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ORIGINAL RESEARCH **PAIN MANAGEMENT**

Wet cupping therapy increases the time withdrawal latency (TWL) and decreases GABA-A receptor expression in the spinal cord in a rat model of neuropathic pain

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Abstract

Background & Objective: Neuropathic pain (NP) is a pain due to a somatosensory lesion. NP leads to disruption of health, work, social relationships, hobbies, sleep, mood, and cognitive function. Up till now, the treatment of NP remains unsatisfactory. It makes many patients seek alternative therapies, including wet cupping therapy (WCT). We aimed to analyze the effects of WCT against NP in chronic constriction injury (CCI) in a rat model by assessing the increase in time withdrawal latency (TWL) and GABA-A receptors expression in the spinal cord.

Methodology: We used CCI as one of the NP models in *Rattus norvegicus* species of rats and treated with WCT. We used 21 four months old male rats, divided into 3 groups (n = 7); P1 (sham CCI group), P2 (CCI group), and P3 (CCI plus WCT group). WCT was given two times every week for three weeks with 5 min of first cupping using −200 mmHg, and followed by ten punctures and then 5 min of second cupping using −200 mmHg. TWL assesment was conducted by using hotplate. Expresion of GABA-A receptor was evaluated with immunohistochemistry.

Results: WCT significantly increased TWL with P = 0.0001 and decreased expresion of GABA-A receptors in the spinal cord of CCI rat model.

Conclusion: This research concluded that wet cupping therapy could increase time withdrawal latency and decrease the expression of GABA-A receptors in the spinal cord in chronic constriction injury rat model. This result suggests a promising method in controlling neuropathic pain. However, further investigations are required to understand the mechanism.

Abbreviations: CCI: Chronic Constriction Injury; CT: Cupping Therapy; DCT: Dry Cupping Therapy; NP: Neuropathic Pain; TWL: Time Withdrawal Latency; WCT: Wet Cupping Therapy;

Key words: Chronic constriction injury; CCI rat model; GABA-A receptors; Neuropathic pain; Pain; Time withdrawal latency; TWL; Wet cupping therapy.

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1. Introduction

Pain, including neuropathic pain, is the major reason for people to visit health care providers.[1] Neuropathic pain (NP) has been defined as pain due to lesion or disease of the somatosensory system. It can be caused by injury as well as lession or disease of nerve, spinal cord, or brain injury. NP is maladaptive response of the somatosensory system lesion and characterized by hyperalgesia, allodynia, spontaneous pain (paresthesia, tingling, burning sensation, pins, and needle sensation, dysesthesia, etc.), and even reduced or loss of sensitivity (anesthesia).[2–4] NP can be classified either based on etiology (painful diabetic neuropathy, trigeminal neuralgia, postherpetic neuropathies, etc), or based on the lesion anatomy (central and peripheral). NP is also classified based on duration as acute (persists for less than 3 months) and chronic (persists for more than 3 months).[5–7]

NP is a global burden. In the general population, 3–17% of people experience neuropathic pain. Exact prevalence of NP within the global population is still unknown. Most studies estimates the prevalence of NP at between 1.5−8%, equalizing to between 100−560 million of people world-wide.[2,8] Chronic debilitating NP responds poorly to analgesic. It causes a decline in quality of life, a lack of productivity, and an increase in health care costs.[2,3,9]

The management of NP includes prevention and therapy. Prevention strategies consist of vitamin E, glutathione, magnesium, and calcium use. Therapy includes pharmacological and non pharmacological. Pharmacological therapies consist of the first-line drugs (gabapentinoids like gabapentin or pregabalin, tricyclic antidepressants, and selective serotonin–norepinephrine reuptake inhibitors like duloxetine or venlafaxine, carbamazepine, oxcarbazepine), the second-line treatment (lidocaine patch, capsaicin, and tramadol) and the third-line treatments (opioids, baclofen, and cannabinoids).[10–12] Invasive treatments such as physical like electrical stimulation of the cortex and acupuncture, psychotherapeutic treatment, and surgical is also used in NP management and numerous studies classified them as non pharmacological therapies. Other non-pharmacological therapies that is much less invasive are also proven to be effective in reducing NP such as hydrotherapy.[10,13,14]

NP is still a major issue in health worldwide. NP is difficult to treat effectively, with only a minority of people experiencing a clinically relevant benefit from any intervention. Pharmacological therapy is still far from being satisfactory or even effective due to its side effects, including somnolence, peripheral edema, weight gain, sleepiness, orthostatic hypotension, and others. These side effects also contribute to its lack of efficacy and limited tolerance. It makes many NP's patients seek alternative treatment.[3,11,15–17] One of the alternative and traditional medicines is cupping therapy (CT). CT is the oldest medical practice both in Western and Eastern countries. It has been used for thousands of years for a variety of objectives, including the treatment of autoimmune illnesses like psoriasis, the reduction of inflammation, the treatment of many musculoskeletal disorders, and the alleviation of pain in pain management.[18–20] There are two types of CT: wet cupping therapy (WCT) and dry cupping therapy (DCT). WCT, a common intervention, has been widely used and proved to be effective in reducing various pains (acute or chronic).[18,21,22]

γ-aminobutyric acid (GABA-A) receptors activation alleviates pain in inflammatory and NP mouse models. The role of GABA-A receptors as an inhibitory neurotransmitter receptors in spinal dorsal horn, one of NP pathways, has been established.[23,24] One of the most widely used animal models in NP research is Bennett and Xie's (1988) unilateral sciatic nerve chronic constriction injury (CCI) that is performed in rats.[2,25] The mechanism of WCT to reduce NP is not clear yet. We hypothesized that WCT could reduce the pain by increasing time withdrawal latency (TWL) and decreasing GABA-A receptors expression in the spinal cord of CCI models.

2. Methodology

This study has been performed in animal research laboratory of school veterinary of Universitas Airlangga, Surabaya, Indonesia. The whole procedures were conducted on animals with approval from the Ethics Committee of Veterinary Medicine of Airlangga University in Surabaya, Indonesia (No. 2.KE.015.01.2018).

2.1. Animals

We carried out this experiment on 21 healthy male *Rattus Norvegicus*, 4 months old and weighing from

220−250 g. The animals were housed for 7 days under standard conditions (26°C constant temperature, 12 h light/dark cycles) for acclimatization and were free to access food (Pelet BR 511 from Comfeed Indonesia) and water. Animals were divided into three groups, with 7 rats each: Group P1 was sham CCI group; Group P2 was CCI group; and Group P3 was CCI plus WCT group. The WCT was performed 2 times/week and lasted for 3 weeks. TWL was counted after the WCT has been completed (6 x WCT), followed by the collection of spinal cords at the next day for GABA-A receptor expression measurement using immunohistochemistry.

2.2. Chronic Constriction Injury (CCI) Procedure

The CCI procedure was carried out according to Bennett and Xie.[2,25] To conduct this procedure, the subject were anesthesized using ketamine, xylazine, and acepromazine. The skin was incised and further surgical procedure was performed to expose the right side of the sciatic nerve at mid-thigh level. The nerve should be freed from the adherent tissue proximal to the sciatic trifurcation. The right sciatic nerve was then ligated loosely in four sites located around it, using chromic catgut (5-0) and each ligation is separated by a distance of 1 mm. This procedure would slightly constrict the nerve diameter, which would not disrupt epineural circulation while producing the desired effect.

2.3. Sham CCI Procedure

The procedure of sham CCI was similar to CCI procedure (exposing sciatic nerve) but in sham CCI procedure it was conducted without the ligation of the nerve.

2.4. Wet Cupping Therapy (WCT)

The WCT was performed on group P3 after 7 adaptation days. The methods of WCT used were cupping, puncture and cupping (CPC) method. Cupping was applied using two cups with 2 cm in diameter. These two cups were placed on bilateral (right and left) paralumbar regions of rats using negative pressure (−200 mmHg) for 5 min and then were removed from the skin. After cupping removal, the skin in the cup application area was then punctured for 10 punctures. The last step was repeating cupping procedure soon after puncturing to allow small quantity of blood withdrawal.

2.5. Time Withdrawal Latency (TWL)

TWL was performed to assess the rat's pain threshold in all groups (P1, P2, and P3) using hot plate exposure (Cold/Hot Plate Cat #35100, Ugo Basile, Varese, Italy). This procedure was counted using a stopwatch from the time the rats were placed on heated surface (51°C) until any pain responses were observed (licking, standing, rubbing, and jumping out of hot plate). The cutoff time to perform TWL was 20 sec to avoid tissue damage. TWL was performed after 6th WCT.

2.6. Determining Of GABA-A Receptors Expression

At the end of experiment, the animals were euthanized using cervical dislocation method and the spinal cords were collected. After the rat's spinal cords were isolated, they were sliced and processed further. Immunohistochemistry method was applied in this research to determine GABA-A receptor expression. Monoclonal antibody that was used to test the expressions of GABA-A receptors on glial cells is anti GABA-A receptors (Cat. No. NBP2-43558) was purchased from Novus Biologicals (Canada) and the counting of positive glial cells (the glial cells that harbour GABA-A receptor expression) was performed under a light microscope (OlympusCX21 from New York, USA).

2.7. Statistical Analysis

This research used a post-test-only control group design. Analyzing normal distribution datas were performed by ANOVA and followed by Least Significant Difference (LSD). Data with an abnormal distribution were analyzed with Kruskall Wallis and followed by Mann-Whitney U test. The $P < 0.05$ was considered significant. All the analysis of the datas were conducted on SPSS version 22.

3. Results

The results in Table 1 and Figure 1 show TWL counting in each group (P1, P2 and P3) in the third week. TWL measurement results were 7.20 ± 1.30 sec for P1 group, 2.57 ± 1.27 sec for P2 group, and 18.20 ± 3.50 sec for P3 group. Analysis using ANOVA showed that TWL count among groups were significantly different with $P = 0.0001$ ($P < 0.05$). Further analysis with the least significant difference (LSD) test revealed that TWL count between P1-P2, P1-P3, and P2-P3 groups were different significantly ($P = 0.003$, $P = 0.0001$, and $P = 0.0001$ respectively).

GABA-A receptors expression were determined by immunohistochemistry. The GABA-A receptors were shown by positive glial cells of spinal cord which were chromogen brown in color, whereas the negative reaction of the expression of GABA-A receptors did not show the chromogen of brown color. Table 2 and Figure 2 have shown that GABA-A receptors expression measured in each group (P1, P2, and P3)

Table 1: Results of time withdrawal latency (TWL) in third week in sec (Mean ± SD)

Groups	Variable	Annova
	TWL	
P1	7.20 ± 1.30[a]	0.0001*
P2	2.57 ± 1.27[b]	
P3	18.20 ± 3.50[c]	

**= statistically significant with P < 0.05 according to ANOVA.*
[a,b,c]= Different superscript letters indicate significant differences (P < 0.05) according to least significant difference (LSD) test.

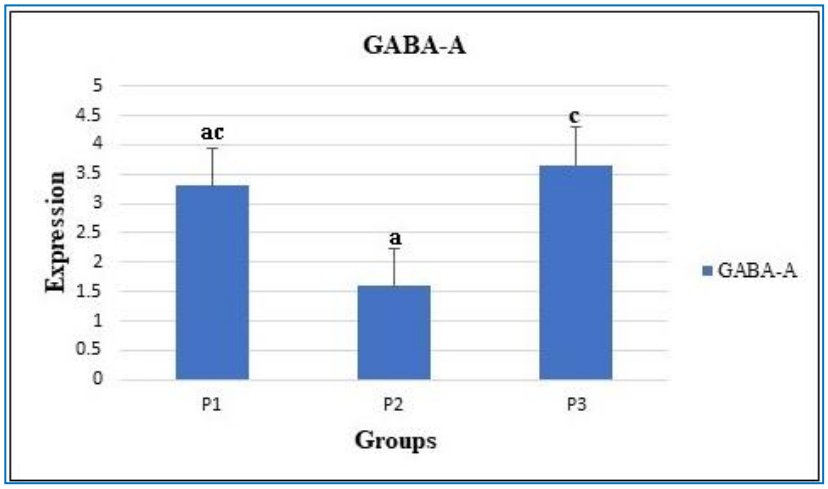

Figure 2: The expression of GABA-A receptors by immunohistochemistry.

and the mean results were 3.31; 1.6; 3.66 respectively. The results revealed that the GABA-A receptor expression mean values of P2 group (CCI group) in 10 expression/ visual field were lower were than P1 group (sham –CCI group) and P3 group (CCI+WCT group).

The distribution of GABA-A receptors expression from P1 to P3 was abnormal, thus Kruskal Wallis revealed significant difference among the groups with P = 0.001 (P < 0.05). It is followed by Mann-Whitney U test. Mann-Whitney U test showed that there were significant differences of GABA-A receptors expression count between the P2-P3 groups with P = 0,001 (P < 0.05).

4. Discussion

Neuropathic pain (NP) have been explained by a lesion or disease of the somatosensory system.[3–5] The somatosensory nerves are found in the skin, joints fascia and muscles, and include mechanoreceptors, thermoreceptors, pruriceptors chemoreceptors, and nociceptors. The signals from these receptors are sent to the spinal cord and finally to the brain

for being processed further involving a thalamic nucleus that receive a sensory signal directed to the cerebral cortex.[9]

Due to its chronicity nature and side effects, the NP treatment is still ineffective and far from ideal.[3,11,15,17] CCI, a well established-animal model of peripheral NP, holds an important contribution in better understanding of NP mechanism. It produces strong of pain hypersensitivity that occur within one week and stable for at least one month after injury.[2,3,25] Our study used CCI model hypersensitivity was seen within 6 weeks following CCI. We saw spontaneous pain behaviour in our study, like: licking, standing, rubbing, and jumping out of the hot plate as NP appearance.

Cupping therapy (CT) has been widely practiced for thousands of years to relieve various types of pain such as muscle sprains, trigeminal neuralgia, rheumatoid arthritis, arthralgia, low back pain, and lumbar disc herniation, radiculopathy, and etc.[18–20] There are two types of cupping include wet cupping therapy (WCT) and dry cupping therapy (DCT). Both of them are pulling the skin up to the cup. In DCT it is not followed by drawing blood, but in WCT the skin is punctured leading to the blood suction into the cup.[21,22] WCT has been proven for reducing pain than DCT. WCT has ability to restore the normal physiology and eliminate the causative pathological substances (CPS), whereas DCT diluted and redistributed the CPS to new sites.[20,26]

A current systematic review included five trials, two

Table 2 : GABA-A receptor expression by immunohistochemistry

Variable	Category	Group			Kruskal-Wallis
		P1	P2	P3	
GABA-A receptor	Mean	3.31	1.6	3.66	0.017
	Median (min−max)	3.2 (2−4.8)	1.4 (1−3.2)	3.8 (1−6.2)	

randomized clinical trials (RCTs) and three controlled clinical trials (CCTs), on musculoskeletal problems found that WCT is effective to treat low back pain.[5] Other study, RCT conducted in Iran has shown that 3 times of WCT with three days interval resulted in reducing pain intensity.[26]

Some changes in the dorsal horn following nerve injury play important roles in encouraging and maintaining NP.[23] Yet, the precise alterations that play significant role in the generation and/or maintenance of NP are still unclear. One of these is the alteration of GABA signaling which is the main inhibitory neurotransmitter in mammalian CNS, mediating its effects via activation of GABA-A and GABA-B receptors.[17] GABA mediates anti-hyperalgesic effects in NP rats.[27] GABA and GABA-A receptors binding particularly opens up a Cl^- permeant anion channel and then the influx of Cl^- ions make the cell being hyperpolarized. GABA and GABA-A receptors binding mediates anion efflux that allows depolarization of the primary afferent. The consequent shunting of incoming action potentials resulting in reducing of excitatory transmitter which release from nociceptive terminal endings (presynaptic inhibition) plays an important role in controlling neuronal hyperexcitability in the spinal dorsal horn.[17]

GABA-A receptors, predominate within superficial layers, receive nociceptive input from primary afferents. Behavioral studies using comparative pharmacology studies in rats and genetically-modified mice have recently concluded α2- and α3- subunits (and may possibly α5 subunit)-containing GABA-A receptors as main contributors of the spinal disinhibition that occurs following inflammatory or neuropathic lesion. Taken together, all of these complimentary approaches have shown that it is the restoration of post-synaptic actions of GABA on intrinsic dorsal horn neurons which mediates analgesia.[17]

Spinal GABA-A receptors are main elements for pain nociception and contribute to chronic pain status and thus are specific targets of analgesia.[28,29] GABA affects the inhibition of the dorsal horn spinal cord both pre-synaptic and post-synaptic. Loss of synaptic inhibition in the dorsal horn of spinal cord is thought to contribute significantly in pathological pain.[29] Previous studies that investigated the GABA-A receptor agonist, namely NP260, concluded its ability to reduce neuronal hyperexcitability and relieve allodynia in mouse model of NP. [24]

There have been no previous studies examining the expression of GABA-A receptors in the spinal cord after wet cupping treatment in the CCI model. Previous studies have shown that benzodiazepines (BDZ) bind to the GABA-A receptor and upon therapeutic administration of HZ166, a partial BDZ agonist, suggesting a dose-dependent antihyperalgesia effect of the CCI model. When comparing between HZ166 and gabapentin, a drug widely used for neuropathic pain treatment, the effectiveness of both drugs showed similar results in reducing CCI-induced pain.[30] The explanation of GLT-1 expression decrement by application of WCT requires more investigation in the future.

The decrease of dorsal horn contents of GABA synthesizing enzyme glutamic acid decarboxylase (GAD) 65 kDa ipsilateral to the lesion and neuronal apoptosis which is detected by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling staining in identified neurons are induced by partial nerve lesion. These processes could lower the presynaptic GABA contents and encourage functional loss of GABAergic transmission in the superficial dorsal horn.[31]

An increase of GABA-A receptors expression would positively correlate with a rise of TWL in pain threshold test. The major inhibitory neurotransmitter γ-aminobutyric acid (GABA) has a regulatory control on the levels of neuronal excitability.[8–10] Thus, reduced GABAergic activity can result in increased neuronal activity in the anterior cingulate cortex (ACC) and is associated with neuropathic pain.

γ-aminobutyric acid (GABA) is one of the most important neurotransmitters and plays role as a regulator to control the levels of neuronal excitability and the primary inhibitory neurotransmitters in the central nervous system (CNS). Studies in human showed that admnistration of GABA agonist has potent effect in pain management.[32–34] GABA is also widely associated with chronic neuropathic pain where it arises from lesion to the peripheral and central nervous system. The association of GABA with chronic pain syndrome is supported by a number of studies such as the anatomic distribution of GABA receptors and the ability of GABA agonists to modify nociceptive responses.[25] Other studies have also shown that the loss of inhibitory pain control in the spinal dorsal horn has been shown to replicate the occurrence of chronic pain status.[35]

5. Conclusion

Since research into pain has not yet progressed to a desirable level, it is still a widespread concern today. A wet cupping is different form of therapy to lessen pain and it appears promising.

6. Data availability

The data generated is available with the authors.

7. Conflict of interest

Authors declare no conflict of interest.

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This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

9. Authors' contribution

HBH: conception of the work, literature research, manuscript drafting

EQ: literature research, performed the analysis, manuscript editing

W: conception of the work, literature research, manuscript writing

SK: literature research, manuscript writing

VAP: literature research, manuscript reviewing and revising

RDS : literature research, manuscript reviewing and revising

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