8 × 10⁹</td>
</tr>
<tr>
<td>EPE</td>
<td>24</td>
<td>1.01 ± 0.03</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.98 ± 0.14</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 2: Means ± SEM of serum TNF α values following intranasal infection of *Pasteurella multocida* in mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>PMA</th>
<th>EPE+PMA</th>
<th>EPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂des</td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.721</td>
<td>0.252</td>
<td>0.237</td>
</tr>
<tr>
<td>Concentration of TNF α (pg/ml)</td>
<td>2963.6±2261.8</td>
<td>910.2±651.8</td>
<td>844.1±1264.0</td>
<td>247.0±440.4</td>
</tr>
</tbody>
</table>

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Discussion

Mouse models of *P. multocida* serotype A infection provide a well established experimental model to study immunopathologic responses to infection. *Echinacea purpurea* is widely used as a self-prescribed agent against upper respiratory tract infections such as the common cold (O’Hara *et al.*, 1998). To characterize *in vivo* antibacterial and potential immunomodulatory effects of *Echinacea*, a mouse model of live *P. multocida*

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**Fig. 1:** Different sections of lung, liver and spleen of PMA and EPE+PMA mice 24 h post infection (H&E, Bar=20 µm). A: Lung from mouse in PMA group. Note thickened inter-alveolar septa which are due to infiltration of neutrophils (arrows) in alveolar septa and around bronchioles and vessels and hyperemia. B: Lung from mouse in EPE+PMA group. Low infiltration of neutrophil (arrows) is obvious. C: Liver from mouse in PMA group. The section showing engorged sinusoids with red blood cells and several neutrophils (arrows). D: Liver from mouse in EPE+PMA group. Reduced number of neutrophils is clear. E: Spleen from mouse in PMA group. Note numerous neutrophils around the white pulp. F: Spleen from mouse in EPE+PMA group. Decreased numbers of neutrophils are observed.
infection was used in the present study. In this model, clinical signs, lung and liver bacterial cultures, histopathological lesions and serum TNFα levels were studied.

In this research, the experimental challenge was the intranasal administration of P. multocida in mice. The pathological study 24 and 48 h post infection showed acute bronchopneumonia which is in agreement with other studies (Praveena et al., 2010; Pors et al., 2011).

Clinical observation of the EPE+PMA group showed better conditions in comparison with the PMA group. In addition, the bacterial count of the EPE+PMA group had significant differences with that of the PMA group (P<0.05). This result was also supported by histopathological observations in that the EPE+PMA group showed fewer lesions in comparison to the PMA group. This finding indicated that the improvement may be due to bactericidal or immunomodulatory effects of E. purpurea extract, a finding which is in agreement with previous reports (Sharma et al., 2010). According to many in vitro studies, E. purpurea stimulates macrophage activity, leading to the stimulation of non-specific immune responses. Goel et al. (2002) reported an evident increase in TNFα and nitric oxide (NO) release by the alveolar macrophages following in vitro stimulation with LPS. A predominant mechanism by which alveolar macrophages destroy infectious agents is releasing cytokines such as NO. Alveolar macrophages also produce a variety of cytokines, such as IL1, IL6, TNFα and cytopotoxic products which can be effective against infectious agents (Luettig et al., 1989). It has been made clear that the polysaccharide components of Echinacea increase phagocytosis and chemotaxis in macrophages (Stitimpel, 1984; Luettig, 1989) and neutrophils (Wagner et al., 1991). Goel et al. (2002) reported that phagocytic activity of alveolar macrophages improved with increasing concentrations of Echinacea components.

In this study, serum TNFα concentration showed significant differences between the PMA and the control groups. The level of serum TNFα in PMA and EPE groups was also significant (P<0.01). This data indicated that bacterial inoculation increased secretions of cytokines followed by inflammatory reactions. Similar to the present study, Praveena et al. (2010) reported higher concentrations of TNFα at 12 and 24 h post infection. LPS and porin proteins isolated from P. multocida have been reported to upregulate mRNA expression levels of proinflammatory cytokines in murine splenic lymphocytes (Iovane et al., 1998).

Serum TNFα concentrations in the PMA and EPE+PMA groups were found to be significantly different (P<0.05), indicating the decrease of these cytokines in the EPE+PMA group which was also supported by the diminishing inflammatory reaction in lung sections. The histopathological study of the EPE+PMA group showed less infiltration of neutrophils and hyperemia in lungs, meaning that the production of pro inflammatory cytokine, especially TNFα level, has been modulated. Compared to the EPE group, serum TNFα of the EPE+PMA group increased, but the difference was not statistically significant (P>0.05). This change means that E. purpurea was able to modulate the secretion of TNFα. As mentioned by other researchers (Rininger et al., 2000; Goel et al., 2002; Matthias et al., 2007), the increasing level of this cytokine in the EPE+PMA group compared to the PMA group may be due to the stimulatory effects of bacteria on increasing TNFα levels in the latter group. Thus far, several experiments have demonstrated the controversial effects of this herb on the immune system. Sullivan et al. (2008) showed that activation of peritoneal macrophages by E. purpurea polysaccharides resulted in increased production of inflammatory cytokines (TNFα, IL1α, IL6, and IL12) and nitric oxide (NO). Burger et al. (1997) demonstrated that by using E. purpurea extract in human peripheral blood macrophages IL1, TNFα and IL10 levels can be increased. Goel et al. (2002) showed that the amount of TNFα and NO produced by alveolar and spleen macrophages can be increased by using water-ethanol extracts of E. purpurea. Chicca et al. (2009) showed that ethanolic extracts with alkamide fractions of Echinacea stimulate anti-inflammatory cytokines (IL10) and inhibit the secretion of proinflammatory (e.g., TNFα) cytokines from murine macrophage cell lines. Sharma et al. (2009) demonstrated that ethanolic extracts of E. purpurea caused reductions in ILα and ILα in a virus infected human bronchial epithelial cell line. Zhai et al. (2007) showed that the production of cytokines such as IL1β, TNFα and NO by macrophages infected with Salmonella enterica decreased when cultured with ethanol extracts of E. purpurea.

In an in vitro study, the alkylamides isolated from Echinacea were shown to inhibit 5-lipoxygenase and cyclooxygenase, which are key enzymes for the production of prostaglandins, and important as inflammatory mediators (Muller-Jakic et al., 1994). The anti-inflammatory effects of this extract have been shown in Arsenic induced hepatic toxicity (Heidari et al., 2011). In the present study, decreased inflammation in the EPE+PMA group was obvious which may be due to the anti-inflammatory effects of Echinacea extract. Echinacea plant extract has been used for immune stimulations for many years, but the evidence supporting its therapeutical potential is still controversial. In recent years, much effort has been made to identify the potential components in Echinacea plant extract and to account for its in vitro immunostimulatory effects. Some of these bioactives include polysaccharides, cichoric acid and alkylamides.

In conclusion, intraperitoneal injections of E. purpurea extract for 2 weeks before the induction of pneumonia by P. multocida prevented bacterial growth in the lung and liver, reduced histopathologic lesions in these organs and modulated the concentration of serum TNFα in challenged mice. Thus, E. purpurea extract can be used as an herbal drug for prevention and control of pneumonia due to P. multocida. Considering the widespread bactericidal, immunomodulatory and anti-inflammatory properties of E. purpurea, it could be a
beneficial herbal drug in any respiratory bacterial infection.

Acknowledgements

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Ethical standards

The experimental and animal care protocols were in compliance with the requirements of the Ethics Committee.

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