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INCREASING OF GLT-1 & TIME WITHDRAWAL LATENCY FOLLOWING WET CUPPING THERAPY IN CHRONIC CONSTRICTION INJURY RATS

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Abstract

Objective: Neuropathic pain (NP), a chronic pain, is caused by disease or lesion of somatosensory system. NP is still a big problem in medical treatment, making many patient seek alternative treatment. The more pain, the more frequent seeking alternative treatment. Wet cupping therapy (WCT), also known as bekam, is one of alternative treatment. WCT has been widely used to relief both of acute and chronic pain, but the mechanism to reduce the pain has not clear yet. Recent studies have shown that NP is associated with reducing pain and alteration of GLT-1/EAAT2, on the other hand CT has beneficial role to reduce the pain in various pain models. None of those have applied WCT in chronic constriction injury (CCI) models. Therefore, we investigate the association between WCT and the reducing pain by looking at the increased of GLT-1 and time withdrawal latency (TWL) in rats with CCI.

Methods: The study design was randomized post test only controlled group with total of 21 male rats (*Rattus Norvegicus*) with (CCI) aged 4 months, weighted 220 to 250g, divided in three groups, P1 as (sham CCI group), P2 (CCI group), P3 (CCI group plus WCT). WCT is applied 2 times/ week for 3 weeks to all of the groups in paralumbar region both left and right side. TWL is counted to assess pain treshold of the rats by hot plate and the expression of GLT-1 on glial cells in spinal cord were counted.

Results: There were significant differences on the TWL between groups P1-P2, P1-P3, & P2-P3 (p=0,003, p=0,0001, and p=0,0001 respectively) and GLT-1 increased significant between groups P2-P3 (p= 0.009).

Conclusion: It could be conclude that WCT decrease the pain by increasing the TWL and GLT-1 in CCI models. We suggest that WCT as a promising method to reduce pain in peripheral neuropathic pain models. However, further investigation is still needed to complete its mechanism.

Keywords: GLT-1/EAAT2, neuropathic pain, wet cupping therapy, CCI, TWL.

INTRODUCTION

Neuropathic pain (NP) is caused by disease or lesion of somatosensory nervous system (1). NP may be generated by either the peripheral or central nervous system, or both. Central NP is caused by poststroke pain ('thalamic pain syndrome'), pain due to spinal cord injury, and pain related to multiple sclerosis. Peripheral NP is commonly caused by painful diabetic neuropathy, postherpetic neuralgia, following amputation, thoracotomy, breast surgery and back surgery that is associated with nerve root fibrosis (2). NP is still a serious healthcare problem, often severe and difficult to be managed, resulting in a debilitating chronic condition that negatively affects the overall functioning and quality of life in patients and associated with a high economic burden for the individual and society (1–4).

Pharmacological and non-pharmacological therapies are the most common treatment modalities for patients with chronic pain (5). Pharmacological therapies for NP has divided into first-, second- and third-line drugs. First-line drugs for neuropathic pain include antidepressants [tricyclic antidepressants

(TCA) and serotonin–noradrenaline reuptake inhibitors (SNRI)] and anticonvulsants acting at calcium channels (gabapentin and pregabalin). Second- and third-line drugs for neuropathic pain are topical lidocaine and opioids (4). Non-pharmacological therapies of NP include: physical, psychotherapeutic treatment, and surgical (6).

NP responds worse to painkillers than other pain like visceral or somatic pain (3). The use of opioid will lead to complications such as abuse, diversion, and addiction. The pharmacotherapy of neuropathic pain is still unsatisfied due to the lack of it's effective treatment and its side effects (4). Since more than 30 yr ago, the World Health Organization has decised to develop the traditional medicine. This decision was based on two foundations; first: lack of access of a large number of people (up to 80% in several countries) to primary healthcare and second: dissatisfaction from the outcomes of treatments by modern medicine, especially in relation to chronic diseases and the side effects of chemical drugs (7). Furthermore, unsatisfactory of medical care in managing the pain is the most common reason for seeking therapeutic alternatives and the more severe the pain, the more frequent is the use of such therapies (8).

Cupping therapy (CT), one of alternatives therapy (9), is the oldest medical practice (10). CT has been known as bekam in Indonesia, *Al-Hijama* in Eigypt and Arab, *ventusynge* in central England, *ventoúza* in France (11–17). This therapy also have been used in like Korea (12), Arab, China, central Europe, some parts of Africa (5), the United Kingdom (UK) (13), Eigyptian (8), Taiwan (9), Finland (18), German (19), Indonesia and many others region and countries for various reasons like stroke rehabilitation (20), hypertension, balances the immune, nervous and hormonal systems, increase blood circulation in joints, dyslipidemia, asthma and allergy (19), and reduce the pain (19).

CT has been practiced for thousands of years and recently become increasingly available to be applied for the public (21) and obtained it's popularity and acceptance as a method for treating pain as well as sports injuries for athletes and other medical conditions (8) like low back pain (22), osteoarthritis, (5,19), migrain and other headaches (7), muscular spasm surrounding the joints, gouty arthritis, musculoskeletal pain, cancer pain, trigeminal neuralgia, rheumatic joints (19), carpal tunnel syndrome, brachialgia paraesthetica nocturna, cervicalgia (5), fibromyalgia (23). CT has beneficial effect for reducing chronic pain (19).

The use of effective therapies for reducing pain and its consequences is, therefore, of primary importance (4). The use of CT as suitable treatment for various types of chronic pain is become increasingly, but the action of mechanism of this treatment is still not clear yet. A through understanding to explain the mechanism of WCT in reducing pain is of vital importance (5).

Glutamate is the primary excitatory neurotransmitter of the central nervous system of mammalian, demontrate a pivotal role in normal pain transmission, the induction of central sensitization, the neuronal plasticity underlying pathological pain at the spinal level. Glutamate release in the spinal dorsal horn is obtained following nerve injury or peripheral inflammation. Several of evidence suggest that glutamate transporters is key roles in pathological pain. Functional deficiency or downregulation of glutamate transporter in dorsal horn of the spinal are related with neuropathic pain after CCI (24). GLT-1 has been shown as the most abundant of EAAT and may indicate the major route for the clearance of extracellular glutamate in the spinal cord (24).

We hypothesized that wet cupping therapy could reduce the pain by increasing TWL and GLT-1 in CCI models. An increase in GLT-1 would positively correlate with a rise of TWL in pain threshold test.

MATERIALS AND METHODS

Setting

This experiment has been conducted in animal research laboratory of school veterinary, Universitas Airlangga, Surabaya, East Java, Indonesia. All experiments were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia (Ethics No: 2.KE.015.01.2018).

Animals

21 male rats (Rattus Norvegicus) aged 4 months, with an average weight 220 to 250 g were used as animal models for this study. The animals were acclimatized for 7 days at constant temperature (26 °C) with 12 h light/dark cycle and allowed and were free fed food (Pelet BR 511, Comfeed, Indonesia) and water ad libitum. Subjects were divided into 3 groups (n = 7), P1 (sham CCI group); P2 (CCI group); and P3 (CCI plus WCT group). WCT period lasted 3 weeks, 2 times/ week. After 3 weeks (6x of WCT), the TWL were counted. One day after TWL, the spinal cords were removed then GLT-1 expression was counted using immunohystochmistry.

Chronic constriction injury (CCI) procedure

CCI procedure has described by Bennett and Xie (1988) then modified by Sommmer et al. During ketamin, xylacizine, and acepromazine anesthesia, after skin incision, the right side of the sciatic nerve was surgically exposed at mid-thigh level and freed from the adherent tissue proximal to the sciatic trifurcation. Four loosely-tied ligations (about 1 mm spacing) using chromic gut (5-0) were located around the right sciatic nerve, until the nerve diameter was slightly constricted just tightly enough touching the nerve without interrupting the epineural circulation.

Sham CCI procedure

The sciatic nerve of the sham CCI group was exposed but was not ligatured by chromic gut.

Wet cupping therapy (WCT)

After 7 adaptation days all of groups were applied with WCT using CPC (cupping, puncture, cupping) method. Cupping step is the application of two cups (2 cm in diameter) both of the left and right paralumbar regions of the skin rats & negative pressure (-200 mm Hg) was given for 5 minutes, then the cups were removed. Puncture step is the puncturing the same area of cup application in 10 punctures at each area. Cupping step was repeated with the same way, resulting in a small quantity of blood withdrawal.

Time withdrawal latency (TWL)

The time withdrawal latency (TWL) was counted using a stopwatch to asses the pain treshold of the rats by hot plate (Cold/Hot Plate Cat #35100, Ugo Basile, Varese, Italy) to all groups (P1,P2, and P3). TWL was counted from the time of placing the rat on the heated surface (51 °C) until a pain response, which was demonstrated by licking, rubbing, standing, and jumping out of the hot plate with 20-second cutoff time to prevent tissue damage. TWL was counted after 6 times of WCT.

Determining of GLT-1/ EAAT2 expression

Following the treatment, the animals were sacrificed by cervical dislocation and the spinal cord of the rats were removed, sliced and processed to measure GLT-1/EAAT2 expression by immunohistochemistry method. The expressions of GLT-1/EAAT2 positive glial cells were tested by immunohistochemistry using antibody monoclonal anti GLT-1/EAAT2 (EAAT2 (E1): sc-365634, Santa Cruz Biotechnology, Dallas, Texas, USA). The positive glial cells for GLT-1 expressions were counted under a light microscope (OlympusCX21, New York, USA).

Statistical analysis

The design of present study was a posttest-only control group. Normal distribution data were analyzed by ANOVA and followed by Least Significant Difference (LSD), whereas abnormal distribution data

were analyzed by Kruskall Wallis and followed by Mann Whitney U test. A value of $p<0.05$ was considered to be statistically significant. Data analysis was used SPSS ver. 22.

RESULT AND DISCUSSION

Table 1. Time Withdrawal Latency (TWL)

Variable	P1 Mean±SD	P2 Mean±SD	P3 Mean±SD	SI	ANOVA
TWL	7.20±1,30[a]	2.57±1.27[b]	18.20±3.50[c]	Second	0,0001*

*= Significantly with $p<0.05$
[a,b,c] (different superscript)= significant between groups

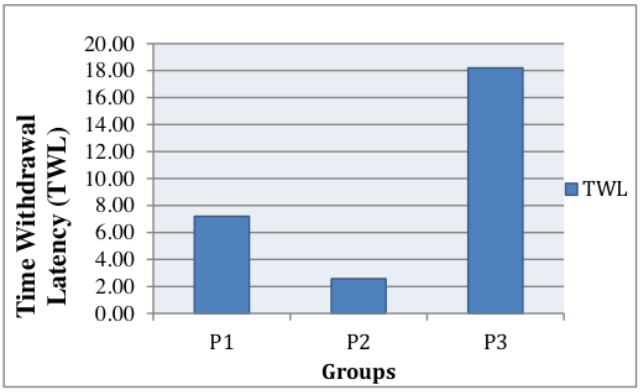

Fig. 1: Time Withdrawal Latency (TWL)

Table 1 and fig.1 showed TWL were counted in each group (P1;P2;P3) in the third week and the results were 7.20±1,30, 2.57±1.27, 18.20±3.50 respectively. Anova test reveals TWL count among group differs significantly with p=0,0001 ($p<0.05$), followed by LSD test with the result showed there were significant differences of TWL count between groups P1-P2, P1-P3, & P2-P3 (p=0,003, p=0,0001, and p=0,0001 respectively).

Table 2. Mean and median of GLT-1 of P1, P2, and P3

Variable	Category	Groups P1	P2	P3	SI	Kruskal Wallis
GLT-1	Mean	8.63[ab]	7.48[a]	10.93[bc]	-	0.029*
	Median	9.4	8.45	11.6		
	Minimum	5.6	1.2	9.2		
	Maximum	10.6	10.4	12		

*= Significantly with $p<0.05$
[ab,a,bc] (different superscript)= significant between groups

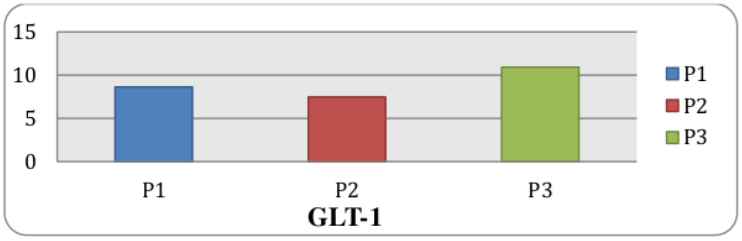

Fig. 2: The expression of glutamate transporter-1 (GLT-1) by immunohistochemistry

The determining of GLT-1 expression by immunohistochemistry was positive glial cells of spinal cord which were chromogen brown in color, whereas the negative reaction of the expression of GLT-1 did not show the chromogen brown color. Table 2 and fig.2 showed that GLT-1 expression were counted in each group (P1;P2;P3) and the mean results were 8.63; 7.48; 10.93 respectively. The distribution of GLT-1 expression from P1 to P3 is abnormal, thus using Kruskal Wallis with the result reveals significant difference among the gropus with p=0.029 (p<0.05), followed by Mann Withney U test with the result showed there were significant differences of GLT-1 count between the P2-P3 group with the results p=0,009 (p<0.05).

The purpose of our study was to evaluate the effectiveness of WCT in reducing neuropathic pain. Animal models are the key for understanding the neuropathic pain mechanism and development of effective therapy for its comprehensive and optimal therapy (25). We used CCI rats, developed by Bennet and Xie, as our peripheral mononeuropathy pain model which has contributed to open new opportunity of research into the mechanisms of all forms of neuropathic pain and the search for effective therapy (25,26). Study of CCI model has led to a better understanding of nociception and the events contributing to the pathogenesis of chronic pain states (26).

The constriction of the sciatic nerve in CCI model is related with focal ischemia, intraneural edema, and degeneration of Wallerian. Previuosly, it had been suggested that sensitization of C-fibers is responsible for the behavioral changes documented following injury or lesion in CCI rats, whereas recently it has been documented that partial lesion of the nerve causes both of A- and C-fibers sensitization, and thus perform an action in generating and preserving pain behavior. Previous study have been documented that the behavioral signs of spontaneous pain like mild to guarding, moderate autotomy, limping of their hind paw in ipsilateral side, excessive licking, and avoidance to place their weight on the injury side. The behavioral changes like thermal and mechanical hyperalgesia, cold allodynia, and chemical hyperreactivity have been documented to occur within one week and the maximal pain-related behaviors and asymmetries of postural rats develop in the second week of post-procedure. These neuropathic pain alterations have been documented to persist for at least 7 weeks after the procedure (25). Our study has shown the similar result that neuropathic pain of the CCI rats develop at third week of post-procedure.

Some studies have demonstrated that cupping therapy is a viable complementary or alternative therapy for treatment of chronic pain (19). There are 2 types of cupping therapy, namely dry cupping therapy (DCT) and wet cupping therapy (WCT) (12,27). We used WCT in our study because the WCT is superior than DCT (11). Following 6 times application of WCT, the neuropathic pain of CCI models has been significant reversed by WCT. Previous study has shown that cupping was performed twice times every week in total 5 sessions with the results can significantly reduce fibromyalgia pain. Cupping in non-specific neck pain was performed 5 times every in two weeks (28). Cupping was performed in various type of cupping (wet or dry cupping therapy), kind of cupping tools (manual or electric; plastic, glass, bamboo, or other materials), the depth of negative pressure, frequency, interval, total of cupping, duration of vacuum, total puncture in every area, and selection of the skin area to be applied with CT.

Although some researchers used various methods and parameters in different setting but the outcomes are always similar: cupping reduces pain. Our study revealed the same result with the previous study. Our study is the first found that WCT reduce the pain in CCI models.

Previously, it has become clear that inflammatory and immune mechanisms both in the central and the peripheral nervous system play a key role in neuropathic pain (29). Previous studies have revealed that the role of glutamate was not only a as neurotransmitter, but also as an important immunomodulator. Some of glutamate receptors and glutamate transporters, including GLT-1/EAAT2, have been widely explained in the central nervous system, include spinal cord, where they, respectively mediate glutamate effects and regulates the levels of glutamate in extracellular (30,31). Reuptake processes of Glu by glutamate transporter will modulate the Glu, and furthermore will modify the pain perception (32). The GLT-1 , on the glutamate transporter, is the most important transporter involved in maintaining the concentration of extracellular glutamate below neurotoxic levels (33). Our study has shown that WCT increase the GLT-1 expression on neuropathic pain models. Previous study hypothesized that WCT may function in a manner similar to acupuncture: it may stimulate particular parts of the body that include the release of neurotransmitters (22), this study confirmed that WCT can increase GLT-1 (transporter of glutamate neurotransmitter). Previous study has revealed that skin has the role as neuroendocrine organ. This concept combines the concepts of endocrinology, neurobiology, and immunology to unravel the multidirectional communications between brain, the endocrine and immune systems, and peripheral organs. The direct stimulation of dermal, adnexal, or subcutaneous cellular components could secondarily lead to the production of biological mediators with definite systemic effects and the activation of skin immune cells can enter the circulation and have distant immunological or regulatory effect (34). The application of WCT in the skin maybe could be explained by “the role of the skin as neuroendocrinology organ” concept. Future investigation is needed to explain the increasing of GLT-1 expression by application of WCT.

CONCLUSION

The result of our study and the studies previously done confirmed the suggestion of beneficial effect of WCT for reducing pain. Although some researchers used other model of pain (rats with inflammatory pain or human with various pain type), other method of WCT (puncture-cupping method), and different in parameters setting but the outcomes are similar: cupping therapy may reduce the pain. Our study revealed WCT reduce the pain significantly and increase the count of GLT-1 in CCI neuropathic pain model. Therefore future investigation is warranted to reveal its importance to explaine the mechanism of decreasing neuropathic pain.

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