Acupuncture at distant myofascial trigger spots enhances endogenous opioids in rabbits: a possible mechanism for managing myofascial pain

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ABSTRACT

Background and aim Acupuncture applied at myofascial trigger points (MTrPs) of distant anatomical regions, to reduce pain in a patient's area of primary complaint, is one strategy that is available to manage myofascial pain. However, the endogenous opioid-mediated analgesic mechanism of distant acupuncture associated with pain control is still unclear. This aims of this study were to evaluate the changes in enkephalin and β-endorphin in serum, spinal cord, dorsal root ganglion (DRG) and muscle induced by acupuncture at distant myofascial trigger spots (MTrSs, similar to human MTrPs) in rabbits, to explore its underlying remote analgesic mechanism.

Methods Acupuncture at MTrSs of a distant muscle (gastrocnemius) was performed either for one session or five daily sessions in rabbits. The levels of enkephalin and β-endorphin in proximal muscle (biceps femoris), serum, DRGs and spinal cords (L5-S2) were then determined by immunoassay immediately and 5 days after treatment.

Results Immediately after treatment, acupuncture comprising both one dose and five doses significantly enhanced spinal enkephalin expression and serum β-endorphin levels ($p<0.05$). However, only five-dose acupuncture significantly enhanced the β-endorphin levels in the biceps femoris and DRGs ($p<0.05$), while 1-dose acupuncture did not ($p>0.05$). Furthermore, 5 days after treatment, significantly increased levels of spinal enkephalin and serum β-endorphin persisted in animals that received 5-dose acupuncture ($p<0.05$).

Conclusions This study demonstrates that interactions within the endogenous opioid system may be involved in the remote effects of acupuncture treatment and could be a potential analgesic mechanism underlying MTrP pain management.

INTRODUCTION

Acupuncture targeting the primary myofascial trigger point (MTrP) is effective at alleviating myofascial pain.[1–4] However, repetitive and intensive manipulation with acupuncture needles may cause excess damage and increase inflammatory nociception in skeletal muscle fibres.[5] Many recent studies of the myofascial pain syndrome have provided clear evidence that acupuncture at distant MTrPs is effective for pain control. This approach is analogous to the traditional acupuncture method of treating distant acupuncture points to influence the primary sites of pain.[3 6–8]

It is reasonably well established that endogenous opioids play an important role in acupuncture-induced analgesia.[9–12] However, the precise endogenous opioid systems responsible for the remote analgesic effects of acupuncture targeting MTrPs are currently unknown.

The endogenous opioid peptides enkephalin and β-endorphin are well known for their anti-nociceptive actions in the body.[10] Enkephalin is predominantly found on primary afferent terminals in the spinal cord, particularly those of neurons located in the superficial dorsal horn that are activated by noxious peripheral stimuli.[2] β-endorphin is abundant in the neurons of both the central and

peripheral nervous systems, as well as in peripheral tissues.[13] The levels of endogenous opioids may reflect their function in the modulation of pain.[14]

An animal model for MTrP study in rabbits was established by Hong and Torigoe in 1994.[15] In their original study they identified a specific hyperirritable spot (myofascial trigger spot, or MTrS) in the rabbit biceps femoris muscle that is similar to a human MTrP. In the MTrS, local twitch responses can be elicited when the needle tip touches a sensitive locus. As in human MTrPs, spontaneous electrical activity, including endplate noise (EPN) and spikes, are frequently recorded within this sensitive spot.[16 17] This animal model has been used in many previous experimental studies of myofascial pain syndrome.[16 18–22]

To explore the mechanisms of remote acupuncture analgesia, we designed a study to investigate potential changes in endogenous opioids in serum, spinal dorsal horn, dorsal root ganglion (DRG) and proximal muscle (biceps femoris) induced by acupuncture at MTrSs of a distant muscle (gastrocnemius) in rabbits.

METHODS

General design

All animal experiments followed the procedures approved by the Animal Care and Use Committee of China Medical University (reference no. 99-4) in accordance with the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals'. This study also followed two out of the three central principles of the '3Rs' of animal research, namely reduction of the number of animals used (by improved experimental techniques and data analysis) and refinement (improved medical care and living conditions to minimise suffering).

A total of 60 healthy rabbits were randomly and equally divided into four groups (n=15 each, figure 1) allocated to receive either 1 day or 5 days of verum acupuncture (1D and 5D groups, respectively) or sham acupuncture (s1D and s5D groups, respectively). Randomisation was performed using a table generated online (http://www.randomization.com). Assuming moderate variability in the levels of opioid peptides, it was estimated that 7–8 animals per group would be required to detect an effect size of 0.5 at 80–90% power and an α level of 0.05.

In each group, randomly selected animals were sacrificed at one of two different time points for immunoanalysis of opioid peptides. Seven animals from each group were sacrificed by intraperitoneal injection of 100 mg/kg pentobarbital sodium (P3761, Sigma-Aldrich, Missouri, USA) 2 h after acupuncture was performed (day 1 for 1D/s1D groups; day 5 for 5D/s5D groups) and eight in each group were sacrificed 5 days after cessation of acupuncture (day 6 for 1D/s1D groups; day 11 for 5D/s5D groups). Spinal dorsal horn, DRGs and biceps femoris tissues were collected and frozen for the subsequent measurement of enkephalin and β-endorphin protein levels. In addition, serum was sampled for the measurement of circulating β-endorphin levels in five randomly selected animals from each group at three time points: before, immediately after and 5 days after acupuncture, respectively.

Animal care

The 60 adult male New Zealand rabbits (16–20 weeks of age, weighing 2.5–3.0 kg) were housed individually in standard polycarbonate tub cages lined with wood chip bedding in an air-conditioned room (25±1°C) with no more than 40 decibels (dBA) of sound and a 12-h alternating light-dark cycle (06:00–18:00). Food and water were provided *ad libitum*. Each animal was housed and cared for according to the ethical guidelines of the International Association for Study of Pain in animals.[23 24] The general experimental conditions were essentially the same as those previously described.[5 21 25]

Identification of the taut band region containing MTrSs

We used a combination of manual palpation and electrophysiological testing to identify MTrSs in rabbits, as per our previous studies.[7 19 21 26 27] Briefly, before general anaesthesia was provided, the most sensitive spots of the biceps femoris and gastrocnemius muscles were identified by finger pinch. The animal's reactions (such as withdrawal of the lower limb, turning of the head and vocalisation) were observed to confirm the exact location of an MTrS. Then, these painful regions were marked on the animal's skin with an indelible marker. Next, anaesthesia was induced with 2% and maintained with 0.5% isoflurane (AErrane, Baxter Healthcare, Puerto Rico, USA). The muscle at each marked site was grasped and gently rubbed between the fingers to locate a taut band. A monopolar fine needle electrode (37 mm disposable Teflon-coated model), connected to an electromyography (EMG) unit (Ivanovo, Russia), was inserted into this taut band to aid identification of MTrSs by detection of EPN. When the needle approached a MTrS locus, continuous electrical activity with amplitudes of 10–50 μV was recorded (data not shown)[7 21] and EPN-confirmed taut bands were designated for acupuncture and immunolabeling studies. All procedures were performed by an experienced clinician (CZH), who was blind to the group assignment and not involved in acupuncture or biochemical assessments.

Acupuncture at MTrSs

An MTrS of the gastrocnemius muscle on a randomly selected side was treated with predetermined doses of manual acupuncture (MA). A sterile single-use stainless steel acupuncture needle (300 μm diameter, 1.5 inches length, Yu-Kuang Industrial Co., Ltd., Taiwan) was first inserted through the skin perpendicularly at the centre of the marked spot, and then advanced

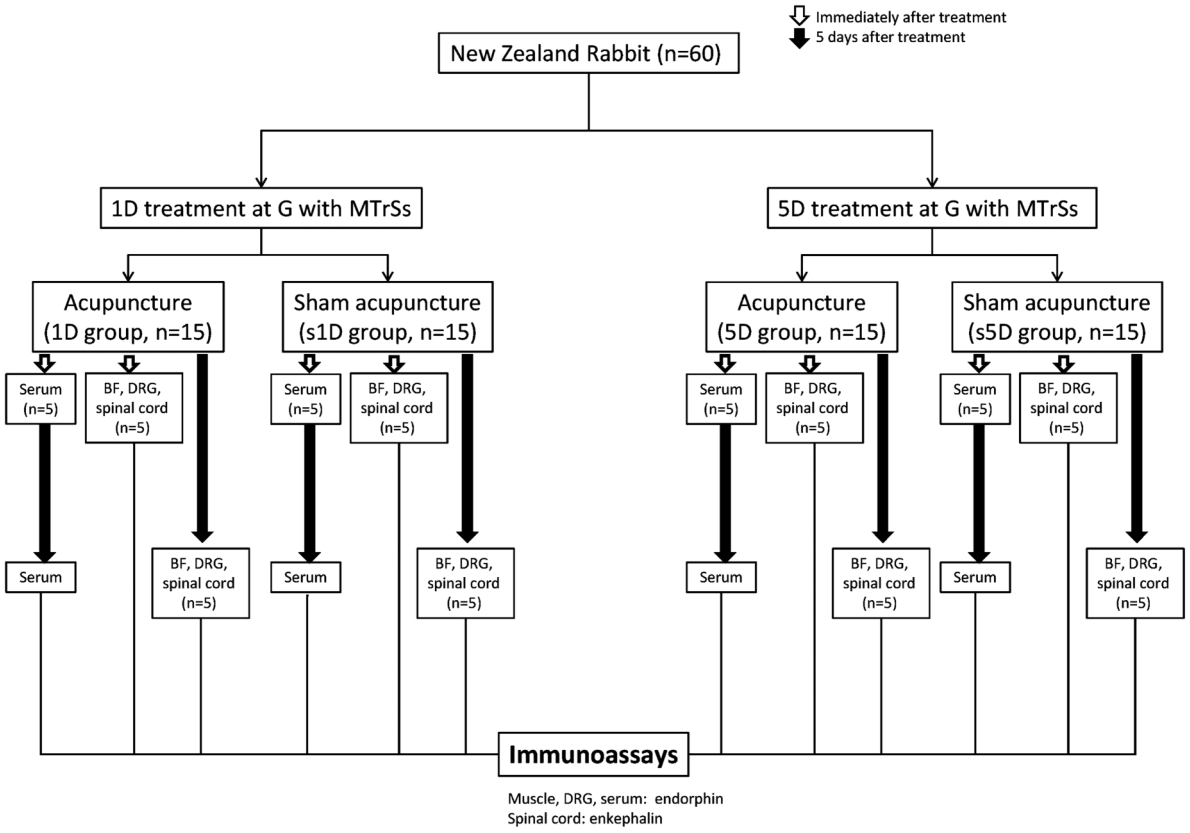

Figure 1 Flow chart illustrating the experimental design. Sixty rabbits were sacrificed immediately after or 5 days following one or five daily sessions of verum acupuncture (1D and 5D groups, respectively, n=15 each) or sham acupuncture (s1D and s5D, respectively, n=15 each). Abbreviations: BF, biceps femoris; DRG, dorsal root ganglion; G,gastrocnemius; MTrS, myofascial trigger spot.

slowly and gently into the muscle with simultaneous needle rotation for 30 s. In the sham acupuncture groups, the needle was inserted into the subcutaneous layer of the marked region to a depth approximately 1–2 mm from the skin surface without penetrating the muscle tissues, and left in place for 30 s.

ELISA for serum β-endorphin

Blood was collected from the marginal veins of the earlobe in a subgroup of five animals at each of the following three time points: (1) baseline; (2) immediately after acupuncture (day 1 or 5 for 1D/s1D and 5D/s5D groups, respectively); and (3) 5 days after completion of treatment (day 6 or 11 for 1D/s1D and 5D/s5D groups, respectively). Serum was separated by centrifugation (15800 g for 80 s) and 1 mL from each animal was stored in Eppendorf tubes at −80°C pending ELISA. The levels of β-endorphin in serum were measured using ELISA kits (E0806Rb, EIAab Science Co., Ltd, Wuhan, China) according to the manufacturer's instructions. Concentrations of β-endorphin were assessed with a reader (Thermo Scientific MultiskanEX, Vantaa, Finland) using a 450-nm filter. Data were then analysed using Ascent Software (Thermo Scientific Ascent Software, Finland) and a four-parameter logistics curve-fit.

Western blot analysis for β-endorphin

Animals were sacrificed by intraperitoneal injection of 100 mg/kg pentobarbital sodium 2 h after the completion of treatments (1 or 5 days of verum or sham acupuncture). Tissue was removed from the taut band of the bilateral biceps femoris and DRGs. Protein was extracted by adding T-PER tissue protein extraction reagent (78510, Thermo Scientific, Illinois, USA) and centrifuging at 10 000 g for 5 min. Equal amounts of protein were loaded onto 10% Tris-Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp, Bedford, Massachusetts, USA) and immunoblotted overnight with primary antibodies against β-endorphin (ab8907, Abcam Inc, Cambridge, UK) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ab8245, Abcam Inc, Massachusetts, USA), followed by horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pennsylvania, USA). The immunoblots were visualised using an enhanced chemiluminescence detection system (Fujifilm LAS-3000 Imager, Tokyo, Japan) and analysed using computer-based densitometry Gel-Pro Analyser V.6.0 (Media Cybernetics, Inc, Rockville, Maryland, USA).

The relative intensity of the band for each protein was normalised to the level of GAPDH protein and presented as a percentage.

Immunohistochemistry for enkephalin and quantitative analysis

Frozen spinal cord tissues from L5–S2 were cut serially and coronally into 4-µm-thick sections with a freezing microtome. Immunohistochemical analyses was performed on 10 alternate sections per rabbit, selected using a systematic-random series with a random start.[5 7] Briefly, the sections were incubated overnight at 4°C with mouse monoclonal anti-enkephalin antibody (1:200, MAB350, Millipore, Temecula, California, USA) followed by biotinylated anti-mouse IgG (115-005-003, Jackson Immunoresearch) and 3,3′-diaminobenzidine (Pierce DAB Substrate Kit, Pierce Chemical, Rockford, Illinois, USA).

Three randomly selected fields of the superficial dorsal horn were examined and photographed using a light microscope (BX43, Olympus America Inc, New York, USA) and a cooled digital colour camera with a resolution of 1360×1024 pixels (DP70, Olympus America Inc). The digital images were analysed using computer-based morphometry (Image-Pro Plus 4.5 software, Media Cybernetics, Silver Spring, Maryland, USA). The number of pixels with strongly positive enkephalin immunoreactivity staining was expressed as a percentage of all stained pixels in the superficial lamina of the spinal dorsal horn and analysed. All biochemical assessments were performed by two research assistants in a blinded manner.

Statistical analysis

Data were expressed as mean±SD. The various outcome parameters were assessed by two-way analysis of variance (ANOVA) with duration of acupuncture and time of sample collection as between- and within-subject factors. Post-hoc comparisons between groups were examined using Scheffe's method. A p value of <0.05 was considered statistically significant. All data were analysed using IBM SPSS V.20.0 for Windows (IBM Corp, Armonk, New York, USA).

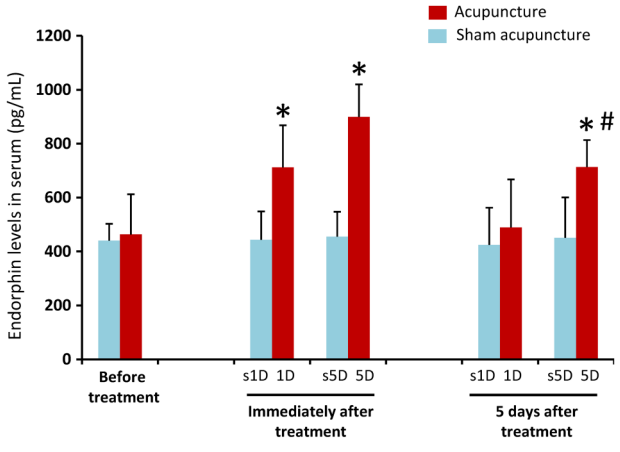

Figure 2 β-endorphin levels, quantified by ELISA, in serum sampled from a subgroup of rabbits before, immediately after and five following one or five daily sessions of verum acupuncture (1D and 5D groups, respectively, n=5 each) or sham acupuncture (s1D and s5D, respectively, n=5 each). Data are mean±SD. *p<0.05 vs sham groups (s1D and s5D). #p<0.05 vs 1D group.

RESULTS

Protein levels of β-endorphin in serum, DRG and muscle

The serum β-endorphin levels, determined by ELISA, before and after treatment for all groups are shown in figure 2. There were no significant differences in levels of serum β-endorphin before animals received either verum or sham acupuncture (p=0.456). However, serum β-endorphin levels in the 1D and 5D groups increased significantly immediately after acupuncture when compared with baseline values (p<0.01). Furthermore, serum β-endorphin levels in

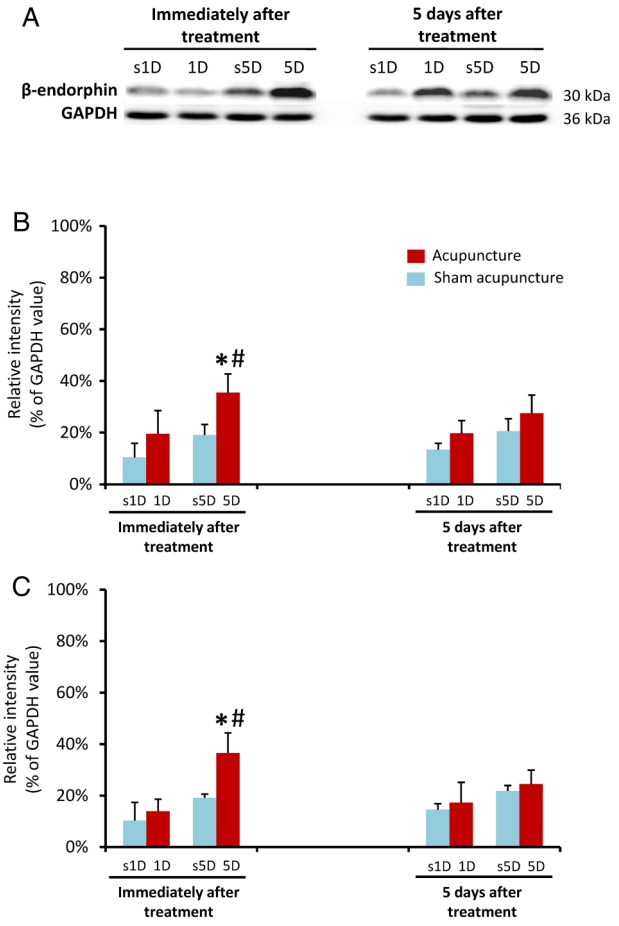

Figure 3 Representative Western blot photographs (A) and relative β-endorphin expression in the ipsilateral (B) and contralateral (C) dorsal root ganglia (DRGs) sampled from 60 rabbits immediately after and 5 days following one or five daily sessions of verum acupuncture (1D and 5D groups, respectively, n=15 each) or sham acupuncture (s1D and s5D, respectively, n=15 each). Data are mean±SD. *p<0.05 vs sham groups (s1D and s5D). #p<0.05 vs 1D group.

both the verum acupuncture (1D and 5D) groups were significantly increased compared to their respective sham acupuncture (s1D and s5D) groups immediately following treatment on day 1 or 5, respectively (both p<0.01). Five days after treatment, on day 11, compared with the s5D group, serum β-endorphin levels in the 5D group were still markedly increased (p<0.01). By contrast, there was no significant difference in β-endorphin levels between the 1D and s1D groups 5 days following treatment on day 6 (p>0.05).

The protein expression of β-endorphin in the DRG and biceps femoris muscle after treatment for all groups are shown in figures 3 and 4, respectively. Immediately after treatment, protein levels of β-endorphin in the bilateral DRGs and biceps femoris muscles were markedly higher in animals of the 5D group compared to those in their respective sham (s5D) group (p<0.001) and also the 1D group that had only received one day of acupuncture treatment (p<0.001). There were no significant differences between the 1D and s1D groups in either tissue on either side (p>0.05). Five days after treatment, there were no differences between the four groups in levels of β-endorphin in the bilateral DRGs (overall ANOVA p=0.079 for the ipsilateral side; p=0.069 for the contralateral side) or the biceps femoris muscles (p=0.289 for the ipsilateral side and p=0.287 for the contralateral side).

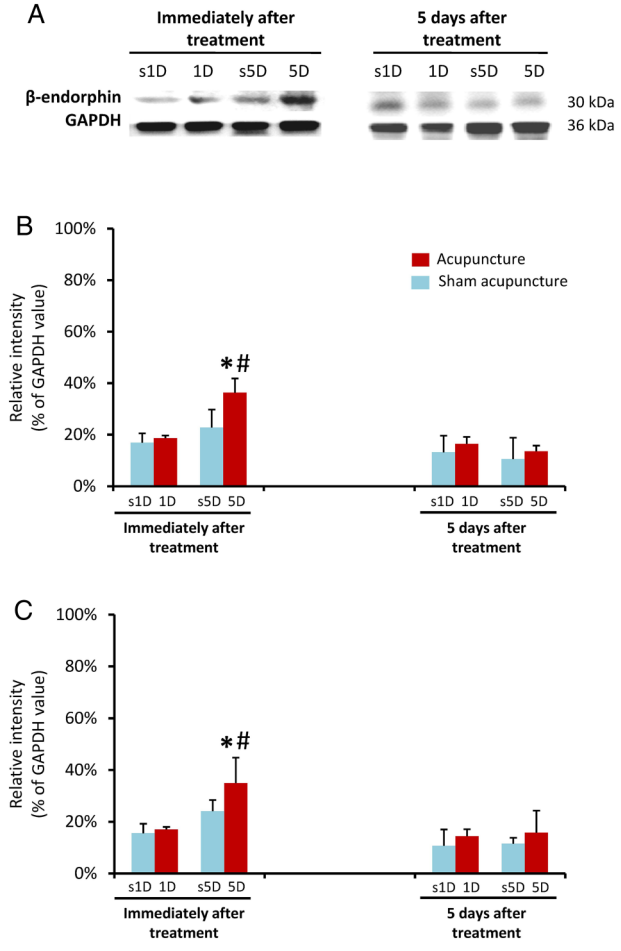

Figure 4 Representative Western blot photographs (A) and relative β-endorphin expression in the ipsilateral (B) and contralateral (C) biceps femoris sampled from 60 rabbits immediately after and 5 days following one or five daily sessions of verum acupuncture (1D and 5D groups, respectively, n=15 each) or sham acupuncture (s1D and s5D, respectively, n=15 each). Data are mean±SD. *p<0.05 vs sham groups (s1D and s5D). #p<0.05 vs 1D group.

Enkephalin-like immunoreactivity of spinal dorsal horn

Qualitative analysis of the enkephalin-like immunoreactivity in the superficial laminae (I–II) of the L5–S2 dorsal horns showed different patterns of reactivity between the verum and sham groups. In animals treated with sham acupuncture, enkephalin-like immunoreactivity was rarely expressed bilaterally in neurons of the superficial laminae. By contrast, in animals receiving acupuncture, neurons stained wtih enkephalin-like immunoreactivity were visualised as brown precipitates, along with some cytoplasmic staining. Most of the enkephalin-like immunoreactive cells were distributed bilaterally in the superficial laminae of the dorsal horns (figure 5A). Quantitative analysis revealed that, immediately after treatment, there was increased expression of enkephalin-like immunoreactivity in the bilateral superficial laminae in the 1D and 5D groups compared with the s1D and s5D groups, respectively (p<0.001 each; figure 5B, C). Five days after treatment, on day 11, there remained higher enkephalin-like immunoreactivity in the 5D group compared to both the s5D and 1D groups (p<0.001 each), however there were no significant differences between the 1D and s1D groups five days post-treatment on day 6 (p>0.05).

DISCUSSION

Acupuncture (or dry needling therapy) at distant MTrPs produces potent pain relief associated with reduced irritability of proximal MTrPs, an observation that has been demonstrated both in clinical trials[3 6–8] and animal models.[7 21] These findings mirror the traditional acupuncture approach of needling distant acupuncture points in order to influence anatomically remote pain.[28] In this study, we have demonstrated that the remote effects of acupuncture may be mediated through modulation of endogenous opioids in the spinal cord and peripheral tissues, consistent with previous reports.[4 6 10]

Opioid peptides play a pivotal role in the descending pain inhibitory control system, which has been implicated in inhibition of nociceptive input at the level of the spinal cord[29 30] The dorsal horn regions contain opioid receptors and enkephalinergic interneurons with input from the rostral ventromedial medulla.[31] Previous studies have demonstrated that acupuncture analgesia is established through a central

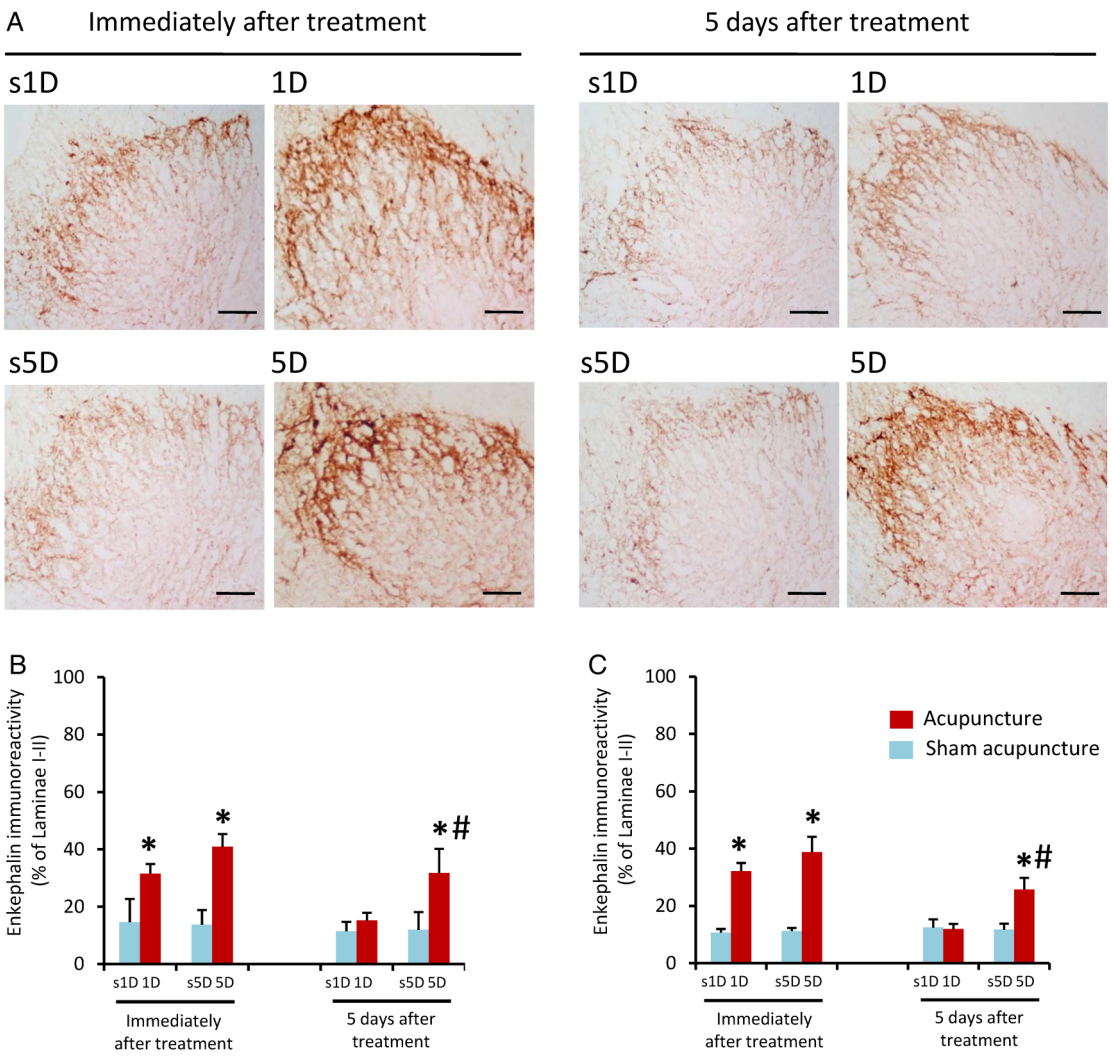

Figure 5 Representative photomicrographs (A) and quantification of immunohistochemical labelling for enkephalin in the ipsilateral (B) and contralateral (C) superficial laminae at L5-S2 in spinal cord sections sampled from 60 rabbits immediately after and 5 days following one or five daily sessions of verum acupuncture (1D and 5D groups, respectively, n=15 each) or sham acupuncture (s1D and s5D, respectively, n=15 each). Data are mean±SD. Scale bar=100 μm. *p<0.05 vs sham groups (s1D and s5D). #p<0.05 vs 1D group.

mechanism involving opioidergic neurohumoral pathways.[4 10]

In this study, we observed that the expression of enkephalin-like immunoreactivity was increased in spinal laminae I-II, corresponding to the innervation of the proximal muscle, following acupuncture at MTrSs of a distant muscle. The results of our study and those of others[11] suggest that the distant antinociceptive effects of trigger point acupuncture involve the spinal release of enkephalin.

We have recently showed that both one and five acupuncture treatments at distant MTrSs suppressed substance P levels in the biceps femoris and spinal dorsal horns when measured immediately after treatment.[7] In this study, induction of raised levels of spinal enkephalin and serum β-endorphin was found immediately after treatment in groups receiving either one or five doses of acupuncture, while β-endorphin expression in the DRG and biceps femoris was only demonstrated in the group that had five doses. Furthermore, 5 days after cessation of acupuncture, the higher expression of spinal enkephalin and serum β-endorphin levels were only seen among animals that had received five doses of acupuncture, suggesting that the endogenous opioids are involved in the remote effects of acupuncture in a dose-dependent manner. The findings of the present experiment and our previous study[7] suggest that repetitive distant needling may provide better and more prolonged analgesic effects by reducing substance P levels and elevating opioid levels, thus helping control myofascial pain.

In our previous study, we found that the remote needling effect was mediated via an intact afferent connection from the site of stimulation to the spinal cord and was dependent on normal spinal cord

function including certain inhibitory circuits at the level corresponding to the innervation of the distant muscle. Other central inhibitory mechanisms may also be involved.[21] Therefore, the neurophysiological basis for the remote effects of acupuncture may be a consequence of certain neural connections including activation of the parasympathetic nerves,[32 33] inhibitory interneurons and/or the opioidergic descending pain control system.

To our knowledge, this is the first study showing the immediate and relatively more prolonged effects of short- and medium-term use of acupuncture at distant MTrSs on levels of endogenous opioids in a variety of tissues. The most critical limitation of this study is the inherent difficulty confirming that the subjective pain sensation originated from rabbits' MTrSs. There is evidence that spontaneous electrical activity (ie, EPN) of muscle fibres can be recorded in the MTrP region.[15–17 21 34] Although animals cannot coherently communicate the nature or site of their pain, we are quite confident that MTrSs can be identified with a reasonable degree of precision based on animals' nocifensive behaviour in response to the pinching of taut bands in muscle, combined with the occurrence of EPN when the electrode approached the MTrS region.

CONCLUSION

In summary, our findings showed protein levels of endogenous opioids in serum, spinal dorsal horn, DRG and proximal muscle can be enhanced by acupuncture at MTrSs of a distant muscle in rabbits. Accordingly, acupuncture needling at some distance from the painful site may facilitate antinociception through segmental, heterosegmental or central analgesic mechanisms by activating opioid systems. Better understanding of the mechanisms underlying the remote analgesic actions of acupuncture may lead to new therapeutic strategies to treat myofascial pain syndrome.

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Contributors All authors contributed to the study conception/design, data collection, and data analysis/interpretation, were involved in drafting and revising the manuscript, approved the final version of the manuscript for publication, and take responsibility for the accuracy and integrity of all aspects of the research. C-ZH, Y-LH and C-CY are the grant holders.

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Competing interests None declared.

Ethics approval The protocol for this study was approved by the ethical review committee of China Medical University, Taichung, Taiwan.

Provenance and peer review Not commissioned; externally peer reviewed.

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Acupuncture at distant myofascial trigger spots enhances endogenous opioids in rabbits: a possible mechanism for managing myofascial pain

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