

# Effects of holothuria extract on pain behaviour and Fos like immunoreactivity (FLI) in formalin pain

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## ABSTRACT

**Introduction:** The aim of this study was to determine the effects of gamat extract on pain behaviour and Fos like immunoreactivity (FLI) expression in the ventral posterolateral thalamus using the acute pain model. **Materials & Methods:** Fourteen Sprague-Dawley male rats (220-300 gram) were given intraplantar injection of 0.05ml formalin (1%) followed by intraperitoneal administration of either 4 mg/kg gamat extracts (*Holothuria* spp.) or saline (control). Behavioural changes were observed and rats were sacrificed 2 hours post-formalin injection. Immunohistochemistry testing was done on the brain sections. FLI was examined using a light microscope attached to an image analyser. The behaviour and FLI data were analysed using repeated measure analysis of variance and independent t-test respectively. Significance level was taken as  $p < 0.05$ . **Results:** The control group has significantly higher pain scores compared to holothuria group ( $F(1) = 13.635$ ,  $p = 0.003$ ). There was significant reduction in the pain behaviour score in the holothuria group when compared to the control group in phase 1 ( $t(14) = 2.9$ ,  $p = 0.012$ ) and most of the time from 15 to 60 minutes post-formalin injection ( $t(12) = 3.535$ ,  $p = 0.004$ ). There was a significant reduction ( $P < 0.05$ ) in the number of FLI on the contralateral aspect of the ventral posterolateral thalamic nucleus in the group that received 4mg/kg of holothuria extract ( $63 \pm 3.18$ ) compared to control group ( $84 \pm 6.36$ ). **Conclusion:** This study showed that administration of holothuria extract significantly suppressed the pain behaviour and reduced the number of FLI in formalin injected rats compared to control.

**KEYWORDS:** Pain, behaviour, holothuria

## INTRODUCTION

C-fos and its protein products are expressed in the neurons in response to noxious stimulation and they induce conversion of information encoded in the c-fos gene to messenger RNA rapidly within minutes after a particular stimulus and transiently.<sup>1,2</sup> Further, c-fos is widely used as a tool in pain research and is a marker for neuroplasticity.<sup>3,4</sup> Neuroplasticity is initiated by afferent input generated by intense noxious stimuli that trigger an increased excitability of nociceptive neurons in the central nervous system.<sup>5</sup> Neuroplasticity is expressed clinically as pathological pain e.g. hyperalgesia and allodynia. It is important to prevent neuroplasticity from occurring as 'pathological pain' is more difficult to treat.<sup>6,7</sup>

There are evidences for the important role of the ventral posterolateral thalamic nucleus in pathological pain responses.<sup>8,9,10</sup> Studies have shown that the ventrobasal thalamic neurons had lower thresholds to pain and their peripherally-evoked responses were enhanced following hindpaw inflammation and nerve injury.<sup>9,10,11</sup> Furthermore, the N-methyl-D-aspartate (NMDA) receptors in the thalamus contributed to hyperalgesia in the rat model of inflammatory pain.<sup>12</sup> Studies have shown that c-fos is expressed in the central nervous system in this acute pain model.<sup>13,14</sup> Inhibition of c-fos expression in the central nervous system has been shown by modern medication e.g. morphine and ketamine.<sup>15,16</sup>

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A traditional medication, gamat (*Holothuria* spp) was shown to promote tissue healing and it was widely used for wound healing in the postpartum period.<sup>17,18,19,20</sup> Another report has demonstrated the antinociceptive property of gamat.<sup>21</sup> Gamat administration in mice has inhibited the abdominal contraction induced by acetic acid.<sup>21</sup> However, up to date, very little is known regarding the effects of gamat on c-fos expression in the central nervous system. The main aim of the present study was to determine the effects of gamat extract on pain behaviour and c-fos expression in the thalamus using the formalin pain model. Results from this investigation could throw

some light as to the possible interaction of the gamat extract with function of the nervous system involved in pain modulation.

**MATERIALS AND METHODS**

**Animals**

Fourteen adult male Sprague-Dawley rats weighing between 220-300g were involved in the study. The rats were housed in individual cages and allowed adaptation for at least three days in the Physiology Department laboratory. They were maintained in a 12-hour light dark cycle and allowed free access to food and water. Investigations were conducted between 0800 and 1600 in the Physiology Department laboratory. Animals were obtained from Laboratory Animal Research Unit, Universiti Sains Malaysia (LARUSM). The present study was approved by the Animal Ethics Committee of Universiti Sains Malaysia.

**Preparation of holothuria extracts**

A dissection was made on the gamat and the internal organs were removed. An air oven (Binder BD 115) was used to dry the gamat tissues at 58°C for two weeks. Following this, the tissues were then blended to produce powder material. Petroleum ether was mixed with the powder material before the solution was then poured into a funnel. The material was collected in a beaker and was left to be separated into organic component (upper portion) and non-organic component (lower portion). The organic component was left to dry for 12 hours at -48°C and 200atm us-

ing freeze dry process (Ilshin Lab. Co. Ltd).<sup>22</sup> These processes produced powder extract which was stored in a refrigerator until used.

**Drugs Used and Experimental Groups**

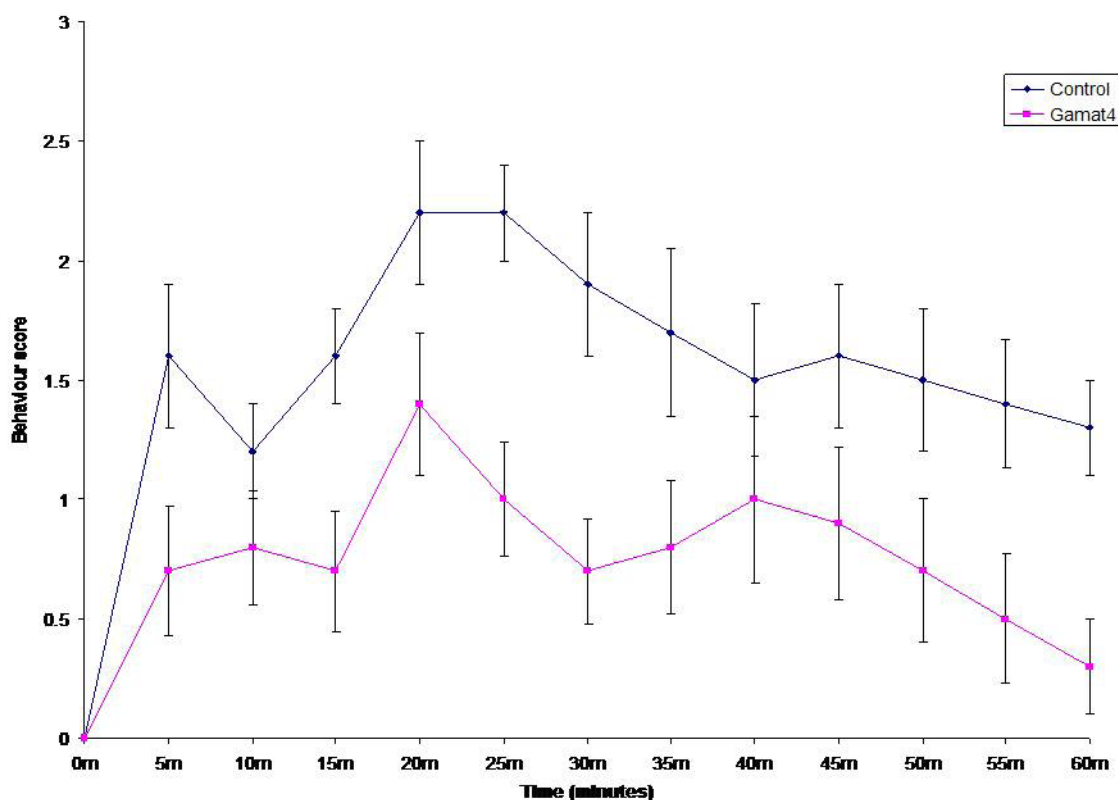
The rats were divided into two groups (gamat and saline) with 7 rats in each group. The experimental group (gamat) was given 4 mg/kg holothuria extracts while control rats were given saline. Holothuria extracts and saline were given at a volume of 1 ml/kg and administered intraperitoneally.

**Behaviour testing**

Both groups of rats were given intraplantar injection of 0.05ml formalin (1%) followed immediately by either intraperitoneal holothuria extract or saline according to their respective groups. They were then observed for behavioural changes for an hour before being sacrificed and the brain removed for immunohistochemistry testing as described below.<sup>23</sup> The behavioural data were analysed by two observers blinded to the treatment of each rat and behaviour pain score was tabulated at each minute and averaged at 5-minute intervals.<sup>23</sup> The quantification was based on the total time spent in 4 behavioural categories.<sup>23, 25</sup>

**Sacrifice of Animals and Perfusion-fixation of thalamus**

All rats were sacrificed with an overdose of sodium pentobarbitone intraperitoneally 120 minutes after formalin injection.<sup>26</sup> Thoracotomy was performed and



**Figure 1:** Behaviour score in the group receiving saline (control) and 4 mg/kg holothuria extracts (gamat 4) (n=7 for both groups). Values are means±S.E.M ( \*p<0.05; \*\* p<0.01) comparison between holothuria 4 mg/kg and control groups)

the heart was exposed immediately following the loss of their pinch reflex. An 18G cannula was inserted into the apex of the left ventricle and the right atrium was snipped with a pair of scissors.<sup>23</sup> Perfusion was performed by gravity method using first phosphate-buffered saline (PBS) until the fluid ran clear, followed by 500ml of cold 4% paraformaldehyde in phosphate buffer (PB) 0.1 mol/litre (pH = 7.4).<sup>23,27,28</sup> The brains were then removed from the cranial cavity.

#### Dissection of Brain and Cryostat Sectioning

The brains were post-fixed in fresh perfusion solution (4% PFA in PB 0.1 M) at 4°C for 4 hours, and cryoprotected overnight in 20% sucrose in phosphate buffer 0.1 M at 4°C.<sup>29</sup> The brains were next embedded in tissue freezing medium (Jung) and sliced into 20-µm sections using a cryostat. 27 Sections were transferred using a paintbrush to a 24-well multiwell plate containing 500µl of PBS in each well.

#### Immunohistochemistry

A three-step peroxidase avidin-biotin complex (ABC) method (purified primary antibody, biotinylated secondary antibody, and ABC with DAB) was used to stain the brain sections for localising fos protein.<sup>30</sup> The immunohistochemistry test was performed as described previously by Asma Hayati et al.<sup>23</sup> Following the test, all sections were mounted on gelatin-subbed slides and air-dried overnight. Slides were then dehydrated with absolute ethanol for 15 minutes, mounted with Styrolyte Mounting Medium and protected with a cover slip.<sup>23,31</sup>

#### Counting of fos like immunoreactivity (FLI) labelled neurons

Sections were examined using light microscopy attached to an image analyzer (Leica QWin) and ventral posterolateral thalamic nucleus was determined using the rat's brain atlas.<sup>32</sup> Immunohistochemically detected nuclear-associated reaction product was referred to as FLI.<sup>33</sup> FLI labelled neurons were counted by two blinded investigators and the counts were then averaged for each rat. Counts that were questionable in

either quantity or quality were excluded. Images of the brain sections were captured at x25 to determine the area and at x50 to localize FLI labelled neurons.

#### Statistical Analysis

Pain behaviour scores by formalin test were analyzed using one-way repeated measures ANOVA with one within subjects factor (time: 13 levels) and two between subjects' factors (drug: 2 levels, holothuria extract and saline). Independent t-test was used to analyze the effects of Phase 1 formalin test (mean score at 5 minutes) and Phase 2 (mean of scores from 10 to 60 minutes) between saline and holothuria extract and significance level was accepted at  $p < 0.05$ .

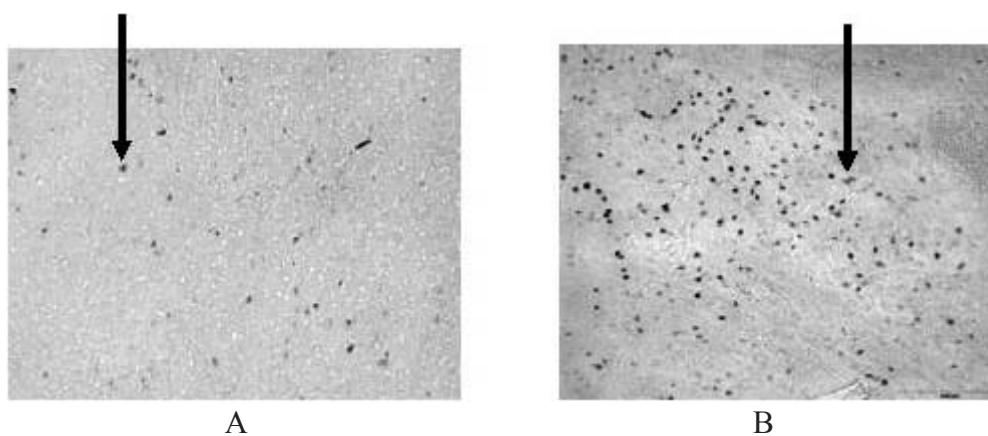
#### RESULTS

##### *Effects of holothuria extract on pain behaviour.*

In the control group, there was an increase in the pain behaviour score in the first five minutes (phase 1) followed by 5 to 10 minutes of reduced nociceptive behaviour (figure 1). Following this, increased pain behaviour was noted from 15 minutes to sixty minutes post formalin injection (phase 2). The control group has significantly higher pain scores compared to holothuria group ( $F(1) = 13.635$ ,  $p = 0.003$ ). There was significant reduction in the pain behaviour score in the holothuria group when compared to the control group in phase 1 ( $t(14) = 2.9$ ,  $p = 0.012$ ) and most of the time from 15 to 60 minutes post-formalin injection ( $t(12) = 3.535$ ,  $p = 0.004$ ).

##### *Effects of holothuria extract on FLI expression in the ventral posterolateral thalamic nucleus.*

There was significant reduction ( $p < 0.05$ ) in the number of FLI on the contralateral aspect of the ventral posterolateral thalamic nucleus (figure 2) in the group that received 4mg/kg of holothuria extract ( $63 \pm 3.18$ ) compared to control group ( $84 \pm 6.36$ ). However, the number of FLI on the ipsilateral side was not significantly different in both of the groups (control:  $26.4 \pm 2.96$ ; holothuria:  $21.4 \pm 1.50$ ).



**Figure 2:** Photomicrographs showing FLI in contralateral side (left side) of ventral posterolateral thalamic nucleus in the (A) holothuria and (B) saline groups (50× magnification). Arrows indicate the dark staining FLI.

## DISCUSSION

In the present study, the effects of intraperitoneal administration of holothuria extracts on pain behaviour in an acute pain model were investigated. The group receiving 4 mg/kg of holothuria extracts demonstrated a significant reduction in pain behaviour compared to control in phase 1 and most of the time in phase 2 ( $p < 0.01$ ). The analgesic property of gamat is in agreement with a report by Ridzwan et al. which demonstrated the ability of gamat extract to inhibit the abdominal contraction in acetic acid induced writhing test.<sup>21</sup> In the present study, the formalin pain model was used. The formalin pain model was chosen as the pain mechanisms in this model are well-known; phase 1 is contributed by peripheral mechanism while phase 2 is mainly due to central nervous system hyperexcitability.<sup>34</sup>

Intraperitoneal administration of holothuria extracts (4 mg/kg) has significantly inhibited the pain behaviour in both phases. Phase 1 involves chemical stimulation of nociceptors and activation of C fibers in the periphery while hyperexcitability of central nervous system is involved in phase 2.<sup>23,25,34,35</sup> The nociceptive behavior in phase 1 was partly contributed by the formation of prostaglandin that generated the signs and symptoms of inflammation.<sup>36</sup> The activation of NMDA receptors that leads to hyperexcitable dorsal horn neurons as well as local inflammatory changes during the second phase, are necessary for the full manifestation of the second phase.<sup>34</sup>

The inhibition of pain behaviour in phase 1 and phase 2 of the present study suggests that holothuria extract

has the ability to modulate the peripheral and central mechanisms and leads to reduction in the pain behaviour. The peripheral effects of holothuria extract could be attributed by several possible mechanisms including inhibition of release of prostaglandin, substance P or bradykinin in the inflamed peripheral tissue.<sup>37</sup> Following holothuria extract administration, marked difference was observed in the effects of formalin injection on the FLI expression. FLI expression was markedly reduced on the contralateral side of the formalin injection. The reduction of FLI in the contralateral ventral posterolateral thalamic nucleus correspond to the significant reduction in the pain behaviour in phase 2 exhibited in the behavioural study. These results indicate that holothuria extract has a central effect and it was able to inhibit FLI expression in the ventral posterolateral thalamic nucleus following formalin injection. The reduction of FLI in the contralateral thalamus correspond to the significant reduction in the pain behaviour in phase 2 exhibited in the behavioural study. The present study has demonstrated the analgesic property of holothuria extract and further investigations should be done to assist in the understanding of its actions at the peripheral or central nervous system.

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## REFERENCES

- Harris JA. Using c-fos as a neural marker of pain. *Brain Res Bull* 1998; 45(1):1-8.
- Asma Hayati A, Zalina I. C-Fos and Its Consequences in Pain. *Malaysian J Med Sci* 2002; 9:3-8.
- Dubner R, Ren K. Brainstem mechanisms of persistent pain following injury. *J Orofac Pain* 2004; 18: 299-305.
- Kosai K, Tateyama S, Ikeda T, et al. MK-801 reduces non-noxious stimulus-evoked Fos-like immunoreactivity in the spinal cord of rats with chronic constriction nerve injury. *Brain Res* 2001; 910:12-8.
- Ji RR, Woolf CJ. Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. *Neurobiol* 2001; 8:1-10.
- Woolf CJ. A new strategy for the treatment of inflammatory pain. Prevention or elimination of central sensitization. *Drugs* 1994; 47:1-9.
- Woolf CJ, Chong MS. Preemptive analgesia--treating postoperative pain by preventing the establishment of central sensitization. *Anesth Analg* 1993; 77:362-79.
- Abdul Aziz CB, Chapman V, Mason R. Comparison of responses of ventral posterolateral and posterior complex thalamic neurons in naïve rats and rats with hindpaw inflammation:  $\mu$ -opioid receptor mediated inhibitions. *Neuropharmacol* 2005; 48:607-16.
- Guilbaud G, Kayser V, Benoist JM, et al. Modifications in the responsiveness of rat ventrobasal thalamic neurons at different stages of carrageenin-produced inflammation. *Brain Res* 1986; 385:86-98.
- Guilbaud G, Neil A, Benoist JM, et al. Thresholds and encoding of neuronal responses to mechanical stimuli in the ventro-basal thalamus during carrageenin-induced hyperalgesic inflammation in the rat. *Exp Brain Res* 1987; 68:311-8.
- Guilbaud G, Benoist JM, Jazat F, et al. Neuronal responsiveness in the ventrobasal thalamic complex of rats with an experimental peripheral mononeuropathy. *J Neurophysiol* 1990; 64:1537-54.
- Kolhekar RS, Murphy S, Gebhart GF. Thalamic NMDA receptors modulate inflammation-produced hyperalgesia in the rat. *Pain* 1997; 71:31-40.



13. Buritova J, Besson JM, Bernard JF. Involvement of the spinoparabrachial pathway in inflammatory nociceptive processes: a c-Fos protein study in the awake rat. *J Comp Neurol* 1998; 397:10-28.
14. Nackley AG, Makriyannis A, Hohmann AG. Selective activation of cannabinoid CB(2) receptors suppresses spinal fos protein expression and pain behavior in a rat model of inflammation. *Neuroscience* 2003; 119:747-57.
15. Catheline G, Le Guen S, Besson JM. Effects of opioid receptor antagonists on the effects of i.v. morphine on carrageenin evoked c-Fos expression in the superficial dorsal horn of the rat spinal cord. *Brain Res* 1999; 824(1):105-11.
16. Zhang GH, Min SS, Lee KS, et al. Intraarticular pretreatment with ketamine and memantine could prevent arthritic pain: relevance to the decrease of spinal c-fos expression in rats. *Anesth Analg* 2004; 99:152-8.
17. Fredalina BD, Ridzwan BH, Abidin AA, et al. Fatty acid compositions in local sea cucumber, *Stichopus chloronotus*, for wound healing. *Gen Pharmacol* 1999; 33:337-40.
18. Taiyeb-Ali TB, Zainuddin SL, Swaminathan D, Yaacob H. Efficacy of 'Gamadent' toothpaste on the healing of gingival tissues: a preliminary report. *J Oral Sci* 2003; 45:153-9.
19. Chan EH, Liew HC. The sea cucumber. *Nature Malaysia* 1986; 10:8-12.
20. Zainudin M, Chua YT, Syed Zahir I, et al. Air gamat (Sea Cucumber [Holothurian]) extracts does not have B-adrenoceptor agonist or phosphodiesterase inhibiting properties. *J Perubatan UKM* 1986; 1:41-8.
21. Ridzwan BH, Leong TC, Idris SZ. The antinociceptive effects of water extracts from sea cucumbers *Holothuria Leucospilota* Brandt, *Bohadschia marmorata vitiensis* Jaeger and coelomic fluid from *Stichopus hermanii*. *Pak J Biol Sci* 2003; 8:2068-72.
22. Zury A. Assessment of general toxicity and analgesic property of several sea cucumbers species. *Universiti Sains Malaysia* 2004 (unpublished report).
23. Asma Hayati A, Zalina I, Myo T, et al. Modulation of formalin-induced Fos-like immunoreactivity in the spinal cord by swim stress-induced analgesia, morphine and ketamine. *Ger Med Sci* 2008; 6:Doc05.
24. Sun WZ, Shyu BC, Shieh JY. Nitrous oxide or halothane, or both, fail to suppress c-fos expression in rat spinal cord dorsal horn neurones after subcutaneous formalin. *Br J Anaesth* 1996; 76:99-105.
25. Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* 1977; 4:161-74.
26. Hao S, Takahata O, Mamiya K, et al. Sevoflurane suppresses noxious stimulus-evoked expression of Fos-like immunoreactivity in the rat spinal cord via activation of endogenous opioid systems. *Life Sci* 2002; 71:571-80.
27. Gilron I, Quirion R,Coderre TJ. Pre-versus postinjury effects of intravenous GABAergic anesthetics on formalin induced fos immunoreactivity in the rat spinal cord. *Anesth Analg* 1999; 89:414-420.
28. Gogas KR, Cho HJ, Botchkina GI, et al. Inhibition of noxious stimulus-evoked pain behaviors and neuronal fos-like immunoreactivity in the spinal cord of the rat by supraspinal morphine. *Pain* 1996; 65:9-15.
29. Ji RR, Rupp F. Phosphorylation of transcription factor CREB in rat spinal cord after formalin-induced hyperalgesia: relationship to c-fos induction. *J Neurosci* 1997; 17:1776-85.
30. Huang W, Simpson RK. Ketamine suppresses c-fos expression in dorsal horn neurons after acute constrictive sciatic nerve injury in the rat. *Neurosci Lett* 1999; 269:165-168.
31. Jennings E, Fitzgerald M. C-fos can be induced in the neonatal rat spinal cord by both noxious and innocuous peripheral stimulation. *Pain* 1996; 68:301-6.
32. Paxinos G, Watson WC. The rat brain in stereotaxic coordinates. New York: Academic Press, 5th Ed 2004.
33. Nakao S, Miyamoto E, Masuzawa M, et al. Ketamine-induced c-Fos expression in the mouse posterior cingulate and retrosplenial cortices is mediated not only via NMDA receptors but also via sigma receptors. *Brain Res* 2002; 926:191-6.
34. Tjølsen A, Berge OG, Hunskaar S, et al. The formalin test: an evaluation of the method. *Pain* 1992; 51:5-17.
35. Malmberg AB, Yaksh TL. Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. *Science* 1992; 257:1276-9.
36. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 1971; 231:232-5.
37. Shibata M, Ohkubo T, Takahashi H, et al. Modified formalin test: characteristic biphasic pain response. *Pain* 1989; 38:347-52.