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Research Article

Electroacupuncture inhibits apoptosis in annulus fibrosis cells through suppression of the mitochondria-dependent pathway in a rat model of cervical intervertebral disc degradation

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Abstract

The purpose of this study was to investigate whether treatment with electroacupuncture (EA) inhibited mitochondria-dependent apoptosis in annulus fibrosis (AF) cells in a rat model of cervical intervertebral disc degradation induced by unbalanced dynamic and static forces. Forty Sprague-Dawley rats were used in this study, of which 30 underwent surgery to induce cervical intervertebral disc degradation, 10 rats received EA at acupoints Dazhui (DU 14) and Shousanli (LI 10). TUNEL staining was measured to assess apoptosis in AF cells, immunohistochemistry was used to examine Bcl-2 and Bax expression, colorimetric assays were used to determine caspase 9 and caspase 3 activities and RT-PCR and western blotting were used to assess the mRNA and protein expression of Crk and ERK2. Treatment with EA reduced the number of AF-positive cells in TUNEL staining, increased Bcl-2-positive cells and decreased Bax-positive cells in immunohistochemical staining, significantly inhibited the activation of caspases-9 and -3, and enhanced the mRNA and protein expression of Crk and ERK2. Our data show that EA inhibits AF cell apoptosis via the mitochondria-dependent pathway and up-regulates Crk and ERK2 expression. These results suggest that treatment with may be a good alternative therapy for preventing cervical spondylosis.

Key words: annulus fibrosis cells, apoptosis, electroacupuncture, mitochondria.

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Introduction

Intervertebral disc (IVD) degeneration is an important phenomenon in pathological conditions commonly seen in orthopedic practice, such as disc herniation, spinal instability and radiculopathy (Gruber *et al.*, 2005). However, the pathogenesis of IVD degeneration remains unclear (Kasra *et al.*, 2006; Ali *et al.*, 2008). Cell apoptosis is one of the crucial pathological changes in disc degeneration (Zhao *et al.*, 2006; Jones *et al.*, 2008; Tschoeke *et al.*, 2008; Wei *et al.*, 2008). The existence of an apoptotic signal pathway in annulus fibrosis (AF) cells remains controversial. Previous studies of AF focused on the period after AF had ruptured, i.e, when IVD degeneration had occurred, and the methods used to produce AF degeneration cincluded surgical ablation of IVD and biological approaches such as molecular therapy (Kim *et al.*, 2003; Neidlinger-Wilke *et al.*, 2009), gene therapy (Moon *et al.*, 2000, 2008; Yoon *et al.*, 2004), cell transplantation (Sakai *et al.*, 2005), tissue engineering (Mizuno *et al.*, 2004; O'Halloran DM and Pandit, 2007) and traditional Chinese medicine such as acupuncture.

Acupuncture is recognized by the World Health Organization as an effective treatment for pain relieve and for cervical spondylosis. Dazhui (DU 14) and Shousanli (LI 10), which belong to the DU channel and Large Intestine channel, respectively, can dredge meridians and free collaterals, free Qi and stop pain, promote blood circulation and eliminate blood stasis, and are often used for neck stiffness, shoulder pain, upper limb paralysis and lumbar pain. However, the precise mechanism involved in these beneficial effects remains to be elucidated. To date, no study has investigated apoptosis in AF before IVD degeneration. The aims of this study were therefore to investigate the occurrence of apoptosis in AF before IVD degeneration, to determine the apoptotic pathway involved, to examine the relationship between apoptosis and Crk and ERK2 expression and to assess the usefulness of electroacupuncture (EA) for treating cervical intervertebral disc degeneration in rats.

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Materials and Methods

Forty Sprague-Dawley rats (one month old) were provided by the Shanghai Laboratory Animal Center and all animal procedures were approved by the Ethical Committee of Fujian University of Traditional Chinese Medicine. The rats were maintained on a 12 h light/dark cycle at 21 ± 2 °C with free access to food and tap water.

Prior to use, the rats were randomly assigned to two groups: a sham group (n = 10; 5 males and 5 females) in which the rats did not undergo any surgery and a surgical group (n = 30; 15 males and 15 females) that underwent surgery to induce cervical IVD degradation through unbalanced dynamic and static forces, as described by Wang *et al.* (2006). After observation for seven days, the 30 rats that have accepted surgery were randomly allocated into three groups of 10 rats (5 males and 5 females): a control group that was handled identically to the other groups but without acupuncture or electrical treatment, a group treated with meloxicam tablets (MT; Boehringer Ingelheim Corporation, Germany) that served as a positive control and a group treated with EA. For the EA protocol, rats were kept in specially designed holders with their necks and limbs exposed. Acupuncture needles were inserted in turn to depths of approximately 3 mm at acupoint Dazhui (DU 14) and approximately 1 mm at acupoint Shousanli (LI 10) bilaterally (Zhongren Li, 2003) and the rats then stimulated electrically (1 mA in intensity at 2/100 Hz) using a HANS EA Instrument (Model No. 100A, Shijiazhuang Fusai Medical Devices Ltd., China). The EA treatment was applied for 30 min once a day over 14 days (a complete course) with a two-day interval between two courses. In the MT group, meloxicam (0.75 mg/kg) was administered intragastrically for 30 days. All of these rats were euthanized with pentobarbitone sodium (Nembutal®; 100 mg/kg, i.p.; Boehringer Ingelheim, Artarmon, NSW, Australia) and the cervical spines were harvested for analysis.

TUNEL assay for apoptosis

For the quantitative analyses of apoptosis, sections from paraffin-embedded AFs were processed for terminal deoxynucleotidyl transferase-mediated dUTPFITC nick end-labeling (TUNEL) by using an *in situ* apoptosis detection kit (Wako Pure Chemical Industries, Ltd. Osaka, Japan). The assay was done according to the manufacturer's instructions, with minor modifications. TUNEL-positive cells were scored in viable regions peripheral to areas of necrosis in AF sections. The number of TUNEL-positive cells was counted in five random high-power (x400) fields in AF sections from each rat.

Immunohistochemical staining for Bcl-2 and Bax

The slides were processed using standard protocols for deparaffinization and rehydration. Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 for 10 min followed by digestion with 0.01% protease K for 10 min. Non-specific binding sites were blocked by incubation with confining liquid for 10 min after which the sections were incubated with rat polyclonal antibody to Bcl-2 or Bax (Cell Signaling Inc., Danvers, MA) at 4 °C for 12 h. After thorough washing, the sections were incubated with biotinylated goat anti-rabbit IgG at 4 °C for 60 min and then in Streptavidin-HRP for 10 min. The final color reaction was developed by incubation with the chromogenic substrate 3,3'-diaminobenzidine (0.5 mg/mL in Tris). The sections were counterstained with hematoxylin and mounted for examination with an Olympus BX50 microscope coupled to an Image Analysis System (Olympus).

Caspase activities

The activities of caspases 3 and 9 were determined by a colorimetric assay using caspase 3 and 9 activation kits (Invitrogen), according to the manufacturer's instructions. Briefly, AF samples were lysed in lysis buffer for 30 min on ice. The lysed cells were centrifuged at 16,000 x *g* for 10 min and 100 μg of protein was incubated with 50 μL of the colorimetric tetrapeptide Asp-Glu-Val-Asp (DEAD)-p-nitroaniline (pNA) (specific substrate of caspase 3) or Leu-Glu-His-Asp (LEHD)-pNA (specific substrate for caspase 9) at 37 °C in the dark for 2 h after which the plates were read at 405 nm in an ELISA reader (Model EXL800, BioTek, USA). The data were normalized to the caspase activities in control cells (treated with 0.5% DMSO vehicle) and expressed as the fold increase.

RNA extraction and RT-PCR analysis

Total RNA from AF samples was extracted with TRIzol reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. Oligo(dT)-primed RNA (1 μg) was reverse-transcribed with SuperScript II reverse transcriptase (Promega) according to the manufacturer's instructions. The resulting cDNA was used to determine the amount of ERK2 or Crk mRNA by PCR with *Taq* DNA polymerase (Fermentas). GAPDH was used as an internal control. The primers used for amplification were: ERK2 forward 5'-TCCAACCTGCTGCTCAACACCAC-3' and reverse 5'-CACTCGGGTCGTAATACTGCTCC-3'; CRK forward 5-ACTATGTGCTCAGCGTCTCA-3' and reverse 5'-ATTCCACCACTGCTCTTCA-3', and GAPDH forward 5'-GTCACCATGACAACTTTGG -3' and reverse 5'-GAGCTTGACAAAGTGGTCGT-3'.

Western blotting

Western blotting was used to examine the protein expression of Crk and ERK2. The AF samples were lysed in Golden lysis buffer supplemented with protease inhibitors (Boehringer Manheim), 1 mM sodium orthovanadate, 1 mM ethyleneglycol-bis-(β-amino ethyl ether), 1 mM sodium fluoride and 1 μM microcysteine (Sigma). Protein concentrations were quantified with a Coomassie Plus pro-

tein assay kit (Bio-Rad). 30 µg of protein exact were separated by SDS-PAGE and then transferred to a PVDF membrane (NEN Life Science Products Inc., Boston, MA). The blots were probed overnight at 4 °C with either rabbit anti-rat Crk or ERK2 (Abcam, Cambridge, MA). After washing, the membranes were incubated for 1 h at 20 °C with goat anti-rabbit IgG (Oncogen, Boston, MA). Immune complexes were detected using ECL (Amersham, Piscataway, NJ). Membranes were reprobed for GAPDH to confirm equal protein loading.

Results

TUNEL staining

The effect of EA treatment on apoptosis in AF cells was assessed by TUNEL staining. This procedure allows the detection and quantification of apoptosis at a cellular level based on the labeling of free 3-OH terminals created during double-strand and single-strand cleavage of genomic DNA. Figure 1A-D shows the TUNEL staining in the four experimental groups. Qualitatively, TUNEL staining was more intense in the cell nuclei of the control group than in the sham group. In the former group, the nuclei were denser, there was disruption of the nuclear membrane and apoptotic bodies were seen in the cytosol; cells in the EA group showed a relatively intact nuclear membrane. The number of TUNEL-positive cells in the control group was significantly higher than in the sham group, and the number of TUNEL-positive cells in the EA group was significantly lower than the control group but similar to the sham and MT groups (Figure 1E).

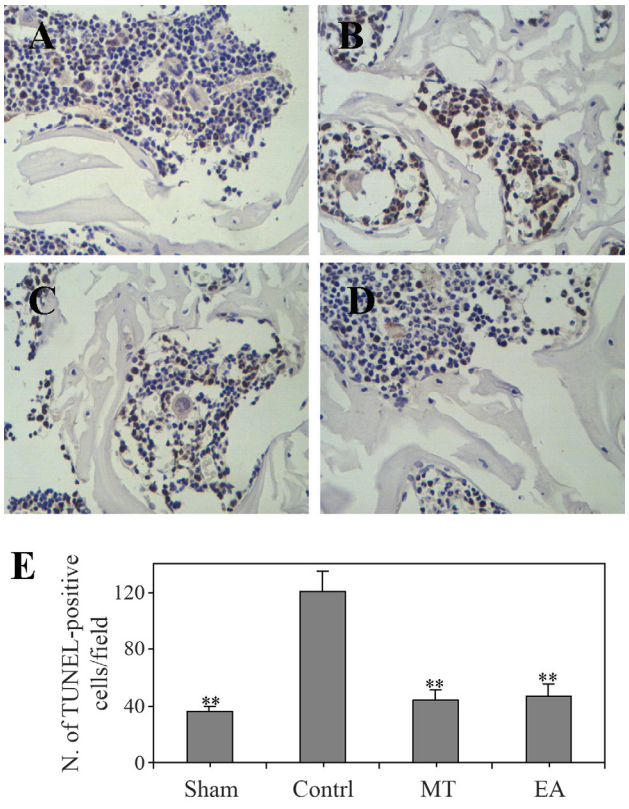

Figure 1 - Effect of EA on TUNEL staining of AF cells. (A) Sham group, (B) Control group, (C) MT group and (D) EA group. (E) Number of TUNEL-positive cells/field in each experimental group. Note the decrease in the number of TUNEL-positive cells in the EA group compared to the control group. The columns represent the mean ± SD (n = 8). **p < 0.01 compared to the control group. Magnification: x400.

Immunohistochemical staining for Bcl-2 and Bax

Bcl-2 family proteins are key regulators of mitochondria-mediated apoptosis and include anti-apoptotic members such as Bcl-2 and pro-apoptotic members such as Bax. Bcl-2 has been implicated in the inhibition of apoptosis. In the present study, Bcl-2-positive cells were detected mainly in the sham, EA and MT groups, although positively stained cells were also seen in the control group (Figure 2A-D). There was a significant increase in the number of Bcl-2-positive cells in the EA group compared to the control group (Figure 2E) and this could explain the decreased apoptosis seen in the former group (Figure 1E).

Unlike Bcl-2, which is anti-apoptotic, Bax induces apoptosis in several cell lines. Bax immunoreactivity was detected mainly in the control group (Figure 3B). Quantitative analysis revealed a significant difference in the number of Bax-positive cells in the control group compared to the other groups (Figure 3E).

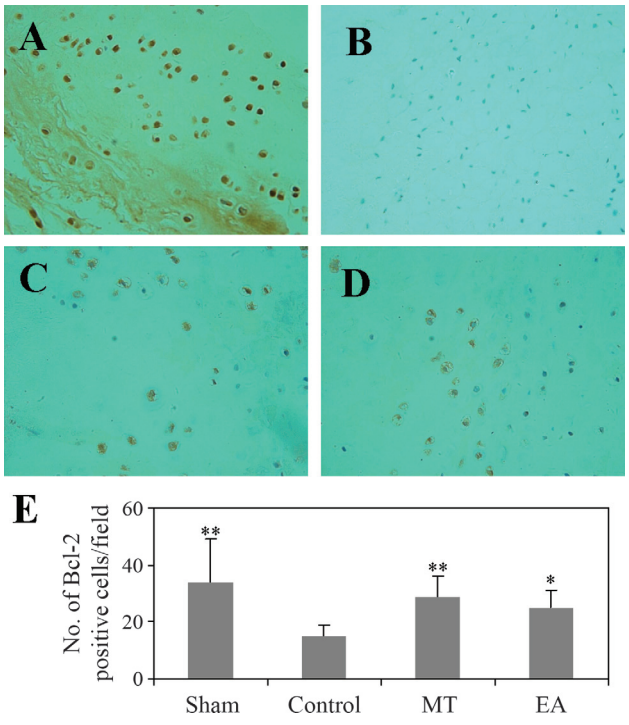

Figure 2 - Effect of EA on immunohistochemical staining for Bcl-2 in AF cells. (A) Sham group, (B) Control group, (C) MT group and (D) EA group. AF cells were stained with anti-Bcl-2 antibody. (E) Number of Bcl-2-positive cells/field in each experimental group. Note the increase in the number of Bcl-2-positive cells in the EA group compared to the control group. The columns represent the mean ± SD (n = 8). *p < 0.05 and **p < 0.01 compared to the control group. Magnification: x400.

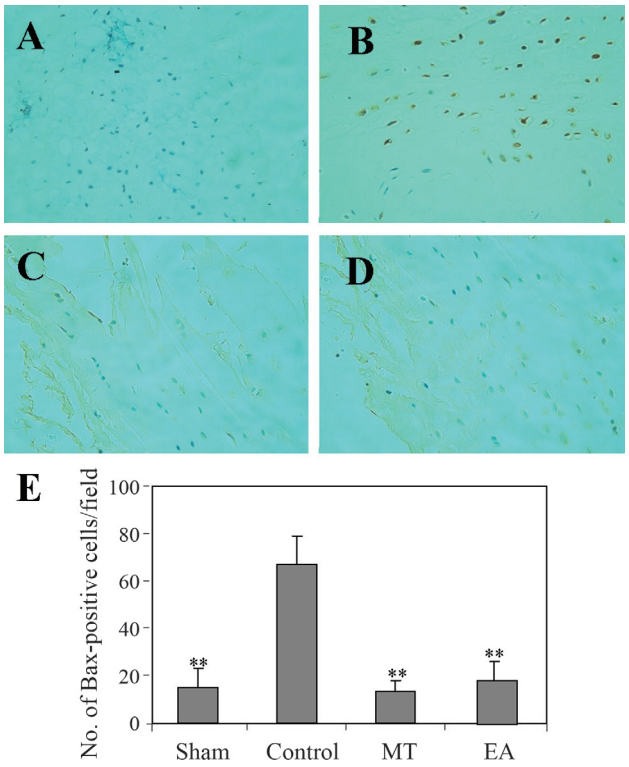

Figure 3 - Effect of EA on immunohistochemical staining for Bax in AF cells. (A) Sham group, (B) Control group, (C) MT group and (D) EA group. AF cells were stained with anti-Bax antibody. Note the decrease in the number of Bax-positive cells in the EA group compared to the control group. The columns represent the mean ± SD (n = 8). *p < 0.05 and **p < 0.01 compared to the control group. Magnification: x400.

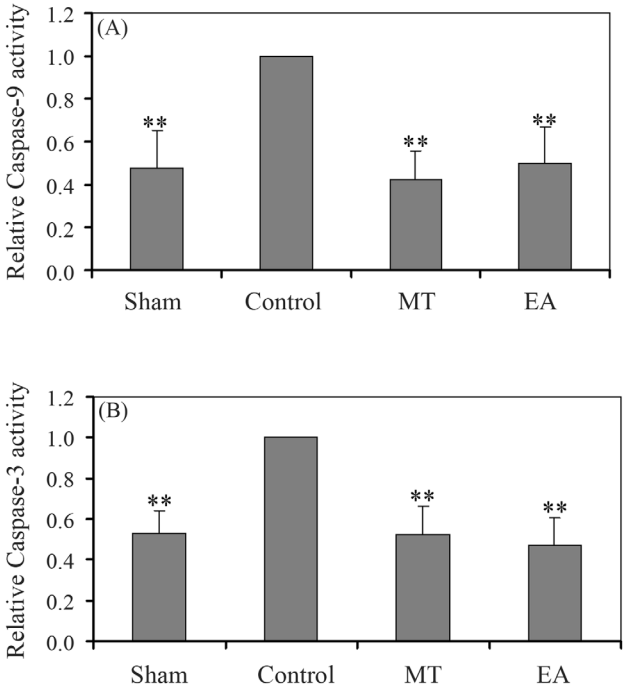

Figure 4 - Effect of EA on caspase activation in AF cells. Caspases-9 and -3 activities were determined by a colorimetric assay. The data were normalized relative to the caspase activities of control group cells. The columns represent the mean ± SD (n = 8). **p < 0.01 compared to the control group.

Assay for caspases 3 and 9

To identify the downstream effectors in the apoptotic signaling pathway, the activation of caspases 9 and 3 was examined by using specific chromophores, *i.e.*, DEVD-pNA (a specific substrate for caspase 3) and LEHD-pNA (a specific substrate for caspase 9). The mitochondria- dependent pathway is the most common apoptotic pathway in vertebrate cells. Permeabilization of the mitochondrial membrane, accompanied by the collapse of the electrochemical gradient across the mitochondrial membrane, is one of the key events during cellular apoptosis. This event leads to the release of numerous apoptogenic proteins, such as cytochrome c, from mitochondria, thereby triggering the activation of caspases 3 and 9 and eventually inducing apoptosis. As shown in Figure 4A,B, treatment with EA significantly inhibited the activation of caspases 3 and 9 in AF cells. These data suggest that EA inhibits apoptosis in AF cells, probably by blocking the mitochondria-dependent pathway.

EA modulates the expression of Crk and ERK2

To enhance our understanding of the anti-apoptotic activity of EA, we used RT-PCR and western blotting to examine the mRNA and protein expression of Crk and ERK2 in EA-treated AF cells. Figure 5A,C and D shows that Crk and ERK2 gene expression in the control group was lower than in the sham group and that EA treatment increased the mRNA expression of these two proteins; the pattern of Crk and ERK2 protein expression was similar to that of their respective mRNA levels (Figure 5B,E and F).

Discussion

Previous studies have shown that apoptosis in AF cells plays a key role in IVD degeneration. Degenerative changes in the IVD can lead to nerve root compression, resulting in radiculopathy (Hacker and Miller, 2003; Chen *et al.*, 2005). To investigate whether electroacupuncture protected AF cells from apoptosis caused by cervical IVD degeneration, we established a rat model of cervical intervertebral disc degradation induced by the application of unbalanced dynamic and static forces, as described in the literature (Wang *et al.*, 2006). TUNEL staining showed that EA treatment could reduce the signs of apoptosis after surgery.

Apoptosis is a complex process by which individual cells undergo self-destruction without inducing an inflammatory response. Caspases, which belong to a family of cysteine proteases, are the key proteins that modulate the apoptotic response. These enzymes occur in a latent form in the cytoplasm and are activated late in the apoptotic pro-

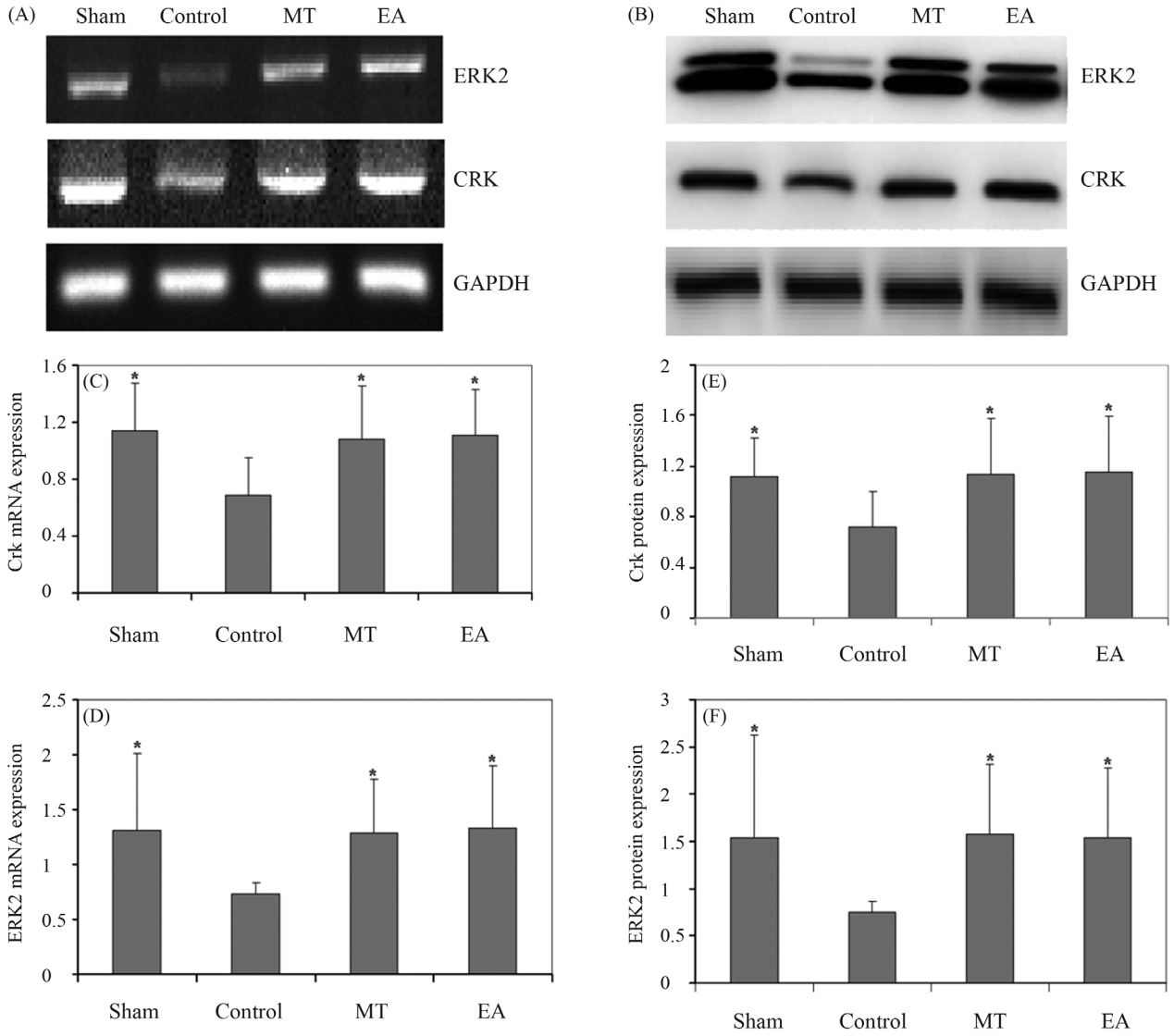

Figure 5 - Effect of EA on Crk and ERK2 expression in AF cells. (A), (C) and (D) Crk and ERK2 mRNA levels of in the four groups were determined by RT-PCR. (B), (E) and (F) Crk and ERK2 protein expression levels were analyzed by western blotting. GAPDH gene and protein expression were used as the internal controls for RT-PCR and western blotting, respectively. The gels and blots are representative of eight specimens. The columns represent the mean ± SD (n = 8). *$p < 0.05$ compared to the control group.

cess. Caspases are organized as a cascade with two major pathways for activation, namely, the mitochondria-mediated pathway and the fas-mediated pathway (Tsujimoto and Shimizu, 2000; Pedersen *et al.*, 2002). Caspase 3 is a key mediator of apoptosis that is activated by an initiator caspase such as caspase 9 during mitochondria-mediated apoptosis. As shown here, EA suppressed the activation of caspases 9 and 3 in AF cells and lead to a reduction in the activities of these two enzymes in these cells.

Mitochondria-dependent apoptosis is regulated mainly by Bcl-2 family proteins, a group of evolutionarily conserved pro- and anti-apoptotic proteins. Bcl-2 is an anti-apoptotic protein that forms channels that stabilize the mitochondrial membrane, thereby preventing the release of cytochrome c, a second mitochondria-derived activator of caspases. Bax is a pro-apoptotic protein that forms heterodimers with Bcl-2, thereby inactivating the latter. Mitochondrial outer membrane permeabilization (MOMP) is thought to involve the formation of pores in the mitochondrial membrane by pro-apoptotic Bax-like proteins; the action of these proteins can be inhibited by anti-apoptotic Bcl-2-like members (Reed, 2000; Cory and Adams, 2002). The ratio of Bax to Bcl-2 is therefore critical in determining the fate of cells. The results described here show that EA treatment increased the number of Bcl-2-positive cells and reduced the number of Bax-positive AF cells. This finding indicates that EA inhibits apoptosis by affecting the Bax/Bcl-2 ratio.

We examined whether changes in the expression of Crk and ERK2 were related to the suppression of apoptosis.

Integrins are adhesion receptors that transmit signals from the extracellular matrix to cells. Of the numerous signals emanating from integrin receptors, Crk-associated substrate and its recruitment of and binding to the adaptor protein Crk are critical events in controlling integrin-dependent processes (Schwartz *et al.*, 1995; Schlaepfer and Hunter, 1998). The amplification of Crk signaling in epithelial cells and fibroblasts suppresses apoptotic mechanisms and may itself be a transforming factor (Cho and Klemke, 2000; Iwahara *et al.*, 2003). Activation of members of the mitogen-activated protein (MAP) kinase family by Crk plays a key role in regulating chondrocyte gene expression.

The Ras-dependent extracellular signal-regulated kinase 1/2 (ERK1/2) pathway also plays a central role in controlling cell proliferation (Meloche and Pouyssegur, 2007). The ERK pathway promotes the transcription of cyclin D (Albanese *et al.*, 1995) and c-Myc, and induces proteasomal degradation of FOXO3a (Yang *et al.*, 2008) and $p21^{Cip1}$ (Hwang *et al.*, 2009) through direct phosphorylation, thereby resulting in cellular proliferation. As shown here for the first time, EA at DU 14 and LI 10 increased the gene and protein expression of Crk and ERK2 in cervical IVD degeneration; the pattern of protein expression agreed with that seen for mRNA. These data indicate that Crk and ERK2 signaling may be associated with AF cell apoptosis during cervical IVD degeneration. However, it is unclear whether these molecules are involved in the signaling cascade of programmed cell death or whether their enhanced expression is simply a non-specific response to apoptosis. Further studies are needed to elucidate the specific role of these molecules in the apoptotic pathway (Nurminskaya *et al.*, 1998; Koike *et al.*, 2003).

Recent work has suggested that EA stimulation can suppress apoptosis induced by surgical trauma stress, possibly by modulating Fas protein expression (Wang *et al.*, 2005), and improve ulcerative colitis in rats, perhaps by promoting neutrophil apoptosis and down-regulating cytokine production by monocytes (Wu *et al.*, 2007). Some studies have also shown that pretreatment with EA can significantly attenuate neuronal apoptosis, preserve neuronal morphology and inhibit caspase 3 activity in the hippocampal CA1 region after exposure to +Gz (Wang *et al.*, 2010). EA may also stimulate endogenous ξ PKC-mediated anti-apoptosis pathways to protect against ischemic damage after focal cerebral ischemia caused by the activation of cannabinoid receptor type 1 (Wang *et al.*, 2011).

In conclusion, our data demonstrate that EA treatment at DU 14 and LI 10 inhibits AF cell apoptosis via the mitochondria-dependent pathway and up-regulates Crk and ERK2. These results suggest that EA may be a good alternative therapy for preventing cervical spondylosis.

Acknowledgments

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