

## Effect of Electroacupuncture on Bronchial Asthma Induced by Ovalbumin in Rats

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### ABSTRACT

**Introduction:** Asthma is a worldwide disabling chronic inflammatory airway disease characterized by an intense eosinophilic inflammatory infiltrate on bronchial mucous membranes. Among the complementary therapeutic approaches to asthma, acupuncture has been widely used.

**Objective:** Here we used a rat pulmonary hypersensitivity experimental model that mimics human asthma in order to address whether electroacupuncture (EA) treatment could reduce the inflammatory process.

**Materials and Methods:** Experimental animals were divided in four groups: control (C), immobilized (I), sham-acupuncture (SA), and acupuncture (A). All rats were sensitized with heat-solidified hen egg white implant. Using clinical acupuncture points, EA treatment began 2 days after antigen priming and was repeated on alternate days for 2 weeks. Subsequently, animals were challenged by inhalation with aggregated ovalbumin and sacrificed 24 hours later when blood samples, bronchoalveolar lavage (BAL), and lungs were collected.

**Results:** Histopathologic analyses showed that peribronchial and perivascular inflammatory cell infiltrates were significantly lower in group A compared to groups SA and I (shown to be similar to group C). Furthermore, BAL total cell count and percentage of polymorphonuclears (as well as the differential counts of neutrophils and eosinophils) were significantly reduced in group A compared to group I. Corticosterone plasma levels were similar in all groups.

**Conclusions:** Taken together these results show that EA efficiently diminishes the bronchial immune-mediated inflammation induced in rats and that this effect is dependent on the choice of specific acupoints.

### INTRODUCTION

The impact of bronchial asthma on public health is dependent on the chronicity and severity of the clinical manifestations aggravated by the increasing prevalence worldwide. Asthma pathogenesis involves complex neuroimmune deregulation that promotes bronchial inflammation resulting in exacerbated mucus production and airway hyper-responsiveness (Jeong et al., 2003; Stacey et al., 2000). It is characterized by airway infiltration of diverse effector cells such as monocytes, mast cells, neutrophils, T

lymphocytes, and remarkably, eosinophils (Bousquet et al., 1995; Donno et al., 2000).

The underlying process that drives and maintains the asthmatic inflammatory process appears to be an imbalance of the equilibrium between T-helper cell type 1 (T<sub>H</sub>1) and T helper cell type 2 (T<sub>H</sub>2) immune response types, with a predominance of the latter (Elias et al., 2003). More strictly, it has been proposed that asthma could be the result of an unbalanced interleukin (IL)-12 secretion, a critical determinant of T<sub>H</sub>1 immune response, and IL-13, a T<sub>H</sub>2 marker response type (Wills-Karp, 2001). The cytokines produced in T<sub>H</sub>2 im-

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mune response (IL-4, IL-5, and IL-13) are associated with allergic reactions and eosinophilia. Exocytosis of cationic proteins from activated eosinophils granules is the cause of damage found on affected tissues (Hamelmann and Gelfand, 2001). Therefore, eosinophilic infiltration is directly associated with the inflammatory process and airway hyper-responsiveness (Erjefalt et al., 2001; Gleich, 2000; Holgate, 2000).

The limited success of conventional antiasthma drugs and the hazards involved with corticotherapy has led many patients to seek alternative or complementary methods for their treatment. Among them, acupuncture, which is traditionally used in China for almost all diseases, is also increasingly being used in Western countries (Ezzo et al., 2000; Xu, 2001). Moreover, acupuncture has been considered useful as an adjunct approach for asthma management (Davis et al., 1998; National Institutes of Health, 1998) and also as treatment procedure for other inflammatory and allergic processes (Blank et al., 2002; Bonta, 2002; Zunhui, 1995).

During recent years, some of us observed that children with asthma who were enrolled in the Chinese Medicine Division of UNIFESP and who were treated with acupuncture presented a significant clinical improvement compared to the control group using  $\beta_2$ -adrenergic receptor agonists. The parameters used to evaluate the physiologic effects of acupuncture were: increase in height and body-mass index, intensity and duration of asthmatic episodes, infection rate, use of conventional oral bronchodilators, need of corticotherapy, and reduction in the use of  $\beta_2$  receptors agonists (Carneiro et al., 1997a, 1997b, 1998, 1999). Thus, we took advantage of an animal experimental model to better address some of the biologic effects of acupuncture.

It has been demonstrated experimentally that the physiologic effects of acupuncture are consequences of neural and humoral signaling (Han et al., 1999; Tabosa et al., 2002; Zhao et al., 2000). However, in contrast to many human diseases, allergic asthma does not occur in animals. To mimic bronchial asthma in an experimental setting a wide variety of models have been developed but none is identical to the condition found in human disease (Szélenyi, 2000). Some years ago, an interesting model suitable for asthma studies was described, first in mice (De Siqueira et al., 1997; Facincone et al., 1997) then in rats (Carvalho et al., 1999). The novelty of the antigen immunization without adjuvant was considered a significant improvement over the existing methods. It depends on priming the animals by a subcutaneous implant of heat-coagulated hen egg white (HEW) followed by a single inhalation challenge with aerosol-aggregated ovalbumin. This procedure results in an intense pulmonary eosinophilic hypersensitivity reaction and bronchial hyper-reactivity, which are features of human asthma. Also, the isotypic profile of serum immunoglobulins is typical of  $T_H2$  type immune response and the histopathologic findings resemble those found in asthmatic individuals

(Russo et al., 1997). This model of bronchial immune-mediated inflammation induced in rats has proven to be suitable to address here the effects of acupuncture in asthma.

## MATERIALS AND METHODS

### *Animals*

Male Wistar rats weighing 200–250 g were supplied by the animal care facility of the Federal University of São Paulo. They were housed under controlled conditions of lighting (12:12 hours light–dark cycle with lights on at 7:00 AM) and temperature ( $24 \pm 1^\circ\text{C}$ ) with free access to water and food. The Experimental Research Committee of UNIFESP (Federal University of São Paulo) approved all procedures involving these animals.

### *Antigens preparation*

**Heat-coagulated HEW.** Hen eggs were boiled in a water bath at  $100^\circ\text{C}$  for 30 minutes. Fragments of solidified egg white (HEW) were rinsed in phosphate-buffered saline (PBS; pH 8.0), dehydrated in 100% ethanol for at least 24 hours, and cut into small fragments of 400 mg each. Before being implanted in the subcutaneous tissues of rats HEW fragments were rehydrated in sterile PBS (pH 7.4) for 1 hour (De Siqueira et al., 1997).

**Heat-aggregated ovalbumin.** Aggregated ovalbumin used for aerosol challenge was prepared according to Titus and Chiller (1981). Briefly, type II ovalbumin was diluted (2.5 mg/mL) in sterile saline and incubated for 1 hour at  $80^\circ\text{C}$ . After centrifugation at 2400 g for 10 minutes the supernatant was discarded and the precipitated material was resuspended in 100 mL of sterile saline.

### *Sensitization procedure and antigen challenge*

Rats were actively sensitized by a subcutaneous implant of 400 mg of HEW in the dorsal region. After 14 days the animals were exposed for 20 minutes to an aerosol of grade II heat-aggregated ovalbumin solution generated by an ultrasonic nebulizer (ICEL US-800, São Paulo, Brazil).

### *Bronchoalveolar lavage and total and differential cell count*

Twenty-four (24) hours after antigen challenge animals were anesthetized intraperitoneally with chloral hydrate (10 mg/kg) and their tracheae cannulated with a polyethylene tube connected to a syringe. The lungs were washed with  $3 \times 10$  mL of PBS at  $4^\circ\text{C}$  and bronchoalveolar lavage fluids (BAL) were recovered and centrifuged at 2400 g for 10 minutes. The supernatants were discarded and the cell pellets resuspended

in 2.5 mL of PBS. One volume of a solution containing 0.5% crystal violet dissolved in 30% acetic acid was added to nine volumes of the cell suspension. Total cell number was determined with the aid of a Neubauer chamber. Differential cell counts were performed in BAL preparations stained with Instant Prov Kit (Newprov, Brazil) upon cytocentrifugation at 500 g (a Shandon Cytospin 3 centrifuge [Shandon Scientific Limited, Pittsburgh, PA]). A minimum of 400 cells were counted and classified according to morphologic criteria.

### *Histopathologic analysis*

The lungs were removed and fixed for 24 hours in 10% paraformaldehyde solution. The organs were then cut into small fragments, dehydrated through an ethanol series (70%–100%), cleared in xylol and embedded in paraffin. The fragments were sliced into 5- $\mu$ m thick sections and stained with hematoxylin-eosin. Histologic evaluation was based on the intensity of the inflammatory infiltrate in randomly selected areas around vessels and bronchioles. Histopathologic changes in the lung were graded by a pathologist according to the degree of inflammation using the following scale: light (1), moderate (2), intense (3), and very intense (4) and numbers represent what was called inflammation score (IS). Results were expressed as mean values of IS for each experimental group.

### *Acupoints and sham acupoints*

The selected acupoints were VG-14 (*Dazhui*), BL-13 (*Feishu*), MDC-1 (*Ding chuan*), in the dorsal region; and L-1 (*Zhongfu*), VC-17 (*Danzhong*), ST-36 (*Zusanli*) and SP-6 (*Sanyinjiao*), in the ventral region. These acupoints are considered of choice to treat asthma (Ngh, 1981; Wong, 1995). Localization of these acupoints was based on available acupoints charts for rodents and on anatomical references such as bones and joints (Andersson and Lundberg, 1995; Romita et al., 1997). These acupoints correspond anatomically to their equivalents in humans. The exact location of acupoints was confirmed using an impedometer (Plexus T20; Lautz, São Paulo, Brazil) that emits a sound signal under low electrical resistance and high conductance. The sham points were located at the dermatome immediately next to that of the acupoints, in an area where the sound signal was absent (Zhong-Xiang, 1981). For electroacupuncture (EA) treatment, rats were immobilized in a plastic cylinder that allowed access to all acupoints. Stainless steel 0.25/20 mm needles (needles with iodized cable and inox iron body; Lautz) were inserted into each acupoint at a depth of approximately 3 mm. The intensity of stimulation was determined to be the minimum voltage to cause moderate muscle contraction. EA stimulation was applied for 20 minutes with an electrical current of faradic, bipolar and asymmetrical wave at 52 Hz and 1 mV intensity using AP 195 Micro Process (Lautz).

### *Experimental model*

Male Wistar rats were randomly divided into four groups: acupuncture (A), sham acupuncture (SA), immobilized (I), and control (C) groups. All animals were sensitized with a subcutaneous implant of HEW. After 24 hours and a light ether anesthesia, the skin was trichotomized in some areas and acupoints and sham acupoints were located. Group A underwent manual acupuncture at dorsal points for 30 seconds. The animals were then placed in ventral decubitus, immobilized, and submitted to EA at ventral points for 20 minutes per day, on alternate days for 2 weeks, between 7:30 AM and 9:00 AM. Immediately after the last EA stimulation the animals were challenged with aggregated ovalbumin given as aerosol. After 24 hours the rats were anesthetized intraperitoneally with chloral hydrate (10 mg/kg) and blood samples, BAL, and lungs collected. The SA group underwent to the same treatment as group A but with acupuncture needles implanted at sham acupoints. Group I underwent to the same anesthetic and immobilization procedures with the omission of needle insertion or electrical stimulation. Group C was sensitized with a subcutaneous HEW implant and 14 days after the challenge with ovalbumin aerosol was performed. This group did not receive any needle stimulation or immobilization, and was sacrificed with the other animals.

### *Statistical analysis*

Data were expressed as mean  $\pm$  standard error of the mean (sem). Statistical analysis was performed by Kruskal-Wallis analysis of variance (ANOVA) followed by the Dunn test, for comparisons among three groups (immobilized, sham acupuncture, and acupuncture). When two groups were compared (control and immobilized) the Mann-Whitney test was used. The significance was set at  $p \leq 0.05$ .

## RESULTS

To start the proposed investigation it was necessary to verify if the immobilization procedure *per se* could induce in the rats either alterations either in corticosterone plasma levels or changes in the BAL inflammatory cell score. This first evaluation is summarized in Table 1. No significant differences were observed in corticosterone levels or in the number of BAL inflammatory cells when groups I and C were compared. Furthermore these results were corroborated by the inflammation score (IS) that was also identical in both experimental groups. These data demonstrated that immobilized rats (group I) and the control group (group C) were equivalent.

The first result demonstrating the efficiency of EA using acupoints in reducing the immune-mediated inflammation was the histopathological analyses of the lung sections, as depicted in Figure 1. The inflammatory infiltrates observed

TABLE 1. CORTICOSTERONE PLASMA LEVELS, BAL CELLULAR COMPOSITION AND TISSUE INFLAMMATION SCORE IN CONTROL (C) AND IMMOBILIZED (I) EXPERIMENTAL GROUPS

| Groups      | Plasma                | BAL                     |                       |                       |                       |                       | Tissue               |
|-------------|-----------------------|-------------------------|-----------------------|-----------------------|-----------------------|-----------------------|----------------------|
|             | Cortico <sup>a</sup>  | TCC <sup>b</sup>        | Poly <sup>c</sup>     | Neutro <sup>c</sup>   | Eosino <sup>c</sup>   | Mono <sup>c</sup>     | IS <sup>d</sup>      |
| Control     | 14.8<br>± 4.2<br>(09) | 290.4<br>± 31.3<br>(10) | 45.5<br>± 5.2<br>(10) | 12.9<br>± 1.9<br>(05) | 35.5<br>± 6.1<br>(05) | 60.3<br>± 5.6<br>(11) | 3.3<br>± 0.3<br>(11) |
| Immobilized | 12.4<br>± 4.7<br>(08) | 288.2<br>± 29.1<br>(10) | 34.9<br>± 3.9<br>(11) | 12.9<br>± 1.1<br>(05) | 26.8<br>± 4.8<br>(06) | 65.3<br>± 3.9<br>(11) | 3.2<br>± 0.3<br>(11) |

<sup>a</sup>Corticosterone plasma concentration (Cortico— $\mu\text{g/dL}$ ).

<sup>b</sup>BAL analyses as total cell count (TCC,  $\times 10^4/\text{mL}$ ); and

<sup>c</sup>BAL cell composition as percentage of polymorphonuclears (Poly), neutrophils (Neutro), eosinophils (Eosino), and mononuclears (Mono).

<sup>d</sup>Inflammatory score (IS). Data are shown as mean  $\pm$  SEM. The number of animals is given in parenthesis.

BAL, bronchoalveolar lavage.

in acupuncture-treated animals (group A) were much smaller than those found in group I. These findings were quantitated using IS and the obtained results are shown in Table 2. A statistical significance on the reduction of the inflammatory infiltrate was found when acupuncture-treated animals (group A) were compared to sham-treated (SA) and only immobilized (I) animals ( $p = 0.00006$ ,  $H = 19.27$ ).

The animal model also provided an opportunity to qualify and quantify the cellular composition of BAL, to further characterize the inflammatory cells infiltrate induced in the lungs. Data presented in Table 2 demonstrate that the total cell count was significantly lower in group A when compared to group I ( $p = 0.007$ ,  $H = 9.70$ ). Similar results were observed when total polymorphonuclears ( $p = 0.0019$ ,  $H = 12.48$ ), neutrophils ( $p = 0.026$ ,  $H = 7.26$ ) and eosinophils ( $p = 0.015$ ,  $H = 8.34$ ) were scored. However, a relatively high percentage of mononuclears was obtained in the BAL of EA-treated animals using acupoints compared to controls ( $p = 0.0021$ ,  $H = 12.34$ ). All results obtained for the acupuncture group were shown to be significantly different from those of the group of immobilized animals.

The corticosterone plasma levels were determined at the time of sacrifice (14th day) of EA treated animals (groups SA and A) and compared to group I. Table 2 shows that no differences among the three experimental groups were found ( $p = 0.518$ ,  $H = 1.31$ ).

## DISCUSSION

According to Traditional Chinese Medicine, health depends on an adequate energy homeostasis. Diseases and their

clinical symptoms occur when the energy homeostasis of the body is disrupted and an energy imbalance is established. Some of our clinical experience (Carneiro et al., 1997a, 1997b, 1998, 1999) has shown that acupuncture can restore the energetic equilibrium, as it alleviates and/or cure symptoms, as also stated by others (Vickers et al., 2002; Ziment and Tashkin, 2000). In recent years, these observations motivated us to evaluate the biologic effects of acupuncture in animal models to reject a placebo effect, as suggested by some authors (Streitberger and Kleinhenz, 1998; Vicent et al., 1989).

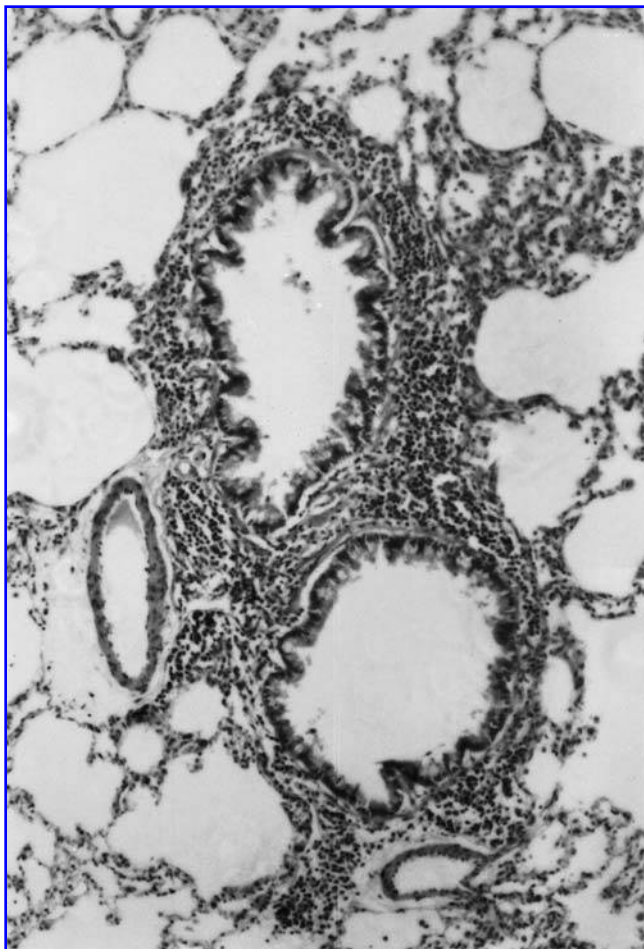
In this regard, experiments in our laboratory have demonstrated that a single EA session using two acupoints specific for inflammatory diseases promoted an anti-inflammatory effect on carrageenan paw edema in rats (Yanaguizawa et al., 2000). It was subsequently verified that the administration of catecholamine antagonists to EA-treated animals abolished the obtained anti-inflammatory effects of acupuncture (M.M. Yabuta and V.L.F. Silveira, unpublished results).

These previous experiences together with other reports concerning the anti-inflammatory action of acupuncture (Pintér and Szolcsányi, 1996; Son et al., 2002) led us to examine if similar results could also be demonstrated in an experimental model of asthma. Thus, EA treatment was tested in rats that underwent an immune-mediated lung inflammation, which resembles human asthma (Russo et al., 1997).

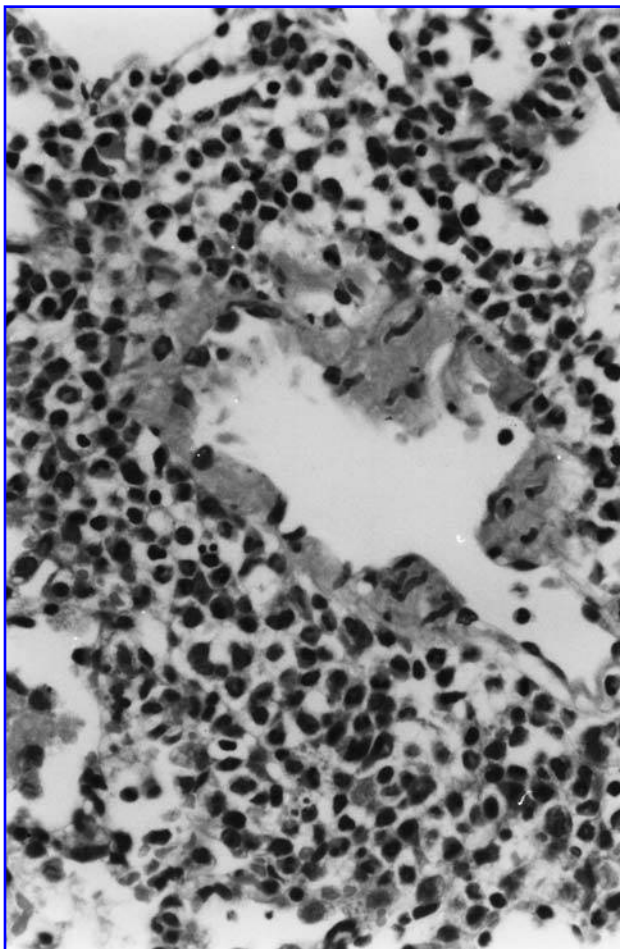
In acupuncture research there is a great difficulty in standardizing an ideal control where the stimulation of sham acupoints do not have any effect on somatic and or visceral neural afferences from the electrical stimulation of acupoints (Ernst and White, 2001). However, in the studies described herein, it is clearly depicted that lung inflammation was ev-

**FIG. 1.** Photomicrography of lung tissue of rats immunized with coagulated egg white implant and challenge with ovalbumin (OVA). Experimental groups: immobilized (A and B) and acupuncture (C and D). Observe the intense peribronchial inflammatory reaction composed predominantly by eosinophils and mononuclear cells in Immobilized group. In the acupuncture group the peribronchial inflammatory infiltrate is reduced. Hematoxylin-eosin staining. Magnifications: in A and C (100 $\times$ ), in B (400 $\times$ ) and in D (200 $\times$ ).

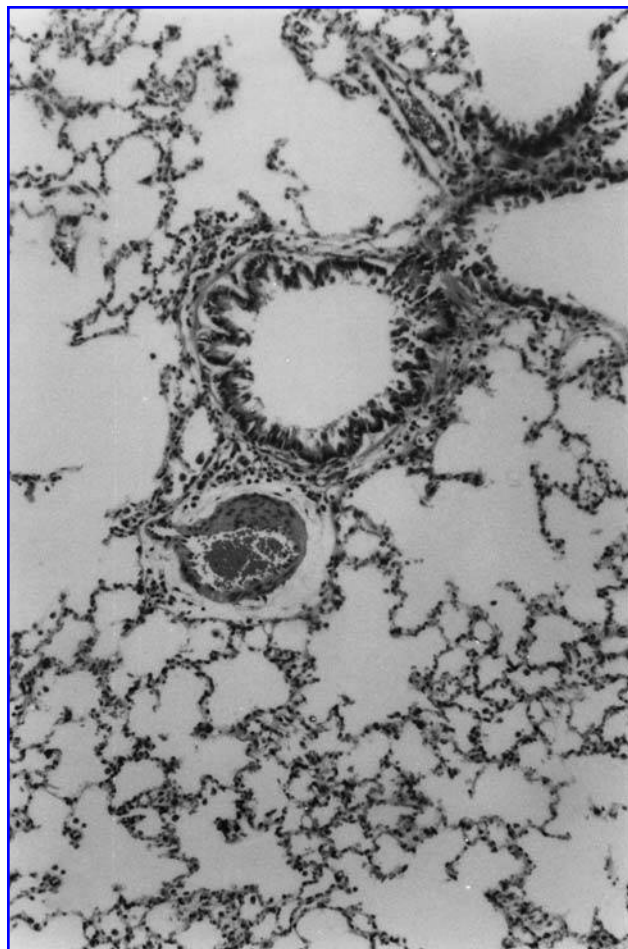
A



B



C



D

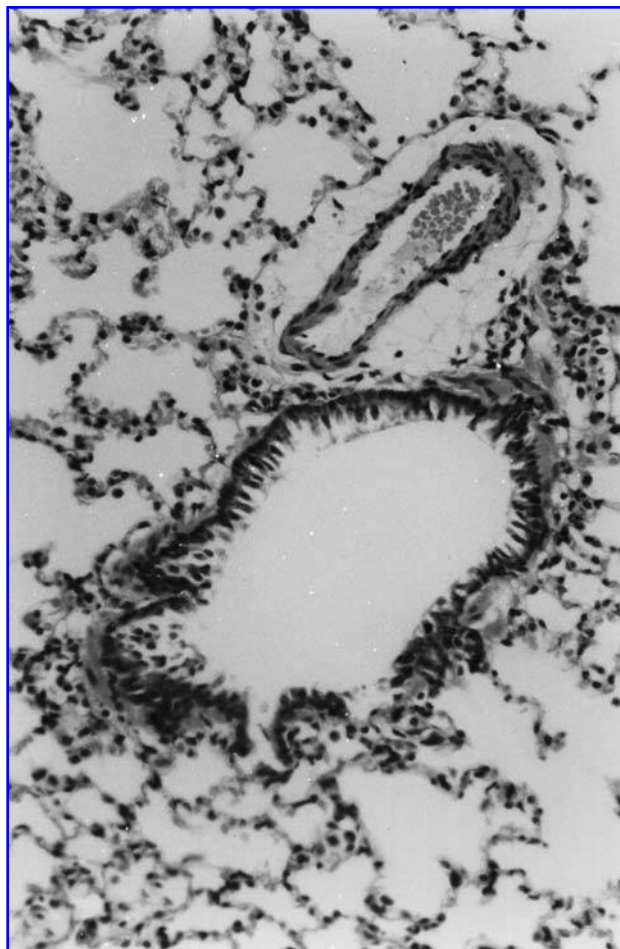


TABLE 2. CORTICOSTERONE PLASMA LEVELS, BAL CELLULAR COMPOSITION AND TISSUE INFLAMMATION SCORE IN ACUPUNCTURE, SHAM ACUPUNCTURE, AND IMMOBILIZED EXPERIMENTAL GROUPS

| Groups           | Plasma                | BAL                                  |                                    |                                   |                                   |                                    | Tissue                              |
|------------------|-----------------------|--------------------------------------|------------------------------------|-----------------------------------|-----------------------------------|------------------------------------|-------------------------------------|
|                  | Cortico <sup>a</sup>  | TCC <sup>b</sup>                     | Poly <sup>c</sup>                  | Neutro <sup>c</sup>               | Eosino <sup>c</sup>               | Mono <sup>c</sup>                  | IS <sup>d</sup>                     |
| Acupuncture      | 14.3<br>± 4.8<br>(10) | 151.6 <sup>e</sup><br>± 20.1<br>(11) | 13.7 <sup>e</sup><br>± 1.7<br>(11) | 5.9 <sup>e</sup><br>± 1.3<br>(06) | 8.9 <sup>e</sup><br>± 1.6<br>(06) | 86.3 <sup>e</sup><br>± 1.7<br>(11) | 1.3 <sup>e,f</sup><br>± 0.1<br>(11) |
| Sham-acupuncture | 12.1<br>± 2.5<br>(08) | 197.4<br>± 35.8<br>(11)              | 20.2<br>± 4.1<br>(10)              | 9.7<br>± 2.1<br>(06)              | 17.3<br>± 3.4<br>(05)             | 77.6<br>± 4.8<br>(11)              | 2.3<br>± 0.1<br>(11)                |
| Immobilized      | 12.4<br>± 4.7<br>(08) | 288.2<br>± 29.1<br>(10)              | 34.9<br>± 3.9<br>(11)              | 12.9<br>± 1.1<br>(05)             | 26.8<br>± 4.8<br>(06)             | 65.3<br>± 3.9<br>(11)              | 3.2<br>± 0.3<br>(11)                |

<sup>a</sup>Corticosterone plasma concentration (Cortico— $\mu\text{g/dL}$ ).

<sup>b</sup>BAL analyses as total cell count (TCC,  $\times 10^4/\text{mL}$ ).

<sup>c</sup>BAL cell composition as percentage of polymorphonuclears (Poly), neutrophils (Neutro), eosinophils (Eosino), and mononuclears (Mono). Inflammatory score (IS). Data are shown as mean  $\pm$  SEM.

<sup>d</sup>The number of animals is given in parenthesis.

<sup>e</sup>Significantly different ( $p < 0.05$ ) from I group.

<sup>f</sup>Significantly different ( $p < 0.05$ ) from SA group.

BAL, bronchoalveolar lavage; SEM, standard error of the mean; SA, sham acupuncture.

idently reduced in acupoint-treated group compared to both sham-acupoint-treated and immobilized groups. Moreover, the reduction on the inflammatory cells in the BAL was statistically significant when group A was compared to the control animals. Also, those animals presented less polymorphonuclears (neutrophils and eosinophils), the tissue damaging cells, than the immobilized group. By contrast, the relative percentage of mononuclear cells in BAL was increased in animals treated with EA in comparison with the immobilized controls, a finding that warrants further investigation.

Taken together, these data show that EA reduced bronchial inflammation and that this result was dependent on the specificity of the selected acupoints for asthma treatment. To our knowledge, this is the first experimental demonstration of the beneficial effects of acupuncture, applied during the sensitization period, in rats undergoing a pulmonary immune-mediated eosinophilic hypersensitivity model.

The experimental conditions of this study implied in stressful situations for the rats during 2 weeks, which could modify their corticosterone levels. It has been reported that corticosterone plasma levels are significantly increased when animals undergo a single immobilization session, compared to chronic stressful conditions (Dhabar et al., 1997; Strausbaugh et al., 1999). Furthermore, it has been shown that chronic immobilization stress does not modify either catecholamine or corticoids plasma levels, indicating an adaptation of the neuroendocrine circuit (Amario et al., 1984; Baldwin et al., 1996; De Boer et al., 1990). Here we show that corticosterone plasma levels did not differ among the three experimental groups. This strongly suggests that the animals had adapted at the time of the experiment, and that the anti-inflammatory effects obtained by EA treatment,

using either acupoints or sham acupuncture, could not be attributed to the action of this hormone.

We speculate that the beneficial effects of acupuncture in asthma could be linked to an inhibitory action on the release of cytokines such as interleukin (IL)-5 and IL-13, primary determinants of eosinophil differentiation and activation (Anderson and Coyle, 1994; Cara et al., 2000). Corroborating this idea, an immunomodulatory effect of acupuncture has been demonstrated in inflammation animal models (Son et al., 2002; Wu et al., 1999). Additionally, acupuncture was also shown to modify cytokines' profile in patients with airways inflammatory diseases (Joss et al., 2000; Petti et al., 2002). Also, a direct effect on the human central nervous system has already been shown (Cho et al., 1998; Haker et al., 2000) supporting the action of acupuncture on the neuroimmune axis.

Hence, we suggest that the anti-inflammatory effect observed in our study occurs by a modulator effect of acupuncture on the imbalance of  $T_H2/T_H1$  immune responses with involvement of central nervous system. This hypothesis is presently under investigation in our laboratory.

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