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Molecular Evidence: EA May Inhibit the Muscle Atrophy

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1. Introduction

Aging is of critical interest in the medical, health, and social domains, especially in developed countries and newly industrializing countries. Because muscle atrophy in elderly individuals can cause falls, its prevention is important. Moreover, prevention of aging-related reduced skeletal muscle mass may allow a higher quality of life in the elderly, because reduced muscle function is linked to the occurrence of several chronic diseases (Handschin & Spiegelman, 2008).

High-intensity resistance training effectively maintains muscle mass and strength, but rigorous training is difficult for elderly people (Seynnes et al., 2007).

Acupuncture is a well-known traditional technique in eastern Asia that is used to maintain health and cure many diseases. Major acupuncture techniques utilize penetration of the skin by thin, solid metallic needles, which are manipulated manually or are stimulated electrically. This electrical needle stimulation is called electroacupuncture (EA) (Klein & Trachtenberg, 1997). EA is effective not only for pain but also for muscle problems, such as stiffness, exhaustion, and atrophy, in many patients including elderly people (Zhang, 2003).

Acupuncture studies have reported the nerve routes of acupuncture signal transmission, effects via the spinal reflex, and reactions in the brain (Cho et al., 1998; Murase & Kawakita, 2000; Uchida et al., 2000). Figure 1 is a schematic diagram showing the routes of EA stimuli between treated points and organs. In a previous investigation on acupuncture, only a neural mechanism of pain reduction was clear; endogenous opioid (beta endorphin and enkephalin) is induced under the acupuncture anesthesia (Chung & Dickenson, 1980).

However, the molecular mechanisms of other effects of acupuncture were as yet not defined (Acupuncture, 1997). Scientific evidence of efficacy is an important as for the CAM research, as for research in Western medicine. The enhancement of blood flow in target organs of acupuncture treatment, which is a major reason for the effectiveness of acupuncture (Niimi & Yuwono, 2000), cannot sufficiently explain the recovery of muscle from exhaustion because it is not clear how the supplied oxygen and nutrients would be used during the cellular recovery process. Many cellular and physiological processes are regulated at the transcription level of gene expression. The identification of genes specifically modulated during the process of acupuncture would provide an initial step toward elucidation of the underlying mechanisms of this technique.
Fig. 1. Schematic diagram of transmission of Electroacupuncture signals. Solid arrows indicate the nervous system; broken arrows, the extraneural system; gray arrows, organ responses. Modified from Takaoka et al. (Takaoka et al., 2007).

Fig. 2. Electrical voltage and current were measured with a digital oscilloscope (LS140; LeCroy, Chestnut Ridge, NY) connected to circuits as shown. Electrical current was calculated on the basis of voltage across a 500-k resistor.
We suggested that EA (Fig. 2) may be an appropriate choice for effective prevention of muscle atrophy, on the basis of our results from a transcriptome study (Fig. 3), which provided molecular evidence obtained from skeletal muscle of wild-type mice: EA suppressed expression of the myostatin gene (Fig. 4), an endogenous inhibitor of muscle growth and satellite cell-related muscle regeneration, and EA induced a proliferative reaction of muscle satellite cells (Fig. 4) (Takaoka et al., 2007). Thus, in view of our previous basic research, EA may be an effective method for retaining muscle mass.

To investigate the effect of EA on muscle atrophy, we used a hindlimb suspension model. Hindlimb-suspended (HS) rodents are a commonly used animal model for pathological studies of the loss of muscle mass, such as disuse muscle atrophy (Däpp et al., 2004; J.F. Desaphy et al., 2001; Dupont-Versteegden et al., 2006; Gallegly et al., 2004; Pisani & Dechesne, 2005; Stelzer & Widrick, 2003). In this model, mainly postural muscles such as the soleus demonstrate reduced mass (Thomason & Booth, 1990). In addition, studies of HS mice suggested that the cross-sectional muscle area was reduced (Nguyen & Tidball, 2003).

The aims of our study were to evaluate the effect of EA according to the measures of muscle mass and myofibre diameter in a murine HS model and in this murine model after repeated EA treatments, and to analyse expression of the myostatin gene and the ubiquitin ligase genes muscle RING finger 1 (MuRF-1), muscle atrophy F-box (MAFbx), and Casitus b-lineage lymphoma-b (Cbl-b) of insulin-like growth factor I (IGF-1)/thymoma viral proto-
oncogene (AKT) pathway, as related to disuse muscle atrophy (Bodine et al., 2001; Centner et al., 2001; Keane et al., 1995; Sandri et al., 2004; Takaoka et al., 2007). This investigation corresponds to a preclinical stage in translational research, that is, preclinical development after basic research that we previously performed. In addition, this study had the potential to acquire direct experimental evidence from an EA-treated animal model.

2. Materials and methods

We first compared the effects of EA, as evidenced by muscle mass and myofibre diameter, in HS mice and HS mice treated with EA (EA/HS). Then we used real-time quantitative RT-PCR to analyse myostatin and ubiquitin ligase gene expression in atrophic muscles of HS mice and in muscles of EA/HS mice. In this research, all mice used were treated according to the Standards Relating to the Care and Management, etc. of Experimental Animals (Ministry of the Environment, Tokyo, Japan). This study was approved by the Institutional Animal Care and Use Committees, at the University of Tsukuba (Permission number 200) and Kobe University (Permission number P080913) and was carried out according to the Animal Experimentation Regulations of these Committees. It was also approved by the Committee for Safe Handling of Living Modified Organisms at Kobe University (Permission number 17-21) and carried out according to the Guidelines of the Committee.
2.1 Murine hindlimb suspension model and EA stimulation

We used 8-week-old Crlj:CD1(ICR) male mice (each weighing 30–35 g; Charles River Japan, Yokohama, Japan) for three groups: control, 7 days of HS, and 7 days of EA/HS. HS mice were prepared by using a modified version of the apparatus of Miyazaki et al. (Miyazaki et al., 2006). A sigmoid hook connected to a metal fitting was fastened to the tail with adhesive tape, so that hindlimbs could not touch the floor. The suspension height was adjusted for the forelimbs so that the mice could move. All groups of mice had *ad libitum* access to food and water for the duration of the experiment.

For EA stimulation, EA/HS mice were anesthetized by means of an intraperitoneal injection of pentobarbital sodium (2.5µg/g); control and HS mice were similarly anesthetized. Stainless-steel acupuncture needles (two needles, each 40 mm long and 0.16 mm in diameter; Seirin Co. Ltd., Shizuoka, Japan) were then inserted into the anesthetized EA/HS mice at the origin and insertion of the soleus muscle. The needles were stimulated with an electrical stimulator (Ohm Pulser LFP4000A; Zen-iryoki Co. Ltd., Fukuoka, Japan), as in our previous study (Takaoka et al., 2007). Every other day, on days 1, 3, 5, and 7, mice received EA for 30 min with 10-Hz pulse wave repetitions. After 7 days, animals were evaluated (Fig. 5).

Fig. 5. Experimental design of EA stimulation

2.2 Relative soleus muscle mass and Histochemical analysis

All mice (*n* = 10, for each time point for each group) were dissected after determination of body weight. For each mouse, both soleus muscles were excised and their wet weights were measured. Then, the relative muscle mass (wet weight per body weight) was calculated to allow groups to be compared.

For histochemical analysis of muscle fibres, soleus cryosections, after having been frozen in 2-methylbutane, were sectioned with a cryostat (CM3050; Leica, Wetzlar, Germany). Frozen sections were stained with hematoxylin and eosin (H&E) according to our previous report (Takaoka et al., 2004). They were then examined with a light microscope (BX51 Research Microscope; Olympus, Tokyo, Japan) equipped with a digital camera (MicroPublisher 5.0; Roper Japan, Tokyo, Japan). To obtain myofibre diameters, an image-based software (MicroAnalyzer; Nihon Poladigital, K.K., Tokyo, Japan) was used. These values are given as means ± S.D. Soleus mass and myofibre diameter for each time point for the three groups are given as percentages of the control at day 0. Student’s *t* test was used for statistical analysis, with *P* values of <0.05 considered statistically significant.

2.3 Gene expression analysis

To examine myostatin gene expression in three experimental groups (control, HS, and EA/HS; *n* = 7 for each time point for each group), total RNA was extracted from soleus
muscle. Total RNA (5-μg samples) was reverse-transcribed into cDNA by using SuperScript RT (SuperScript Preamplification System; Gibco/BRL, Gaithersburg, MD), according to the company’s instructions. Expression levels were compared by using real-time quantitative RT-PCR analysis with gene-specific primers as follows: myostatin: sense: 5'-GACAAAAACACGAGGTACTCC-3'; antisense: 5'-GATTCAGCCCATCTTCTCC-3'; MuRF-1: sense: 5'-CTCCTTGTGCAAGGTTTGG-3'; antisense: 5'-CCAGCATGAGATGCAGTTA-3'; MAFbx: sense: 5'-AGCGCTTCTTGGATGAGAAA-3'; antisense: 5'-ACGTCGTAGTTCAGCTGCTT-3'; and Cbl-b: sense: 5'-TCATTCAGAAAGGCATCGTG-3'; antisense: 5'-CGGGAGTGGTTTGTCTTGTT-3'. Real-time quantitative PCR was performed by using Power SYBR Green PCR Master Mix and StepOne (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. The reaction mixture consisted of 2 μl of SYBR Green, 4 μl of cDNA, and each primer at 5 pmol, plus water to a final volume of 20 μl. The PCR conditions were 95°C for 10 min followed by 50 cycles for 95°C for 15 s, 60°C for 60 s, and then 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. Three internal control genes for this research were chosen from six housekeeping genes, β-actin (Actb), β2-microglobulin (B2m), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-glucuronidase (Gusb), transferrin receptor (Tfrc), and 18S rRNA, by using the method of Vandesompele et al. (Vandesompele et al., 2002). Then the relative expression levels were calculated by means of comparison with the geometric mean of the expression of the three internal control genes. These values are given as means ± S.D. Data related to myostatin gene expression are presented as percentages of control. Student’s t test was used for statistical analysis, with P values of <0.05 considered statistically significant.

3. Results

In view of our previous research indicating that EA-induced myostatin gene suppression may help prevent muscle atrophy, we continued our investigations of this effect of EA by using a pathological animal model in a preclinical study: hindlimb-suspended (HS) mice in the disuse muscle atrophy model. We first compared the effects of EA, as evidenced by muscle mass and myofibre diameter, in HS mice and HS mice treated with EA (EA/HS). We found that EA/HS mice maintained a soleus muscle mass that was not significantly different from that of control mice, whereas HS mice had significantly reduced muscle mass. Also, the diameters of myofibres in EA/HS mice, which were not significantly different from control values, were significantly larger than those in HS mice. We then used real-time quantitative RT-PCR to analyse myostatin and ubiquitin ligase gene expression in atrophic muscles of HS mice and in muscles of EA/HS mice. Repeated EA treatment suppressed expression of these genes in skeletal muscle of EA/HS mice but induced expression of them in HS mice.

3.1 Relative wet weight of the soleus muscle

To determine the relative wet weight of the soleus muscle, we measured the wet weight of the soleus muscle and the body weight of each mouse (total n = 30; n = 10 for each group). The HS group showed a significant reduction in relative wet weight of the soleus muscle when compared with the control mice (P < 0.005) (Fig. 2a). These data suggest that hindlimb suspension led to muscle atrophy. Comparison of EA/HS mice with the control group showed no significant differences in relative soleus muscle wet weight at 7 days. EA/HS mice had a significantly higher relative soleus muscle weight than did HS mice (P < 0.01) (Fig. 6a). These results suggest that EA prevented muscle atrophy.
Fig. 6. Changes in relative soleus muscle mass and myofibre diameters after EA. a, The measure of wet weight of the soleus muscle per body weight shows an effect of EA on muscle atrophy. b, Effect of EA on prevention of muscle atrophy as shown by changes in myofibre diameters. Open circle, control mice; closed squares, HS mice; open squares, EA/HS mice; **P < 0.01; ***P < 0.005; NS, not significant; n = 10.

Fig. 7. Logarithmic histogram of expression levels of six housekeeping genes. An approximately 100-fold expression difference is apparent between the most and least abundantly expressed gene, as well as treatment-specific differences in expression levels for particular genes (for example, Actb). Genes are as follows: Actb, β-actin; B2m, β2-microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gusb, β-glucuronidase; Tfdc, transferrin receptor; and 18S rRNA, 18S ribosomal RNA.
3.2 Diameters of muscle fibres in cross section
Soleus muscles from all groups of mice were stained with H&E and muscle fibre diameters were measured. HS mice had significantly reduced cross-sectional muscle fibre diameters when compared with control mice. EA/HS mice had significantly larger muscle fibre diameters than HS mice (Fig. 6b). These findings suggest that EA prevented the muscle atrophy that was caused by hindlimb suspension. EA maintained muscle diameter sizes similar to those of control mice. No abnormality was observed in H&E-stained muscle tissues (data not shown).

3.3 Real-time quantitative RT-PCR analysis
To identify proper internal control genes for real-time quantitative RT-PCR analysis, we analysed the expression of six housekeeping genes according to a previous report (Vandesompele et al., 2002). We first investigated the gene expression level of these housekeeping genes via real-time quantitative RT-PCR in samples prepared from the same amount of RNA for each gene. The result revealed that no gene showed a constant expression level in both HS and EA/HS groups (Fig. 7).

![Gene expression related to hindlimb suppression and EA effect](https://www.intechopen.com)

Fig. 8. Gene expression as related to hindlimb suppression and EA effect. Real-time RT-PCR analysis of expression of Mstn (myostatin) (a), MuRF-1 (b), and MAFbx (c) genes in control, HS, and EA/HS mice (for each group, n = 7). Relative transcript levels are shown (means ± S.D.). Open circle, control mice; closed squares, HS mice; open squares, EA/HS mice; *P < 0.05; **P < 0.01; ***P < 0.005; NS, not significant.

Because we found no housekeeping gene with expression stability in all groups, we calculated the gene expression stability of these genes by using geNorm (http://medgen.ugent.be/~jvdesomp/genorm/) and found that the geometric mean of the expression levels of three genes—β-actin, GAPDH, and transferrin receptor—was the optimal value for normalization factors. Thus, we used the geometric mean of these three genes to calculate the relative gene expression level.

We next analysed changes in myostatin and ubiquitin ligase gene expression in HS mice to examine the effect of EA in HS mice and compared the expression level with the control. Figure 8a shows that the myostatin gene was induced in HS mice but that its expression was significantly suppressed in EA/HS mice. In addition, the two ubiquitin ligase genes, MuRF-
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1 and MAFbx, were induced in HS mice but their expression was significantly suppressed in EA/HS mice (Fig. 8b and c). Expression of Cbl-b was induced at 13 days in HS mice but was significantly reduced in EA/HS mice compared with control and HS mice (data not shown).

4. Discussion

The HS model is characterized by a muscle disorder—disuse muscle atrophy—that was previously established in rabbits (Anzil et al., 1991; Sancesario et al., 1992), rats (Morey-Holton & Globus, 2002; Riley et al., 1990; Thomason & Booth, 1990), and mice (Stelzer & Widrick, 2003; Thomason & Booth, 1990). For the present preclinical study, we confirmed the molecular evidence of the effect of EA by using a pathological murine model of disuse muscle atrophy involving hindlimb suspension. Our previous basic research indicated that EA suppresses myostatin gene expression in skeletal muscle and causes a satellite cell-related proliferative reaction (Takaoka et al., 2007). In this study, the myostatin gene and three ubiquitin ligase genes were evaluated by means of real-time quantitative RT-PCR analysis.

First, to examine the possibility that EA prevented muscle atrophy, we determined relative soleus muscle mass and cross-sectional diameters of soleus myofibres. Relative wet weights of muscles and myofibre diameters in EA/HS mice were significantly larger than those in HS mice (Figs. 6a and b). In addition, values of both relative wet weights and cross-sectional myofibre diameters for EA/HS mice were not significantly different from those of control mice. These results indicate that EA prevented muscle atrophy induced by hindlimb suspension.

Then, to investigate the molecular mechanisms governing the effect of EA in prevention of disuse muscle atrophy, the expression of the myostatin gene and three ubiquitin ligase genes (MuRF-1, MAFbx, and Cbl-b) in these HS models was analysed by using real-time RT-PCR. We chose these genes because they play key roles in hindlimb suspension and are therefore appropriate for investigations of the effect of EA on HS mice. Figure 4a shows that expression of the myostatin gene was induced in HS mice and was significantly greater than that in control mice, but this effect was not observed in EA/HS mice, as in our previous study of EA-treated mice (Takaoka et al., 2007). This induction of myostatin gene expression in HS mice is consistent with the presence of muscle atrophy. Application of EA significantly suppressed myostatin gene expression in EA/HS mice at 13 days (data not shown). This result is consistent with prevention of muscle atrophy by EA. The consequence of this suppressed gene expression is increase in proliferation of satellite cells, that is, skeletal muscle stem cells, and prevention of muscle atrophy.

For microgravity-induced muscle atrophy, the ubiquitin-proteasome pathway plays the most important role in the protein degradation system (Ikemoto et al., 2001). Therefore, this ubiquitin-proteasome-related protein degradation system can be rate-limiting for degradation of proteins in the muscle atrophy found in our HS mice. Indeed, expression of the three ubiquitin ligase genes that we examined in this study was reportedly up-regulated in HS rats (Haddad et al., 2006; Nikawa et al., 2004). In our experiment, these genes except Cbl-b showed significant difference in expression at 7 days in HS mice (Fig. 4b and c), and had significantly greater expression at 13 days compared with three genes of control mice (data not shown). In EA/HS mice, expression of MuRF-1 and expression of MAFbx were significantly lower than those of control mice at both 7 and 13 days and were significantly suppressed compared with those of HS mice at both time points. EA/HS mice also had
significantly lower Cbl-b gene expression at 13 days compared with control mice and HS mice (data not shown).

Our results from the myostatin and ubiquitin ligase gene expression study were mostly consistent with results from previous reports on HS mice and rats (Haddad et al., 2006; Kawada et al., 2001; Nikawa et al., 2004; Stevenson et al., 2003)), except for the finding for HS mice at 7 days which showed no significant difference from the control. The differences between our findings and those of previous HS studies may result from differences in the kinds of rodents or mouse strains. For example, our previous studies of transgenic mice showed amyloid deposition in C57BL/6 mice (Takaoka et al., 2004) but not in C57BL/6 × C3H F1 mice (Sasaki et al., 1986). In our HS ICR strain mice with the phenotype of muscle atrophy, ubiquitin ligase genes were expressed at 7 days at the level of controls, whereas myostatin gene expression was significantly greater than that of controls (Fig. 8a). This result suggests that the muscle atrophy was caused because the myostatin gene expression was induced, which led to suppression of satellite cell proliferation and a degree of protein degradation was not changed from that of controls.

Fig. 9. Molecular mechanisms of EA efficacy in prevention of muscle atrophy. Summary of a model of molecular mechanisms of EA efficacy in prevention of muscle atrophy, including the role in the IGF-1/AKT pathway and myostatin gene expression. a, HS mice. b, EA/HS mice. Our experiment has not yet elucidated the details of the signaling pathway leading from EA to gene suppression.

In this study, the molecular evidence that EA suppressed induction of the expression of the myostatin gene and the three ubiquitin ligase genes in HS mice was consistent with the phenotype, and EA prevented muscle atrophy. Figure 9 provides schematic diagrams of the molecular mechanisms of the effects of EA on inhibition of disuse muscle atrophy, as based on the previous reports (Sandri et al., 2004; Takaoka et al., 2007; Workman et al., 2006) and our data from this study. After the myostatin gene induction in our HS mice, the expression of ubiquitin ligase genes of the IGF-1/AKT pathway was facilitated (Fig. 9a and b). When HS mice received EA every other day, expression of the myostatin, MuRF-1, and MAFbx genes was significantly suppressed at 7 days; at 13 days, expression of Cbl-b was also
significantly suppressed. With regard to expression of the three ubiquitin ligase genes, our data suggest that EA-induced suppression of MuRF-1 and MAFbx at 7 days did not involve the IGF-1/AKT pathway and that another signaling pathway may exist for regulation of these genes. In addition, the finding that at 7 days the Cbl-b gene showed no significant expression after EA suggested that the effect of EA on gene suppression differs for Cbl-b and the other two ubiquitin ligase genes. The results for 13-day EA/HS mice suggested that induced satellite cell proliferation via the suppressed myostatin gene and reduced protein degradation via suppression of ubiquitin ligase gene expression neutralized each other because the relative soleus muscle mass and myofibre diameters were not significantly different from control values. This result suggested that a more effective EA method for treatment of muscle atrophy may be developed by analysing the changes in expression of these genes. These molecular findings and muscle phenotype thus support the suggestion that EA may be an effective technique for prevention of muscle atrophy.

In the present study, we used 10-Hz pulses for EA, not 1-Hz pulses as in our previous study, because we determined that in the range of 1–40 Hz, 10 Hz provided the best myostatin gene suppression (Fig. 10). Onuma et al. (Onuma et al., 2008) reported on the effect of electrical stimulation applied by using electrodes on the skin (no needles were inserted) to prevent muscle atrophy. They compared electrical pulse sizes and found that 20 and 30 Hz effectively prevented atrophy. Another research group reported, however, that electrical stimulation at 50 Hz caused muscle atrophy (Kanno et al., 1999). These data suggest the existence of other more effective stimulation conditions than 10 Hz, which we chose for our experiment, to prevent muscle atrophy. Understanding the relationship between EA microcurrent pulse conditions and reactions of skeletal muscle is important, and investigations to determine the best stimulation conditions are needed.

Fig. 10. Mstn gene suppression by various EA stimulations (each n=5). *P < 0.05; **P < 0.01; ***P < 0.005.
Certain physical therapy techniques involve types of pulse stimulation that differ from EA stimulation. In rehabilitation medicine, electrotherapies such as transcutaneous electrical nerve stimulation (TENS) and electrical muscle stimulation (EMS) are used for relief of pain, reduction of inflammation, and improvement of muscle function (Hurley & Bearne, 2008; Maffiuletti et al., 2003). In these electrotherapies, pulses pass from the skin surface through motor nerves to skeletal muscles to cause muscle contractions (Collins et al., 2002). In EA, a stainless-steel needle, which is inserted into a muscle, stimulates and electrifies the muscle directly by means of a pulse wave of a low-frequency microcurrent (−0.14 to +0.30 mA in mouse muscle), the result being muscle contraction (Takaoka et al., 2007). Indeed, unlike TENS and EMS, EA can clearly electrify a targeted muscle that has had an acupuncture needle inserted and can also stimulate tissues distant from the skin (Ishimaru et al., 1995). In addition, the direct microcurrent may induce growth signal transduction in the cell (McCaig et al., 2005). Therefore, EA probably induces cell growth (Takaoka et al., 2007) by direct application of electrical current, which is superior to the electrical stimulation through the skin such as that provided by TENS and EMS. Thus, EA is likely to enhance muscle function during rehabilitation.

Long-term rehabilitation is needed for complete recovery from muscle atrophy. For example, complete recovery after a 2-week hindlimb suspension required 3–4 weeks (J. Desaphy et al., 2005). In view of this finding, preventing muscle atrophy is important, to avoid the need for such rehabilitation. Although the efficacy of resistance training for disuse muscle atrophy has been reported (Kannus et al., 1998; Suetta et al., 2008), development of methods other than exercise to prevent muscle atrophy is important for individuals such as elderly people and patients on long-term bed rest after surgery.

5. Conclusion

In this study, we demonstrated that EA was an effective option to prevent muscle atrophy, as evidenced by molecular data showing suppression of myostatin and ubiquitin ligase gene expression. Additional research is now under way to analyse EA-related differences in functions such as muscle contraction and relaxation.

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7. References


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Acupuncture is growing in popularity world-wide. Acupuncture and related techniques are useful tools for treating a spectrum of diseases. However, there are still many areas of controversy connected to it due to the fact that mechanisms of action of acupuncture are not entirely clear. Another debilitating element is the absence of a convincing model of sham acupuncture for a control group in clinical trials. Therefore, there are still inappropriate prejudice and unfamiliarity regarding acupuncture. I hope this book can contribute to guide the advance of this ancient medical art. The reader will here find texts wrote by authors from different parts of the world. The chapters cover strategic areas to collaborate with the consolidation of the knowledge in acupuncture. The main objective is to share elements to make acupuncture more and better offered at health systems worldwide.

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