Pain Relief with Wet Cupping Therapy in Rats is Mediated by Heat Shock Protein 70 and ß-Endorphin

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Abstract

Background: Wet cupping therapy is a complementary therapy in pain management. The mechanism of this therapy, however, needs further elucidation. Cells injured by wet cupping therapy seem to stimulate the expression of heat shock protein 70 (HSP70). Its benefit in pain reduction could be mediated by the expression of ß-endorphin. This study aimed at determining the correlation between HSP70 and ß-endorphin after wet cupping therapy.

Methods: Sixteen male Wistar rats were divided into control (CG; n=8) and treatment (TG; n=8) groups. The rats in both groups were injected with complete Freund’s adjuvant (CFA) at the footpad. In the TG, wet cupping therapy was done at the left and right paralumbar regions 48 hours after the CFA injection. Twenty-four hours after therapy, the hot plate test was done to assess pain threshold. Thereafter, immunohistochemistry from the skin subjected to wet cupping therapy was conducted for HSP70 and ß-endorphin.

Results: The expression of HSP70 was significantly higher in the keratinocytes of the TG (20.25±3.53; P<0.001) than in the keratinocytes of the CG (10.50±2.44; P<0.001). The expression of ß-endorphin was significantly higher in the keratinocytes of the TG (22.37±3.52; P<0.001) than in the keratinocytes of the CG (5.12±1.72; P<0.001). The results also revealed a high correlation between HSP70 and ß-endorphin (β=0.864; P<0.001). Pain threshold after wet cupping therapy was significantly higher in the TG (22.81±6.34 s; P=0.003) than in the CG (11.78±3.56 s).

Conclusions: The benefit of wet cupping therapy in terms of pain reduction in rats could be mediated by the expression of HSP70 and ß-endorphin.

Keywords • Pain • Rat • HSP70 heat-shock proteins • ß-endorphin • Complementary therapies

What's Known

• Wet-cupping therapy is a complementary and alternative therapy. Many studies have shown that cupping therapy can relieve pains such as headache, low back pain, brachialgia paraesthetica nocturna, carpal tunnel syndrome, and cervicalgia. The mechanism is still unclear.

What's New

• In an animal model of pain, wet-cupping therapy reduced pain. This study showed that wet-cupping therapy expressed heat shock protein 70 and ß-endorphin. We conclude that the mechanism of wet-cupping therapy in reducing pain was mediated by heat shock protein 70 and ß-endorphin.

Introduction

Pain prevalence ranges from 8% to over 60% worldwide and as such constitutes a major clinical, social, and economic problem.¹,² Chronic pain reduces health-related quality of life³ by creating disturbances in sleep, sexual activity, social activity, and work.⁴,⁵
The most common treatment modalities for patients with chronic pain are pharmacological and non-pharmacological therapies. Pharmacological therapies draw upon nonsteroidal anti-inflammatory drugs (NSAIDs), steroids, opioids, and herbal medicines. Until now, NSAIDs have been used as common drugs for the treatment of chronic pain. However, prolonged treatment with NSAIDs will lead to complications such as dyspepsia and peptic ulcer. Non-pharmacological therapies include aerobic exercise, cognitive behavioral therapy, balneotherapy, and cryotherapy. Complementary and alternative medicines, including cupping therapy, have been utilized for many a year in different regions and countries such as China, Arab world, Central Europe, and some parts of Africa. There are different types of cupping therapy such as needle cupping, moving cupping, medicinal (herbal) cupping, and bleeding cupping (wet cupping). Wet cupping therapy is the most common cupping therapy and has been demonstrated to be beneficial in headache, brachialgia paraesthetica nocturna, carpal tunnel syndrome, and low back pain. As the election of a suitable treatment for various types of chronic pain is becoming increasingly based on the mechanism of the treatment, a thorough understanding of the mechanism of wet cupping therapy in reducing pain is of vital importance.

Heat shock protein 70 (HSP70) is a chaperone protein that is expressed in response to stress. Wet cupping therapy can induce stress due to cell injury (Asea et al. [2007]). HSP70 binds to protein substrates and stabilizes them to avoid denaturation or aggregation until the condition improves. Stress can trigger the expression of heat shock proteins, including HSP70, as well as corticotropin-releasing hormone (CRH). CRH binds to corticotropin-releasing hormone receptor-1 (CRHR1) and stimulates the transcription of the proopiomelanocortin (POMC) gene and the expression of adrenocorticotropic hormone as well as β-endorphin. β-endorphin is the main endogenous opioid and is coded by the POMC gene. β-endorphin has been known to have analgesic effects. Its mechanism occurs in both peripheral and central nervous systems by binding to opioid receptors, particularly of the mu subtype.

We hypothesized that wet cupping therapy could confer pain relief and that its analgesic effects could be mediated by the expression of HSP70 and β-endorphin due to cell injury. An increase in HSP70 would positively correlate with a rise in β-endorphin. Accordingly, we sought to determine the correlation between HSP70 and β-endorphin after wet cupping therapy.

### Patients and Methods

The present study was a posttest-only control group design. The study protocol was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (Ethics #189-KE). Sixteen 3-month-old male Wistar rats were randomized into 2 groups: Control (n=8) and treatment (n=8). In the control group, the rats were treated only with complete Freund’s adjuvant (CFA) (Sigma-Aldrich, Saint Louis, Missouri, USA). In the treatment group, the animals were treated with CFA and wet cupping therapy.

#### Animal Experiment

The rats were housed in cages (4–5 per cage) for 7 days under a 12:12 hour light/dark cycle (lights off at 19:30 hours) and at constant temperature (24 °C). Food (Pelet BR 511, Comfeed, Indonesia) and tap water were available ad libitum.

CFA (100 μL) was injected into the ventral surface of the right hind paw centered in the footpad of the rats in both groups. In the treatment group, the animals were treated with wet cupping therapy. The skin of the left and right paralumbar regions of the rats was punctured with a lancet, and then sterile cups (2 cm in diameter) were placed and negative pressure was applied (-200 mm Hg) for 5 minutes. Twenty-four hours after cupping therapy, all the rats were tested for pain threshold by using the hot plate test (Hot/Cold Plate Cat #35100, Ugo Basile, Varese, Italy). Following the pain threshold test, the animals were sacrificed by cervical dislocation and their paralumbar skin was directly dissected for immunohistochemistry.

#### Pain Threshold Test

Pain threshold was assessed by using hot plates under light conditions. Pain threshold was counted from the time of placing the rat on the heated surface (51 °C) until a nociceptive response, which was demonstrated by licking the hind paw or attempting to jump out of the hot plate within a 30-second cutoff time. One measurement was taken using a stopwatch for each animal to obtain paw withdrawal latency.

#### Immunohistochemistry Assay

The effects of wet cupping are presented in figures 1 and 2. The expressions of HSP70- and β-endorphin-positive cells were tested by...
immunohistochemistry using antibody monoclonal anti-HSP70 (HSP70/HSC70 antibody [W27]; sc-24, Santa Cruz Biotechnology, Dallas, Texas, USA) and antibody monoclonal anti-ß-endorphin (anti-endorphin beta antibody, clone 3-E7. MAB5276. Chemicon, Merck Millipore Co., Darmstadt, Germany), respectively. The cells positive for HSP70 and ß-endorphin expressions were counted using a light microscope (OlympusCX21, New York, USA).

**Statistics Analysis**

The Mann–Whitney U-test was used to compare the data between the control and treatment groups for the expression of HSP70 and ß-endorphin as well as for the pain threshold test. The Spearman correlation test was applied to demonstrate the correlation between the expression of HSP70 and ß-endorphin. The significance of the results was set at $P < 0.05$. Statistic package SPSS 17 was employed.

**Results**

**Expression of Heat Shock Protein 70 and ß-Endorphin**

The expression of HSP70 and ß-endorphin according to immunohistochemistry demonstrated positive cells (keratinocytes), which were brown in color, in the treatment groups.
Pain, particularly chronic pain, is a debilitating disease. Patients with chronic pain often tend to exhibit other symptoms such as depression and anxiety, stiffness, sleep disturbances, and other complaints related to quality of life. Needless to say, all these problems affect their daily life activities and working performances.

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The negative reaction of the expression of HSP70 (1A) and ß-endorphin (1C) did not show the dark brown color of keratinocytes. Meanwhile, the positive reaction of the keratinocytes produced a dark brown color both for HSP70 (figure 1B) and ß-endorphin (figure 1D).

Counting the number of the positive cells in the keratinocytes revealed a significantly higher number of cells that expressed HSP70 (P<0.001) and ß-endorphin (P<0.001) in the treatment group than in the control group (figures 2 A and B, respectively).

Correlation of Expression between Heat Shock Protein 70 and ß-Endorphin

A positive correlation was demonstrated between HSP70 and ß-endorphin in the treatment group (β=0.864; P<0.001).

Pain Threshold Test

The pain threshold test was done 24 hours after wet cupping therapy in the treatment group, while it was carried out in the control group 72 hours after the CFA injection. In the treatment group, the rats had longer pain thresholds than the rats in the control group (P<0.001) (table 1).

Table 1: Pain thresholds of the control and treatment groups according to the hot plate test

| Group      | Pain thresholds (s) | P value |
|------------|---------------------|---------|
|            | Mean±SD             | (Min–Max)|         |
| Control    | 11.78±3.56          | (7.50–18.00) | <0.001  |
| Treatment  | 22.81±6.34          | (14.30–30.00) |         |

*P<0.001, Mann–Whitney U-test

Discussion

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Taking together, chronic pain begets problems in many domains, from health to social life and economic matters. Due to the complexity of their symptoms, patients with chronic pain are usually prescribed to have multimodal treatments, which consist of pharmacological and non-pharmacological modalities, including drugs, exercise, and psychological treatment. However, many patients with chronic pain still seek other alternative medicines. Some common complementary or alternative medicines include acupuncture and chiropractice. Another alternative is cupping therapy, which has been practiced for many years now. Wet cupping therapy is the most common type of cupping therapy.

The present study demonstrated that wet cupping therapy reduced pain by increasing pain threshold. To the best of our knowledge, this is the first study of its kind to demonstrate this effect of wet cupping therapy in an animal study. The difference in pain thresholds between our treatment and control groups was almost 50%, which means that the effect was considerable. This marked effect of wet cupping therapy among patients with chronic pain has also been demonstrated in a previous study. The result obtained in terms of pain reduction is in agreement with the benefit of wet cupping therapy in chronic regional pain syndrome, chronic low back pain, and headache. Furthermore, although there have been reports of hematoma as a regular minor adverse effect at the site of the application of glass cupping, we detected no serious adverse events.

The selection of an appropriate treatment modality for different types of chronic pain syndromes is becoming increasingly based on the mechanism of the modality. As the mechanism of this benefit is still far from clear, an understanding of the mechanism of wet cupping therapy in conferring pain reduction is of great significance and needs further elucidation. According to previous studies, wet cupping therapy can lessen pain among patients with neck and shoulder pain by stimulating the peripheral nervous system, removing antioxidants, and decreasing oxidative stress. Since nociceptive activation contributes to chronic pain, it seems that wet cupping therapy reduces pain by its antinociceptive effect. In the current study, wet cupping therapy increased the expression of ß-endorphin and HSP70 in the keratinocytes at the site of cupping.

The expressions of HSP70 and ß-endorphin in keratinocytes had a positive correlation. HSP70 is a chaperon protein that can be upregulated by stress. It seems that negative pressure produced by wet cupping therapy leads to stress to the cells and stimulates the expression of HSP70. Additionally, in wet cupping therapy, a lancet is used to puncture the skin before applying negative pressure. This treatment leads to the damage of the cell membrane of keratinocytes. The damaged tissues trigger an inflammation reaction and release inflammatory mediators.
mediators such as bradykinin, prostaglandin, leukotriene, serotonin, histamine, and substance P. The expression of prostaglandin E2 stimulates the expression of HSP70, and the excess of HSP70 is secreted from the cells. Furthermore, extracellular HSP70 acts as a cytokine and triggers the expression of CRH.

In mammals, the activation of the hypothalamic-pituitary-adrenal (HPA) axis is the main endocrine response to stress, which is signaled by a rise in CRH. In corticotroph hypophyse cells, CRH binds to CRHR1 and stimulates the transcription of the POMC gene. POMC is the progenitor of adrenocorticotropic hormone and β-endorphin. Nuclear factor kappaB (NFkB) is a transcription factor associated with the expression of inflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha (TNF-α). These cytokines are highly expressed in inflammation states. The induction of NFkB in corticotroph hypophyse cells activates the POMC gene via CRH. NFkB also binds to the POMC gene and stimulates the transcription of the POMC. POMC is a precursor protein that produces different types of biologically active peptides, including β-endorphin, via a series of enzymatic steps. β-endorphins are synthesized and stored in the anterior pituitary gland and the immune system; they include T-lymphocytes, B-lymphocytes, monocytes, and macrophages. In the peripheral nervous system, β-endorphins bind to the opioid receptor (particularly of the μ subtype) at both pre- and post-synaptic nerve terminals and exert analgesic effects. In the present study, β-endorphins were determined in keratinocytes, where the wet cupping treatment was located. Interestingly, the expression of β-endorphin and the mu-opioid receptor could also be detected on keratinocytes both at mRNA and protein levels. The interaction between the nervous system keratinocytes and immune cells is mediated by the opiate receptor system. Nonetheless, this interaction, which leads to pain reduction after wet cupping therapy, needs to be studied further. The effect of wet cupping therapy seems to be systemic as it was able to raise pain thresholds in the hot plate test. The systemic effect of pain reduction could involve the central nervous system. Therefore, it would be of interest to determine its mechanism in the central nervous system given that β-endorphin and the mu-opioid receptor are also expressed in the central nervous system.

The current study provides more evidence-based data on wet cupping therapy for pain reduction and its mechanism through the expression of HSP70 and β-endorphin. Nevertheless, this study has several limitations, first and foremost among which is that wet cupping therapy was applied in only a single treatment. (It was applied only 48 hours after the CFA injection.) Therefore, it would be of interest and importance to conduct prospective studies to determine the longer effects bearing in mind that it will also be more relevant for patients with chronic pain. Inflammatory pain was a model for the current study. Thus, it would also be interesting to determine the effect of wet cupping therapy in a neuropathic pain model. Wet cupping therapy is mostly applied to healthy subjects as a preventive strategy; it is, therefore, prudent that the benefit and mechanism of wet cupping therapy as a preventive strategy be determined in an animal model.

Conclusion
Taken together, the present study showed that wet cupping therapy conferred pain relief. We assume that this benefit was mediated by a rise in the expression of HSP70 and β-endorphin.

Acknowledgement
Taken together, the present study showed that wet cupping therapy conferred pain relief. We assume that this benefit was mediated by a rise in the expression of HSP70 and β-endorphin. Imam Subadi and Raghwendra Mishra contributed equally to this work.

Conflict of Interest: None declared.

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