

# Electroacupuncture inhibits apoptosis of splenic lymphocytes in traumatized rats through modulation of the TNF- $\alpha$ /NF- $\kappa$ B signaling pathway

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**Abstract.** Surgical trauma leads to a severe deterioration of the immune system. Electroacupuncture (EA) may improve the immunodeficiency that occurs following surgery; however, the underlying signaling mechanisms require further study. In the present study, 40 rats were equally randomized into four groups: Control; Control + EA; Trauma; Trauma + EA. EA was applied at the ‘Zusanli’ (ST36) and ‘Lanwei’ (Extra37) acupoints, immediately following surgery. The splenic T cells were isolated from the rats 24 h after surgery. The apoptotic rate of the lymphocytes was measured by flow cytometric analysis, and western blotting was used to determine the protein expression levels of caspase-3, caspase-8, tumor necrosis factor (TNF)- $\alpha$  and TNF receptor 1 (TNFR1). The DNA binding activity of nuclear factor (NF)- $\kappa$ B was determined using Trans-AM<sup>®</sup> ELISA-based kits. The results of the present study showed that surgical trauma induced apoptosis of splenic lymphocytes, and significantly increased the protein expression levels of caspase-3 and caspase-8. This was accompanied by increased expression levels of TNF- $\alpha$  and TNFR1, and a marked reduction in the activity of NF- $\kappa$ B in splenic T cells. Administration of EA significantly decreased the expression levels of caspase-3, caspase-8, TNF- $\alpha$  and TNFR1, elevated

the activity of NF- $\kappa$ B, and suppressed the apoptotic rate of the lymphocytes. The data suggests that EA may inhibit the apoptosis of splenic lymphocytes induced by surgical trauma, and ameliorate the postoperative immunosuppression. This may be mediated by the downregulation of TNF- $\alpha$  expression levels and upregulation of the activity of NF- $\kappa$ B.

## Introduction

Surgical and traumatic injury notably affects the innate and adaptive immune responses, and a marked suppression in cell-mediated immunity has been suggested to be responsible for the increased postoperative susceptibility to infections, multiple organ dysfunction, and mortality (1,2). Previous studies have revealed that surgical trauma suppresses the proliferation of splenic lymphocytes and the production of interleukin-2 (IL-2), and initiates the apoptosis of lymphocytes (3-5). It has additionally been shown that excessive splenic lymphocyte apoptosis may lead to a breakdown of host defense mechanisms and ultimately to depression of the immune system (6,7). It thus appears that it may be essential to prevent lymphocyte apoptosis, in order to attenuate postoperative immune deteriorations and ensure survival.

Previous acupuncture research has been carried out with the aim of understanding pain control (8). In addition to analgesic evidence, clinical and experimental evidence has demonstrated that electroacupuncture (EA) treatment is effective for numerous immunological diseases, including infections, autoimmune diseases and immunodeficiency-syndromes (9,10). Previous evidence has suggested that EA may increase splenic lymphocyte proliferation and IL-2 production in rats following surgical trauma (11,12). A previous study indicated that EA administration ameliorated the postoperative impaired immune function, by correcting the imbalance in the production of T helper (Th)1 (IL-2 and interferon- $\gamma$ ) and Th2 (IL-4 and IL-10) cytokines in splenic T cells of traumatized rats (13). However, few studies have observed the effects of EA on the apoptosis of splenic lymphocytes in rats undergoing surgical trauma, nor has the signaling mechanism mediating these effects been elucidated.

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**Abbreviations:** EA, Electroacupuncture; JNK, c-Jun N-terminal kinase; TNF- $\alpha$ , tumor necrosis factor-alpha; NF- $\kappa$ B, nuclear factor-kappa B

**Key words:** electroacupuncture, surgical trauma, lymphocyte apoptosis, TNF- $\alpha$ , NF- $\kappa$ B

Among apoptosis triggering mechanisms, the tumor necrosis factor- $\alpha$ /c-Jun N-terminal kinase/nuclear factor- $\kappa$ B (TNF- $\alpha$ /JNK/NF- $\kappa$ B) pathway has a critical role in numerous pathological conditions. The TNF- $\alpha$ /JNK/NF- $\kappa$ B pathway has been extensively investigated in various *in vitro* and *in vivo* models, as modulation of this pathway may be a novel anti-apoptotic strategy (14). It is considered that the interaction of TNF- $\alpha$  with TNF receptor 1 (TNFR1) induces apoptosis by activating JNK (15,16). JNK is a stress-activated protein kinase, and is capable of inducing cellular apoptosis (17). A previous study reported that TNF- $\alpha$  could also activate NF- $\kappa$ B, which may lead to anti-apoptotic gene expression (e.g., superoxide dismutase) and prevent apoptosis (18,19). Therefore, apoptosis induced by TNF- $\alpha$  is regulated by the balance of JNK and NF- $\kappa$ B activation. TNF- $\alpha$  is one of the initial cytokines released during the inflammatory process, and is the main cytokine with an established correlation with trauma (20,21). In addition, the activity of JNK and NF- $\kappa$ B in splenic T cells has been shown to be decreased following trauma-hemorrhage (13,22). However, the exact roles of the TNF- $\alpha$ /JNK/NF- $\kappa$ B pathway in the apoptosis of splenic lymphocytes in rats undergoing surgical trauma, and whether this pathway is involved in mediating the immunomodulatory effects of EA remain unclear.

It was hypothesized that EA may ameliorate surgical trauma-induced immunosuppression by inhibiting the apoptosis of splenic lymphocytes, and this effect may be mediated via the TNF- $\alpha$ /JNK/NF- $\kappa$ B pathway. Therefore, the aims of the present study were to investigate the effects of EA on splenic lymphocyte apoptosis of rats subjected to surgical trauma, and to evaluate the possible signaling mechanisms.

## Materials and methods

**Subjects.** Male Sprague Dawley rats (weighing  $200 \pm 20$  g) were obtained from the Animal Center of Vital River Laboratories Co., Ltd. (Beijing, China). The rats had access to food and water *ad libitum*, and were acclimated in controlled conditions (12:12 h light /dark cycle,  $23 \pm 0.5^\circ\text{C}$ , 40–60% relative humidity) for one week prior to the surgery.

**Experimental design.** A total of 40 rats were randomized equally using a computer-generated random number table, into the four following groups: Control (I); Control + EA (II); Trauma (III); Trauma + EA (IV). As previously described by Wang *et al* (3), the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA), and were incised longitudinally, 6 cm along the dorsal median line and 5 cm along the abdominal median line. The rats of groups I and II were anesthetized only, without undergoing surgical trauma. All of the animal procedures were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MA, USA) and were approved by the local Animal Research Ethics Committee of the Harbin Medical University (Harbin, China).

**Electroacupuncture application.** The ST36 (located 5 mm below and lateral to the anterior tubercle of the tibia) and Extra37 (located ~3.3 mm below ST36) acupoints were selected.

ST36 is a frequently used point for the study of acupuncture analgesia and immunomodulation, and Extra37 is an adjunct acupoint (13). The EA parameters were set as alternating strings of dense-disperse frequencies (60 Hz for 1.05 sec and 2 Hz for 2.85 sec alternately), and the intensity was adjusted to induce moderate muscle contraction of the hind limb ( $\leq 1$  mA) for 30 min. A pair of needles (diameter, 0.3 mm; length, 50 mm), were inserted into the ST36 and Extra37 acupoints, and connected to electrical acupuncture apparatus (Nerve and muscle stimulator SDZ-II, Suzhou Medical Appliance Factory, Jiangsu, China). The rats of groups I and III were tied for 30 min but did not receive EA. EA was performed by an expert in acupuncture who was blinded to the group assignments, and was not involved in data acquisition or analysis.

**Isolation of splenic T cells.** All of the rats were sacrificed 24 h after surgery, by an overdose of pentobarbital sodium. The spleens were removed aseptically and dissociated into single cell suspensions. Briefly, the splenocytes were isolated by homogenizing the spleen samples in RPMI-1640 media (Sigma-Aldrich), supplemented with 10% fetal calf serum, 10 mM HEPES, 1 mM glutamine and 50 mg/ml penicillin (Sigma-Aldrich). The erythrocytes were lysed using lysing solution. The isolated splenocytes were collected, washed three times with Hank's balanced salt solution, counted and adjusted to  $5 \times 10^6$  cells/ml in RPMI-1640 medium.

**Flow cytometry.** A flow cytometric analysis was performed to calculate the percentage of apoptotic cells, using fluorescein isothiocyanate (FITC) conjugated Annexin V and propidium iodide (PI) staining (BD Biosciences, Franklin Lakes, NJ, USA). The isolated splenocytes were washed twice with ice-cold phosphate-buffered saline (PBS), adjusted to a cell density of  $2 \times 10^5$  cells/ml, and re-suspended in 100 ml of binding buffer, containing 5 ml of FITC-conjugated Annexin V and PI (50 mg/ml). The mixture was incubated for 15 min at room temperature in the dark. The percentage of early apoptotic (FITC positive and PI negative) cells was calculated from the data originating from the flow cytometric analysis (Beckman Coulter, Brea, CA, USA).

**Western blot analysis.** A total of 50  $\mu\text{g}$  of extracted protein was resuspended in sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min. Equal amounts of total protein were loaded onto a 10% SDS-containing polyacrylamide gel and were separated by SDS-PAGE (Sigma-Aldrich), followed by an electrotransfer to polyvinylidene difluoride membranes (Sigma-Aldrich). The membranes were saturated with blocking buffer (Sigma-Aldrich) for 1 h at room temperature and incubated with antibodies against caspase-3 (1:1,000), caspase-8 (1:1,000), TNF- $\alpha$  (1:1,000), TNFR1 (1:1,000), total-JNK (1:500), phospho-JNK (1:500) and  $\beta$ -actin (1:1,000) (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at  $4^\circ\text{C}$  overnight. The membranes were washed three times in 0.3% Tween<sup>®</sup> 20/PBS for 10 min, and were then incubated with an IRDye800 conjugated secondary antibody (Biotrend Chemikalien GmbH, Köln, Germany) in blocking buffer. The protein-antibody complexes conjugated with IRDye800 were visualized on the Odyssey<sup>®</sup> Infrared Imaging System (LI-COR Biosciences GmbH, Bad-Homburg, Germany).

**ELISA.** The DNA binding activity of NF- $\kappa$ B was measured using Trans-AM ELISA-based kits (Active Motif, Carlsbad, CA, USA). The nuclear protein of the splenic lymphocytes was extracted using a Nuclear Extract kit (Active Motif). Biotinylated double-stranded oligonucleotides (Active Motif) containing the NF- $\kappa$ B consensus site were precoated onto 96-well plates. The activated transcription factors, bound to the respective immobilized oligonucleotides, were detected using antibodies against NF- $\kappa$ B p65, followed by a horseradish peroxidase-conjugated secondary antibody in an ELISA-like assay (Active Motif).

**Sample size and statistical analyses.** A power analysis, based on the results of preliminary experiments comparing immune variables among groups, yielded a sample size of  $n = 8$  ( $\alpha = 0.05$  and  $\beta = 0.2$ ), for each group. The data were analyzed using an analysis of variance with Student-Newman-Keuls test, or a Mann-Whitney U test for multiple comparisons. All statistical analyses were performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). A  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**The effects of EA on apoptosis of splenic lymphocytes.** The apoptotic rate of the splenic lymphocytes was measured using flow cytometric analysis (Fig. 1). There were no statistically significant differences between the apoptotic rate of the splenic lymphocytes in Groups I and II ( $4.65 \pm 0.7$  and  $4.28 \pm 0.11\%$ , respectively), 24 h following surgery. The apoptotic rate of the splenic lymphocytes in Group III ( $15.51 \pm 0.89\%$ ) was significantly increased, as compared with Group I ( $P < 0.01$ ). The apoptotic rate of the splenic lymphocytes in Group IV ( $6.52 \pm 0.78\%$ ) was significantly decreased, as compared with Group III ( $P < 0.05$ ). These results indicate that administration of EA significantly alleviated the apoptotic rate of splenic lymphocyte, induced by surgical trauma.

**The effects of EA on the protein expression levels of caspase-3 and caspase-8.** The protein expression levels of caspase-3 and caspase-8 in splenic T cells, 24 h following surgery, were determined by western blotting (Fig. 2). There were no statistically significant differences in the protein expression levels of caspase-3 and caspase-8 between Group I ( $0.25 \pm 0.04$  and  $0.12 \pm 0.02$ , respectively) and Group II ( $0.26 \pm 0.03$  and  $0.09 \pm 0.02$ , respectively). The protein expression levels of caspase-3 and caspase-8 in Group III ( $0.77 \pm 0.06$  and  $0.67 \pm 0.04$ , respectively) were significantly increased, as compared with Group I ( $P < 0.01$ ). The expression levels of caspase-3 and caspase-8 in Group IV ( $0.39 \pm 0.05$  and  $0.37 \pm 0.06$ , respectively) were significantly decreased, as compared with Group III ( $P < 0.05$ ). These results suggest that administration of EA significantly inhibited the protein expression levels of caspase-3 and caspase-8.

**The effects of EA on the protein expression levels of TNF- $\alpha$  and TNFR1.** As shown in Fig. 3, the protein expression levels of TNF- $\alpha$  and TNFR1 in splenic T cells, 24 h following surgery, were determined by western blotting. The protein expression levels of TNF- $\alpha$  and TNFR1 were low in both Group I ( $0.22 \pm 0.02$  and  $0.19 \pm 0.04$ , respectively), and Group II ( $0.21 \pm 0.02$  and  $0.17 \pm 0.05$ , respectively). The protein expression

levels of TNF- $\alpha$  and TNFR1 in Group III ( $0.71 \pm 0.06$  and  $0.46 \pm 0.04$ , respectively) were significantly elevated, as compared with Group I ( $P < 0.01$ ). The expression levels of TNF- $\alpha$  and TNFR1 in Group IV ( $0.27 \pm 0.03$  and  $0.21 \pm 0.04$ , respectively) were significantly decreased, as compared to Group III ( $P < 0.05$ ). These results suggest that administration of EA significantly suppressed the upregulated expression levels of TNF- $\alpha$  and TNFR1.

**Effects of EA on the activity of JNK and NF- $\kappa$ B.** The activity of NF- $\kappa$ B was similar in Groups I and II ( $0.65 \pm 0.02$  and  $0.68 \pm 0.03$ , respectively). NF- $\kappa$ B activity was significantly decreased in Group III ( $0.26 \pm 0.03$ ), as compared with Group I ( $P < 0.01$ ). The activity of NF- $\kappa$ B in Group IV ( $0.57 \pm 0.05$ ) was significantly increased, as compared with Group III ( $P < 0.05$ ). There were no significant changes to the expression levels of phospho-JNK and total-JNK among the groups.

## Discussion

The results of the present study demonstrated that surgical trauma induced apoptosis of splenic lymphocytes, and increased the expression levels of caspase-3 and caspase-8 in splenic T cells. This was accompanied by increased protein expression levels of TNF- $\alpha$  and TNFR1, and a marked reduction in the activity of NF- $\kappa$ B. EA administration significantly decreased the expression levels of both TNF- $\alpha$  and TNFR1, and increased the activity of NF- $\kappa$ B. The apoptotic rate of the splenic lymphocytes and the expression levels of caspase-3 and caspase-8 were markedly suppressed in response to EA administration. These results suggested that the anti-apoptotic effects of EA may be associated with the TNF- $\alpha$ /NF- $\kappa$ B pathway.

A previous study demonstrated that surgical trauma affected leukocyte cellularity, reduced the number of splenic lymphocytes, and increased the susceptibility to postoperative complications (23). It has also been shown that the peripheral lymphocyte CD4 $^{+}$  and CD8 $^{+}$  T cells exhibit a higher frequency of apoptosis following surgery (24). An increase in the apoptotic rate of lymphocytes has been observed in patients undergoing surgical procedures (25,26). In the present study, the results showed that surgical trauma initiated the apoptosis of splenic lymphocytes, and increased the expression levels of caspase-3 and caspase-8 in splenic T cells. Administration of EA markedly reduced lymphocyte apoptosis, and inhibited the expressions of caspase-3 and caspase-8. These results are consistent with a previous study that showed that EA suppressed the increased rate of apoptosis of splenic lymphocytes induced by surgical trauma (3). These results implied that EA may attenuate the apoptosis of splenic lymphocytes, and restore the suppressed immune function following surgery.

To explore the mechanisms underlying the immunomodulatory capabilities of EA, the present study aimed to determine whether the TNF- $\alpha$ /JNK/NF- $\kappa$ B pathway had any role in the anti-apoptotic effects of EA. TNF- $\alpha$  is a proinflammatory cytokine that induces apoptosis of various cells through activation of the JNK pathway. It has previously been demonstrated that TNF- $\alpha$  release leads to the triggering of receptor-dependent apoptotic cell death, and TNF- $\alpha$  may transduce a death signal via TNFR1–TNFR-associated death domain–Fas-associated death domain complexes. This death receptor signal triggers

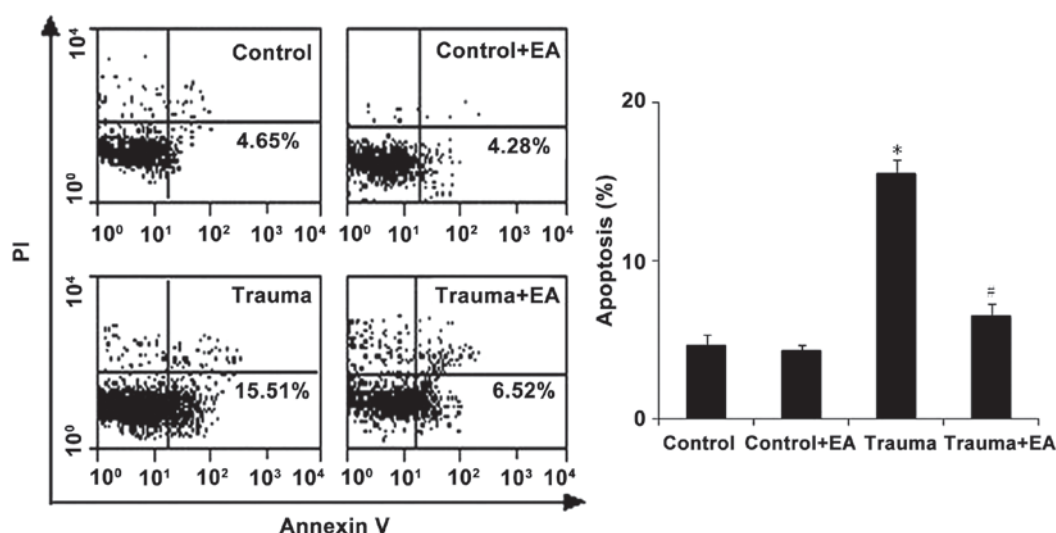


Figure 1. Effects of electroacupuncture (EA) on the apoptotic rate of splenic lymphocytes following surgery. EA was applied at the ST36 and Extra37 acupoints immediately following surgery. The rats were sacrificed 24 h after surgery, and the splenic T cells were isolated and adjusted to  $2 \times 10^5$  cells/ml. The apoptotic rate was determined by flow cytometric analysis using fluorescein isothiocyanate conjugated Annexin V and propidium iodide (PI) staining. The cells staining positive for Annexin V and negative for PI were considered apoptotic. The data represent the means  $\pm$  standard deviation ( $n = 10$ /group). \* $P < 0.01$  vs. Control group; # $P < 0.05$  vs. Trauma group.

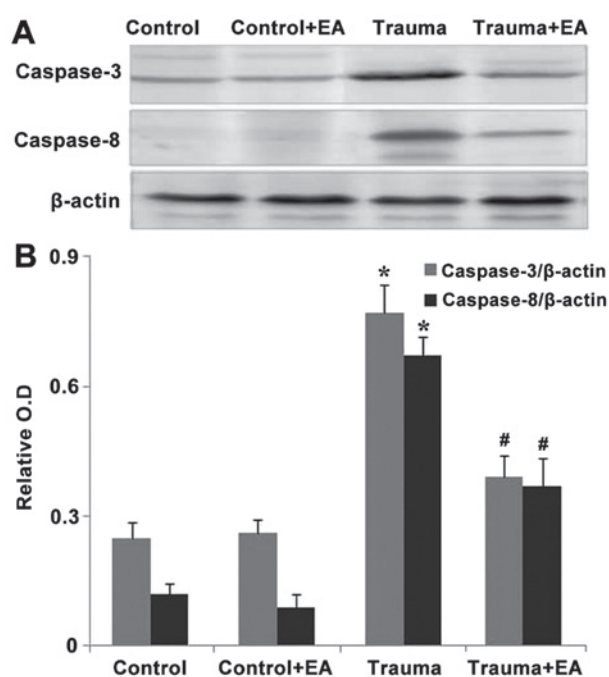


Figure 2. Effects of electroacupuncture (EA) on the protein expression levels of caspase-3 and caspase-8, following surgery. EA was applied at the ST36 and Extra37 acupoints immediately following surgery. The rats were sacrificed 24 h after surgery, and the splenic T cells were isolated and analyzed for the protein expressions of caspase-3 and caspase-8 by western blotting. (A) The protein bands were quantified by laser scanning densitometry, and  $\beta$ -actin was used as a loading control. (B) Statistical analysis of the relative levels of caspase-3 and caspase-8, the densitometric values were normalized to  $\beta$ -actin. The data represent the means  $\pm$  standard deviation ( $n = 10$ /group). \* $P < 0.01$  vs. Control group; # $P < 0.05$  vs. Trauma group. OD, optical density.

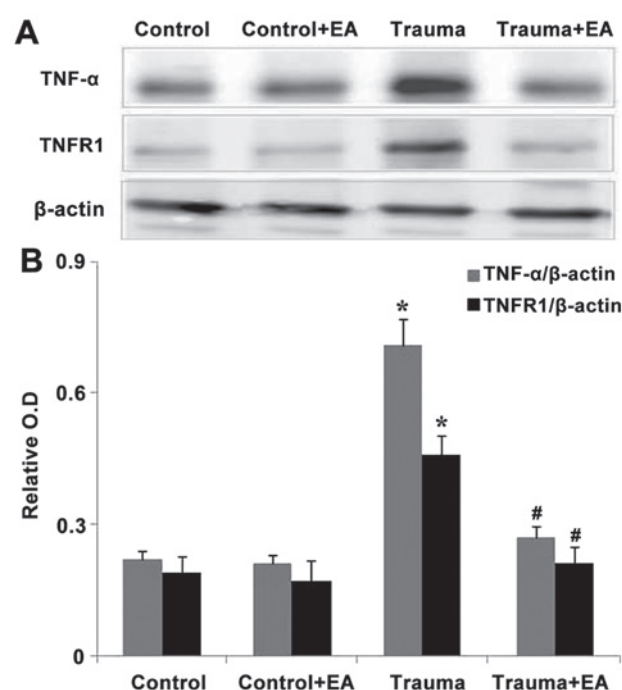


Figure 3. Effects of electroacupuncture (EA) on the protein expression levels of tumor necrosis factor (TNF)- $\alpha$  and TNF receptor 1 (TNFR1) following surgery. The rats were sacrificed 24 h after surgery, the splenic T cells were isolated and analyzed for the protein expressions of TNF- $\alpha$  and TNFR1 by western blotting. (A) The protein bands were quantified by laser scanning densitometry. (B) Statistical analysis of the relative levels of TNF- $\alpha$  and TNFR1, the densitometric values were normalized to  $\beta$ -actin. The data represent the means  $\pm$  standard deviation ( $n = 10$ /group). \* $P < 0.01$  vs. Control group; # $P < 0.05$  vs. Trauma group. OD, optical density.

activation of caspases, leading to apoptosis (27). NF- $\kappa$ B is an intracellular transcription factor that regulates the expression of numerous genes, the products of which are anti-apoptotic, (e.g., survivin, B-cell lymphoma (Bcl)-2, and Bcl-extra large), inflammatory cytokines, and cell cycle regulatory genes (28).

The effects of TNF- $\alpha$  on apoptosis is mainly dependent on the balance of JNK and NF- $\kappa$ B activation. A previous study reported that TNF- $\alpha$  protein expression was detected in significantly high levels in patients with severe trauma (29). Furthermore, NF- $\kappa$ B activity has been shown to be significantly decreased



in the splenic T cells of rats following trauma-hemorrhage (13). The results of the present study, showed that the expression levels of TNF- $\alpha$  and TNFR1, in splenic lymphocytes, were increased, and NF- $\kappa$ B activity was decreased 24 h following surgery; however, administration of EA markedly increased NF- $\kappa$ B activity, and decreased the expression levels of TNF- $\alpha$  and TNFR1. These results suggested that the TNF- $\alpha$ /NF- $\kappa$ B pathway could be targeted by EA treatment, and EA may exert an immunoregulatory role through the TNF- $\alpha$ /NF- $\kappa$ B pathway.

Previous research showed that EA stimulation suppressed the increase of apoptosis induced by surgical trauma, possibly by modulating Fas protein expression (3). In the present study, a novel observation made was that administration of EA inhibited surgical trauma-induced splenic lymphocyte apoptosis, by downregulating the expression levels of TNF- $\alpha$  and upregulating NF- $\kappa$ B activity. There were no significant changes observed in the expression levels of phospho-JNK and total-JNK in splenic T cells. This may be due to the activity of a distinct signaling pathway which may differ in various biological systems. Further work is required to determine the roles of the JNK pathway in triggering the apoptosis of lymphocytes following surgical trauma. To further elucidate the exact roles of the TNF- $\alpha$ /NF- $\kappa$ B pathway, specific inhibitors which interfere with this pathway may be used in further studies.

In conclusion, the present study demonstrated that administration of EA inhibited the increased apoptotic rate of splenic lymphocytes induced by surgical trauma, and attenuated postoperative immunosuppression; these effects are likely mediated via the TNF- $\alpha$ /NF- $\kappa$ B signaling pathway. The results of the present study imply that EA improved surgical trauma-induced immune suppression, and may be a potential therapeutic approach for the treatment of immune dysfunction.

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