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ORIGINAL RESEARCH PAIN MANAGEMENT

Increase in the glutamate transporter 1 and time withdrawal latency following wet cupping therapy in chronic constriction injury in rats

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Abstract

Objective: Neuropathic pain (NP) is a chronic debilitating pain and is caused by disease or lesion of somatosensory system. NP respond worse to the pharmacological drugs leading to this pain still a big problem in medical treatment and furthermore make many patients seek alternative treatment. Wet cupping therapy (WCT) has been widely used to relief both of acute and chronic pain, but the mechanism for reducing pain has not yet been clear. Recent studies have shown that NP is associated with alteration of GLT-1/EAAT2, and WCT has beneficial role to reduce the pain in various pain models. This is the pilot study, no other study has applied WCT in chronic constriction injury (CCI) models, the most commonly employed animal model of NP. Therefore, we investigate the association between WCT and the reducing pain by looking at the increase of GLT-1 and time withdrawal latency (TWL) in rats with CCI.

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

Results: This study revealed that mean ± SD values for P1, P2, and P3 were 7.20 ± 1.30, 2.57 ± 1.27, and 18.20 ± 3.50 respectively. There were significant differences in the TWL between groups P1-P2, P1-P3, and P2-P3 ($p = 0.003$, $p = 0.0001$, and $p = 0.0001$ respectively) and GLT-1 increase was significant between groups P2-P3 ($p = 0.009$).

Conclusion: It can be concluded that wet cupping therapy decreases the pain by increasing the time withdrawal latency and GLT-1 in chronic constriction injury models. We suggest that wet cupping therapy as a promising method to reduce pain in peripheral neuropathic pain models. However, further investigation is still needed to confirm its mechanism of action.

Key words: GLT-1/EAAT2; Neuropathic pain; Wet cupping therapy; Chronic constriction injury; CCI, TWL

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1. 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 post-stroke 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 is 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 have been 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 poorly to pain killers as compared to other pains like visceral or somatic pain.[3] The use of opioids will lead to complications such as abuse, diversion, and addiction. The pharmacotherapy of neuropathic pain is still unsatisfactory due to the lack of it's effective treatment and its side effects.[4] Since more than 30 years ago, the World Health Organization decided 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 treatment by modern medicine, especially in relation to chronic diseases and the side effects of chemical drugs.[7] Furthermore, unsatisfactory 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 the alternative therapies,[9] is the oldest medical practice.[10] CT has been known as bekam in Indonesia, *Al-Hijama* in Egypt and other Arab countries, *ventusynge* in central England, and *ventoúza* in France.[11–17] This therapy has been used in many Asian, African and European countires,[5,8,9,12,13,17-20] for various reasons, e.g., stroke rehabilitation,[21] hypertension, balancing the immune, nervous and hormonal systems, increasing blood circulation in joints, dyslipidemia, asthma and allergy,[20] and reducing the pain.[20]

CT has been practiced for thousands of years and has recently became increasingly available for the public,[22] and achieved popularity and acceptance as a method for treating pain, sports injuries in athletes and other medical conditions,[8] e.g., low back pain,[23] osteoarthritis,[5,20] migraine and other headaches,[7] muscular spasm surrounding the joints, gouty arthritis, musculoskeletal pain, cancer pain, trigeminal neuralgia, rheumatic joints,[20] carpal tunnel syndrome, brachialgia paraesthetica nocturna, cervicalgia,[5] and fibromyalgia.[24] CT has beneficial effects for reducing chronic pain.[20]

The use of effective therapies for reducing pain and its consequences is of primary importance.[4] NP is a chronic debilitating pain, and it responds poorly to pharmacological therapy leading to dissatisfaction and may lead to development of conditions such as allodynia, hyperalgesia and hyperpathia that negatively impact the overall functioning and quality of life of the patients.[3,4,25–27] WCT, a relatively simple, safe and economical of the traditional therapies,[19] has been used for long time as suitable treatment for various types of chronic pain.[20] But the mechanism of its action is still not clear yet.[5]

Glutamate is the primary excitatory neurotransmitter of the central nervous system of mammals, and demonstrates a pivotal role in normal pain transmission, the induction of central sensitization, the neuronal plasticity underlying pathological pain at the spinal level like neuropathic pain.[28,29] Glutamate is released in the spinal dorsal horn following nerve injury or peripheral inflammation. Evidence suggests that glutamate transporters have the key role in pathological pain. Functional deficiency or downregulation of glutamate transporters in dorsal horn of the spinal are related to neuropathic pain after CCI.[28] GLT-1 has been shown as the most abundant of glutamate transporters and may indicate the major route for the clearance of extracellular glutamate in the spinal cord.[28,29]

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.

2. Methodology

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 of 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 cycles 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 immunohistochemistry.

Chronic constriction injury (CCI) procedure:

CCI procedure has been described by Bennett and Xie (1988) then modified by Sommer et al. During ketamine, xylazine (an analogue of clonidine and an agonist at the α_2 class of adrenergic receptor), and acepromazine (acetylpromazine, a phenothiazine derivative antipsychotic drug) 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 catgut (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 catgut.

Wet cupping therapy (WCT):

After 7 adaptation days all of groups were applied with WCT using CPC (cupping, puncture and cupping) method. Cupping is the application of two cups (2 cm in diameter) at left and right paralumbar regions of the rats' skin and negative pressure (-200 mmHg) was given for 5 min, then the cups were removed. Puncture involves puncturing each area of cup application for 10 times. Cupping was repeated in 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 assess the pain threshold 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 cords 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 post-test-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 done on SPSS ver. 22.

3. Results

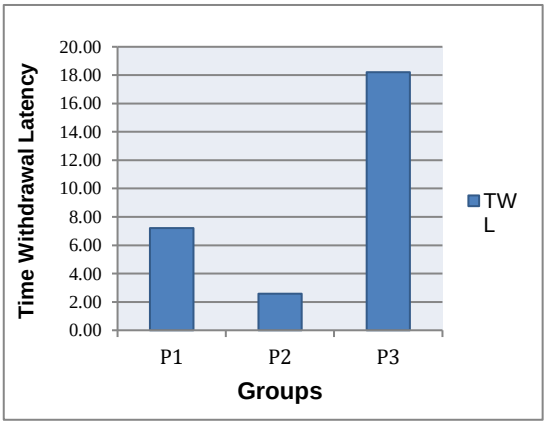

Figure 1: Time Withdrawal Latency (TWL) in sec

Table 1 and Figure 1 show TWL measured in each group (P1, P2 and P3) in the third week and the results were 7.20 ± 1,30, 2.57 ± 1.27, 18.20 ± 3.50 seconds 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, and P2-P3 (p = 0,003, p = 0,0001, and p = 0,0001 respectively).

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 Figure 2 show that GLT-1 expression measured in each group (P1, P2 and P3) and the mean results were 8.63; 7.48; 10.93 respectively. The distribution of GLT-1 expression from P1 to P3 was abnormal, thus using Kruskal Wallis with the result revealed significant difference among the groups with p = 0.029 ($p < 0.05$); followed by Mann-Whitney U test which showed there were significant differences of GLT-1 count between the P2-P3 groups with p = 0,009 ($p < 0.05$).

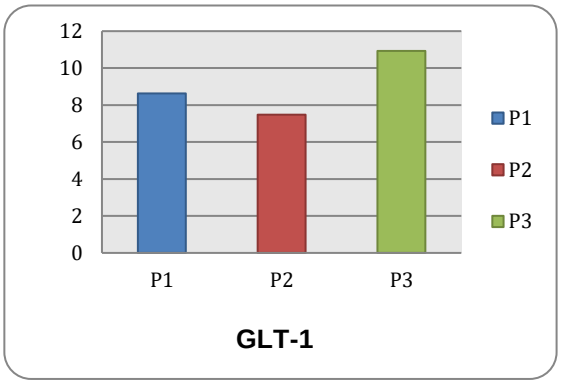

Figure 2: The expression of glutamate transporter-1 (GLT-1) by immunohistochemistry

4. Discussion

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.[30] 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.[30,31] Study of CCI model has led to a better understanding of nociception and the events contributing to the pathogenesis of chronic pain states.[31]

The constriction of the sciatic nerve in CCI model is related with focal ischemia, intraneural edema, and Wallerian degeneration. Previously, 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 studies have documented the behavioral signs of spontaneous pain e.g., mild 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 posture are developed by the rats in the second post-procedure week. These neuropathic pain alterations have been documented to persist for at least 7 weeks

Table 1: Time Withdrawal Latency (TWL) in sec (Mean ± SD)

Variable	Groups			SI	ANOVA
	P1	P2	P3		
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*

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

Variable	Category	Groups			SI	Kruskal Wallis
		P1	P2	P3		
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		

** = Significant with p < 0.05; [ab,a,bc] = significant between groups*

after the procedure.[30] Our study has shown similar result that neuropathic pain of the CCI rats develops at third post-procedure week.

Some studies have demonstrated that cupping therapy is a viable complementary or alternative therapy for treatment of chronic pain.[20] There are two types of cupping therapy, namely dry cupping therapy (DCT) and wet cupping therapy (WCT).[12,32] We used WCT in our study because the WCT is superior than DCT.[11] Following six times application of WCT, the neuropathic pain of CCI models has been significantly reversed by WCT. Previous study has shown that cupping was performed twice every week in total five sessions with the results can significantly reduce fibromyalgia pain. Cupping in non-specific neck pain was performed 5 times every two weeks.[33] 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 similar result to the previous study. Our study has been the first one to find that WCT reduces the pain in CCI models.

Some new studies indicate that immune cells have an important role as pain modulators, not just in inflamed tissues, but also in lesions of peripheral as well as CNS.[34] Previous studies have revealed that the role of glutamate was not only as a neurotransmitter, but also as an important immunomodulator. Some of glutamate receptors and glutamate transporters, including GLT-1/EAAT2, have been widely explained to be abundantly distributed in the central nervous system, including spinal cord, where they mediate glutamate effects and regulate the levels of glutamate in extracellular spave.[29] Reuptake processes of Glu by glutamate transporter will modulate the Glu, and furthermore will modify the pain perception.[35] The GLT-1 on the glutamate transporter, is the most important transporter involved in maintaining the concentration of extracellular glutamate below neurotoxic levels.[36] Our study has shown that WCT increase the GLT-1 expression on neuropathic pain models. A 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,[23] this study confirmed that WCT can increase GLT-1 (transporter of glutamate neurotransmitter). It has been revealed that skin has the role as neuroendocrine immune organ.[37]

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. The application of WCT in the skin could be explained by 'the role of the skin as neuroendocrinology organ' concept. Future investigation is needed to explain the increased GLT-1 expression by application of WCT.

5. Strength & Limitations

This study had strengths and limitations. The used sham-CCI groups in this study is one of the strengths to control CCI groups. A sham-CCI operation was performed to confirm the consequence of nerve injury by exposure of the sciatic nerve without ligation.[39] Inspite of the strengths, the research also had the limitations such as there is no negative control of wet cupping therapy in this study. Future study might consider using acupuncture, dry-cupping, or other alternatives medicine therapy in an RCT design compared with wet-cupping therapy.

6. 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 explain the mechanism of decreasing neuropathic pain.

7. Conflict of interests

This work was supported by Universitas Airlangga, Surabaya, East Java, Indonesia.

8. Authors' contribution

All authors contributed equally.

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