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Original Article

Effects of Warming Acupuncture on Ligament Recovery in Injury-induced Rats

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Object : Warming acupuncture (WA) has been used in Oriental Medicine for the treatment of physical disabilities caused by ligament damage. Here, the effects of WA on injured ligament tissues were investigated using the rat model.

Methods : The rats were induced injury on the right hind ankle, and 4 weeks later, WA was given onto the acupoint GB40 (Quixu) of the injury area on a weekly basis for 6 weeks. Main outcome was measured by levels of Erk1/2, Hoechst nuclear staining and collagen staining in the ligament tissue.

Results : Levels of active form of Erk1/2 kinase were increased in the injured ligament with WA compared with the control ligament induced injury only, and this change correlated with cell number increases in the ligament by WA. Type III, but not type I, collagen mRNA and protein levels were elevated in the injured ligament treated with WA. Moreover, histological staining showed increased re-organization of collagen fibers in the ligament by WA.

Conclusions : The present data suggest that WA performance to the injured ligament may facilitate the healing process via increasing cellular activity.

Key Words : warming acupuncture (WA), injury-induced, ankle sprain, ligament healing, Erk1/2, collagen

Introduction

Ankle sprain is one of the most frequently occurring sprain injuries, caused by inversion or eversion wrench of the joints. In general, patients can recover through clinical treatments, but when the treatments are not performed properly at the early stage, the injury can develop into chronic, relapsing sprain, rendering treatment more difficult[1,2)]. Ligament, along with tendons and aponeuroses, belongs to dense regular connective tissue, and joins bone to bone. When the ligament tissue is physically damaged due to sprain, infections, and other causes, it may undergo either healing or degeneration[3,4)]. The healing process involves pathological stages of inflammation, regeneration, and remodeling. In the inflammation stage, infiltration of leukocytes and macrophages as well as increased production of inflammatory cyt- okines are well observed[5)]. After the ligament injury, growth factors IGF-1, TGF-β , PDGF, bFGF, IL-6, TNF-α , and IL-1β cytokines may be important for the healing process[3,6,7)]. Tissue inhibitors of metalloproteinases (TIMP) 1, 2, 3 isoform mRNA were also up- regulated, suggesting their involvement in remodeling[8)].

Warming acupuncture (WA) refers to a combined therapy of acupuncture and moxibustion. In Oriental Medicine, WA has long been used for the treatment of musculoskeletal diseases such as ligament sprain, traumatic inflammation, cervical spondylopathy, and lumbago[9,10)]. Therapeutic effects of acupuncture are assumed to be enhanced by burning moxibustion which conducts heat through the needle. It is further believed that heat conduction could facilitate the flow of blood circulation and thus help the healing pr-

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ocess[11]. However, the biological basis for the healing effects of WA remains to be investigated.

Here, we investigated cellular responses in the injured ligament tissues by WA. After inducing ankle sprain injury in the rats, WA therapy was given and biochemical and histological changes were investigated in the ligament tissue. Our data demonstrate that WA therapy activates Erk1/2 kinase and induces increased cell proliferation and production of type III collagen protein in the injured ligament.

Materials and Methods

1. Experimental animals and ankle sprain injury

A total of 54 Sprague-Dawley rats (male, 8 weeks old) were used in this experiment. Animals were divided into experimental group (injury plus WA), injury-only group, prolotherapy group as a positive control, and no injury group. The rats were anesthetized with a mixture of ketamine (80mg/kg) and xylazine (5mg/kg). Ankle sprain injury was performed as described previously12) with some modifications. The right hind foot was repeatedly bent in the direction of simultaneous inversion and plantar flexion 60 times for 1 min with gradually increasing force. During the next 1 min period, the same injury paradigm was repeated. The rats were recovered from anesthesia and returned to the cage until further treatment. Authorization of animal experiments was obtained by Daejeon University (Daejeon, Korea).

2. WA and prolotherapy

For WA, a stainless steel acupuncture needle (Zeus Korea Acupuncture Develop Co., Korea, 30 mm length×0.30 mm diameter) was used. The handle portion of the acupuncture needle was fitted with moxibustion in a cylindrical shape (18 mm diameter ×25 mm height weighing 2.0 g) and separated from the surface of the skin by 2.6 ㎝. WA therapy was initiated 4 weeks after ankle sprain injury and continued for the next 6 weeks, once every week. The sharp tip of the needle was inserted into the injured area of the right hind lateral ankle, GB40 (Quixu) at 4 mm depth[13] and held stable during treatment. The needle was warmed by burning the attached moxibustion for 15 min. A sheet of cotton patch was covered over the rat body to protect the skin from heat conducted from the burning moxibustion. For prolotherapy control experiment, 0.1 ㎖ of 12.5% glucose solution was slowly injected using 1 ㎖ syringe into the same point.

3. Immunofluorescence staining, Masson's trichrome staining and Hoechst nuclear staining

Animals were anesthetized and the anterior talofibular ligament and peroneus brevis tendon tissues attached to the bones around the ankle were carefully dissected. Dissected tissues were embedded into the OCT medium, and sections (20 ㎛) were cut using a cryostat and mounted on positively charged slides (Superfrost, Fisher, USA). For immunofluorescence staining, anti-collagen type I or III antibody (Santa Cruz Biotech, USA), anti-Erk1/2 antibody (Santa Cruz Biotech, USA), and anti phospho-Erk1/2 antibody (Santa Cruz Biotech, USA), and fluorescein-goat anti-mouse (Molecular probes, USA) or rhodamine-goat anti-rabbit secondary antibodies (Molecular probes) were used. Immunofluorescence staining was performed essentially as described previously[14]. Before the final washing step following secondary antibody reaction, sections were stained with Hoechst 33258 dye (25 ㎍/㎖in 0.1% triton X-100 in phosphate-buffered saline solution for 10 min) when necessary. All the reagents for Masson's trichrome staining were purchased from Dako Cytomation (Carpinteria, USA) and staining experiment was carried out as recommended by the manufacturer.

4. Western blot analysis

Ligament tissue was sonicated under 50 - 200 ㎖ of triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 25 mM β -glycerophosphate, pH 7.14, 2 mM sodium pyrophosphate, 2 mM EDTA, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 5 ㎍/㎖ leupeptin, 5 ㎍/㎖ aprotinin, 3 mM benzamidine,

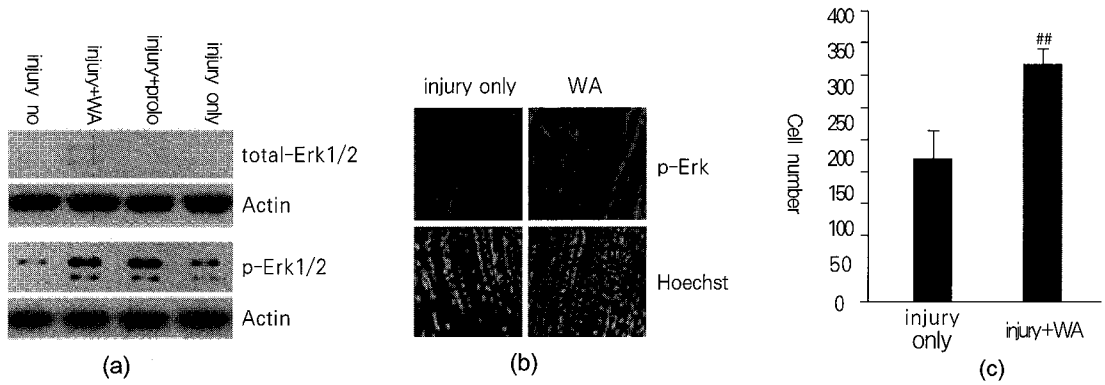

Fig. 1. Expression of Erk1/2 kinase levels and nuclei in the ligament tissue.
(a) Western blot analysis of total Erk1/2 and phospho-Erk1/2 proteins in the ligament tissues after different treatments. Western blot analysis for actin protein was performed as an internal loading control. prolo: prolotherapy.
(b) Immunofluorescence staining of phospho-Erk1/2 protein signal in the ligament tissue. Cell nuclei were visualized by Hoechst staining (blue) in the same sections. Scale bar = 50 ㎛.
(c) Individual nuclei were counted in the defined image field of the ligament section (200 x 250 ㎛) and the mean number of nuclei was compared in the ligaments between the injury-only and injury with WA treatment groups.
n (number of animals) = 6, error bars: SD. **p <0.01 (Student's t-test).

0.5 mM DTT, 1 mM PMSF). Protein (10 ㎍) was resolved in 12% SDS polyacrylamide gel and blot transfer and detection of autoradiographic images were performed[14]. The antibodies used in the present study were anti-actin (ICN Biomedicals, 1:5000 dilution), anti-Erk1/2 and anti-phospho-Erk antibody (Santa Cruz Biotech, 1:5000 dilution).

5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Extraction of total RNA, RT and PCR reactions were performed as described previously[14]. The nucleotide sequences of the PCR primers of rat collagen type I are 5'tatgacttcagcttcctgcc3' for sense primer and 5'gtctgttggtccatgtaggc3' for antisense primer, and for the rat collagen type III are 5'gcaatatgtccacagccttc3' for sense primer and 5'ggtttcacccttctctccat3' for antisense primer. Seq- uences for actin were 5'cacactgtgcccatctatga3' as sense primer and 5'tacggatgtcaacgtcacac3' as anti-sense primer.

Results

1. Effects of warming acupuncture on Erk 1/2 kinase levels

Total Erk1/2 protein levels were almost undetectable in ligament tissues with different treatments including WA (Fig. 1A). However, phospho-Erk1/2, an active form of Erk1/2, was increased in the ligament with WA, and its level was similar to that in the prolotherapy control group. Immunofluorescence staining of ligament tissues using anti-phospho Erk1/2 antibody showed that protein signal intensity in the ligament with WA was higher than that in control group. Hoechst staining of the same set of sections for nuclear visualization revealed significant increases in the number of nuclei in the WA group compared with the injury-only control group (Fig. 1 B, C), suggesting that WA therapy facilitates cell proliferation in the ligament tissues.

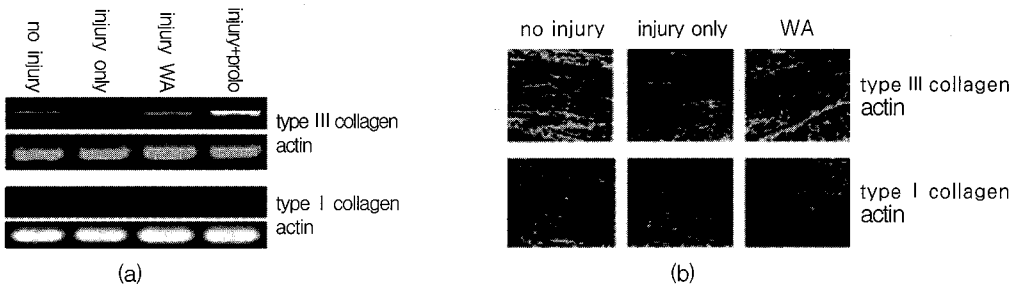

Fig. 2. Expression of collagen type I and type III in the ligament tissue.
(a) RT-PCR analysis of collagen type I and type III mRNA in the ligament (593 bp and 600 bp respectively). RT-PCR of actin mRNA was performed as an internal loading control (409 bp).
(b) Immunofluorescence staining of type I and type III collagen proteins in the ligament tissue. Collagen type I and III proteins were seen as fibrous structure (green) in the ligament. Scale bar = 50 ㎛.

2. Changes in collagen protein expression in the ligament

To investigate regulation of collagen expression in the ligament tissue, type I and type III collagen mRNA were quantitatively analyzed by RT-PCR. Collagen type III mRNA which was increased in the WA group more than in the intact group but was not detected in the injured-only group. Similar increase was observed in the ligament of prolotherapy, suggesting correlative regulation of mRNA expression associated with ligament healing between WA and prolotherapy. Collagen type I mRNA was not detected in the ligaments with any treatments (Fig. 2A). We further examined distribution of collagen type I and III proteins in the injured ligament. As shown in Fig. 2B, collagen type III protein staining was increased in WA ligament compared with the injury-only control group, showing intense fibrous staining structures similar to intact ligament. Collagen type I staining intensity was much lower than type III in all treatments, and did not show any clear difference among different treatments.

3. Masson's trichrome staining of the ligament tissue

To determine changes of collagen fiber structures by WA, ligament tissue was treated with Masson's trichrome reagents that stain collagen fibers with blue color above nuclear and cytoplasmic staining with black and red respectively. As shown in Fig. 3, collagen fibers were clearly seen with blue color. Injury-only group ligament tissue was much weaker than intact in staining intensity of collagen fibers. WA treatment increased fiber staining compared with injury control, suggesting that WA may confer positive effects on the collagen fiber recovery.

Discussion

In the present study, the effects of WA on injured ligament tissues were investigated in rats. Our data showed increased phosphorylation of Erk1/2 kinase and cell proliferation in the injured ligament tissues by WA. Erk1/2 activation along with PI3 kinase/Akt pathway can increase cell proliferation by inactivating pro-apoptotic molecules such as Bad and caspases and activating nuclear transcription factors such as CREB and microtubule associated protein-2 (MAP-2)[15-17]. Thus, increases in active form of Erk1/2 protein by phosphorylation in the injured ligament given WA might be related to increased proliferation of fibroblast cells in the ligament, as has been shown in the periodontal ligament[18].

Another interesting find of the present study was increased synthesis of collagen type III

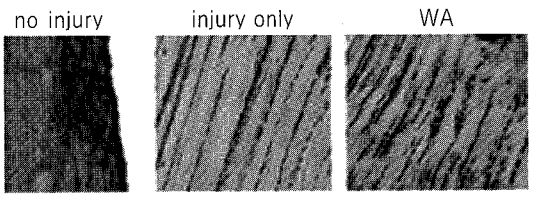

Fig. 3. Masson's trichrome staining of the ligament tissue.
Collagen fibers were stained in blue, fibroblast cell nuclei were stained in black and the cytoplasmic structure in red. Scale bar = 50 ㎛.

protein in WA-stimulated ligament. More than 10 different types of collagen protein were identified from different tissues. Collagen proteins present in the extracellular matrix and connective tissues such as ligament and tendon are synthesized from the fibroblast cells. Three different protein subunits, once synthesized within the cells, are modified for the generation of hydroxyproline before molecular assembly, secreted out of the cells, and form a fibril structure of collagen throughout supramolecular assembly process. Previous studies suggested that different collagen types were found in the ligament tissue before and after the ligament injury; RT-PCR analysis reported proportional increases in both type I and type III collagen mRNA in rabbit medial collateral ligament[4] whereas higher expression of type III collagen than type I collagen protein was shown in human periodontal ligament tissues undergoing healing process[19]. In another study, type I collagen expression was elevated in rabbit medial collateral ligament administered with TGF-β [20]. Variation in type I and type III collagen expression was further reported in cultured ligament fibroblasts with different treatments[21]. In our first report on type I and type III collagen in the rats ankle ligament, collagen type III mRNA was increased in the ligament by WA compared with the injury control group, suggesting selective induction of collagen type III mRNA expression by WA. Similar increases in collagen III mRNA were observed in the tissues with prolotherapy, further suggesting positive correlation of increased collagen III protein with ligament healing. Immunofluorescence staining demonstrated increased expression of collagen type III by WA, supporting RT-PCR results. Yet, type I collagen mRNA was not detected, and immunofluorescence staining also showed no clear collagen type I protein signals in the tissue. Finally, Masson's trichrome staining revealed increased collagen fiber staining by WA. Together, these results suggests that type III collagen may be produced from the fibroblast and constitute the major component in the fiber structures of the regenerating ligament.

In the injured ligament, up-regulation of phospho-Erk1/2 and type III collagen was similarly observed in the ligament tissues with prolotherapy. Prolotherapy has been used for promoting the healing process of injured tissues including ligament. Dextrose or a mixture with other reagents such as glycerin, phenol and lidocaine is usually applied to the injured tissues[22]. Although the reports on the recoveries are still limited and the effects are variable among studies[23], this therapy has been reported to be clinically effective[24]. Since there have been no reports examining prolotherapy in experimental animals, no comparative interpretation with the present data is possible. Nonetheless, prolotherapy was effective for ligament healing process in our experimental system.

In summary, our data demonstrate that WA, as a combined therapeutics of acupuncture and moxibustion, activated a signaling pathway to induce cell proliferation and increased collagen synthesis in the injured ligament. With this experimental approach, identification of related molecular factors may be possible and further provide an insight into understanding the acupuncture mechanism at biological level.

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