Journal of Integrative Medicine 17 (2019) 66–70

Contents lists available at ScienceDirect

Journal of Integrative Medicine

journal homepage: www.jcimjournal.com/jim
www.journals.elsevier.com/journal-of-integrative-medicine

Original Research Article

Effects of Tualang honey in modulating nociceptive responses at the spinal cord in offspring of prenatally stressed rats

Che Badariah Abd Aziz [a,*], Siti Qusyasyiah Ahmad Suhaimi [a], Hidani Hasim [a], Asma Hayati Ahmad [a], Idris Long [b], Rahimah Zakaria [a]

[a] Department of Physiology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[b] School of Health Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

ARTICLE INFO

Article history:
Received 31 January 2018
Accepted 15 October 2018
Available online 15 December 2018

Keywords:
Prenatal stress
Nociceptive responses
Tualang honey
Spinal cord

ABSTRACT

Objective: This study was done to determine whether Tualang honey could prevent the altered nociceptive behaviour, with its associated changes of oxidative stress markers and morphology of the spinal cord, among the offspring of prenatally stressed rats.
Methods: Pregnant rats were divided into three groups: control, stress, and stress treated with Tualang honey. The stress and stress treated with Tualang honey groups were subjected to restraint stress from day 11 of pregnancy until delivery. Ten week old male offspring ($n = 9$ from each group) were given formalin injection and their nociceptive behaviours were recorded. After 2 h, the rats were sacrificed, and their spinal cords were removed to assess oxidative stress activity and morphology. Nociceptive behaviour was analysed using repeated measures analysis of variance (ANOVA), while the levels of oxidative stress parameters and number of Nissl-stained neurons were analysed using a one-way ANOVA.
Results: This study demonstrated that prenatal stress was associated with increased nociceptive behaviour, changes in the oxidative stress parameters and morphology of the spinal cord of offspring exposed to prenatal stress; administration of Tualang honey reduced the alteration of these parameters.
Conclusion: This study provides a preliminary understanding of the beneficial effects of Tualang honey against the changes in oxidative stress and neuronal damage in the spinal cord of the offspring of prenatally stressed rats.

Please cite this article as: Abd Aziz CB, Ahmad Suhaimi SQ, Hasim H, Ahmad AH, Long I, Zakaria R. Effects of Tualang honey in modulating nociceptive responses at the spinal cord in offspring of prenatally stressed rats. *J Integr Med.* 2019; 17(1): 66–70.

1. Introduction

Prenatal stress is a condition where an expectant mother is exposed to stressors [1]. It may lead to changes in the hormonal system of the mother and may affect the brain development of the unborn child [2]. Studies have shown the association of maternal stress with development of abnormal behaviour and alteration of the nociceptive responses in the offspring [3–6]. Offspring of prenatally stressed rats have shown increased nociceptive responses as well as Fos-like immunoreactive neurons in the lumbar dorsal horn following formalin injection to the paw [5,7].

The exact mechanism by which maternal stress can lead to modulation of the nociceptive responses is not clearly understood. During chronic stress, a prolonged activation of maternal hypothalamic-pituitary-adrenal axis leads to high levels of catecholamines and glucocorticoid in the blood, resulting in permanent structural and functional changes in the nervous system [8,9]. Exposure of the foetus to high glucocorticoid may contribute to increased oxidant levels and alterations of the developing brain. The changes may predispose the offspring to development of abnormal behaviour and altered pain responses.

During normal pregnancy, placental 11 β-hydroxysteroid dehydrogenase type 1 will deactivate maternal glucocorticoids and prevent its passage into the foetal circulation. However, during prenatal stress, the higher glucocorticoid level will defeat the deactivation process and expose the foetus to elevated levels of

* Corresponding author.
E-mail address: badariah@usm.my (C.B. Abd Aziz).

https://doi.org/10.1016/j.joim.2018.12.002
2095-4964/

glucocorticoid [10]. Extended exposure to the glucocorticoid will lead to oxidative stress and alter the growth and maturity process of the foetal brain [11–13]. Since oxidative stress contributes to abnormal development of the offspring of prenatally stressed rats, administration of antioxidants might have a protective effect.

Tualang honey is a Malaysian multifloral jungle honey and it is known to have antioxidant activity. It has more phenolic acids and flavonoids compared to Manuka and Gelam honey [14]. Tualang honey's antioxidant property has been shown to oppose oxidative stress that occurred in diabetic rats and in ageing rats [15,16]. Tualang honey itself has an antinociceptive property when given pre-emptively to adult rats injected with formalin [17]. Our preliminary study showed that administration of Tualang honey inhibited the increase in nociceptive responses in the offspring of prenatally stressed rats [5]. However, whether or not the increased nociceptive responses were associated with changes in the morphology and alteration of oxidative stress markers in the spinal cord of these offspring is yet to be determined. This study aimed to determine the alteration in morphology of the spinal cord and oxidative stress markers in the offspring of prenatally stressed rats and whether Tualang honey could confer protective effects on these changes.

2. Materials and methods

2.1. Animals

Thirty-nine adult female Sprague–Dawley rats weighing 180–225 g were purchased from the Animal Research and Service Centre (ARASC), Health Campus, Universiti Sains Malaysia (USM). The rats were maintained on a 12-hour light/dark cycle (light phase 7 a.m.–7 p.m.) with standard laboratory food and water during the entire study. Each animal was used only once. This study was approved by the Animal Research Ethics Committee of USM, [USM/Animal Ethics Approval/2014/(94) (577)] in accordance with the internationally accepted principles for laboratory animal use and care.

2.2. Study design

Pregnant rats were randomly assigned into three groups (n = 13 for each group): control, stress, and stress treated with Tualang honey. Following delivery and maturation, adult male rat offspring (n = 9 for each group) were subjected to nociceptive stimulation using the formalin test, and they were sacrificed 2 h after formalin injection. Behavioural pain scores, oxidative stress markers and morphology of the spinal cord were compared among the three groups.

2.3. Study procedure

Experiments were conducted in the ARASC laboratory, Health Campus, USM between 8:00 and 17:00. The female rats at proestrus stage were caged with mature males overnight in the laboratory. Vaginal smear was done between 9:00 and 10:00 am and if sperm was positive, the day was considered as day 0 of pregnancy [18]. The pregnant female was then separated and kept in an individual cage under standard conditions. Tualang honey (Federal Agricultural Marketing Authority; 1.2 g/kg) or distilled water was given by oral gavage from day 1 of pregnancy until delivery. The dose of Tualang honey was chosen because it had been shown to produce antinociceptive and anti-inflammatory effects in adult rats [17]. Stress was given in the form of restraint stress; 3 times daily for 30 min each time, until delivery [5]. The restrainer was in form of plastic cylinder measuring 23 cm × 6 cm.

2.4. Formalin test

Nine male pups at the age of 8–10 weeks from each group underwent the formalin test. The plantar surface of the right hind paw was injected subcutaneously with 50 μL of 1% formalin using a 1 mL syringe with 27-G needle [5]. Each rat was acclimatised in a testing chamber for about 30 min before the test. The chamber size was 26 cm × 20 cm × 20 cm and there was a mirror mounted at 45° below it which allowed unhindered observation of the formalin-injected paw [19]. The behaviours of each rat were recorded for 1 h using a digital video camera and the tape was reviewed later by two observers blinded to the treatment group [5]. Nociceptive behavioural score was analysed based on the behavioural categories [19,20], where 0, the injected paw is not favoured; 1, the injected paw has little or no weight on it with no toe splaying, indicating mild pain felt; 2, the injected paw is elevated and the heel is not in contact with any surface, indicating moderate pain; and 3, the injected paw is licked, bitten or shaken, indicating severe pain.

2.5. Sacrifice of rats

All the rats were sacrificed 2 h after formalin injection with an overdose of sodium pentobarbitone injected intraperitoneally [5,19]. After losing their righting reflex, rats were placed in a supine position on a bed of ice until loss of the toe pinch response (10–15 min). Rats were then decapitated using a guillotine and the spinal cords were removed for histological examination and oxidative stress parameter measurement.

2.6. Histology of spinal cord

The lumbar region of the spinal cord tissue was harvested and weighed using a digital analytical balance. The tissue was fixed in 10% formalin solution and stored at room temperature. Following fixation, the tissues were dehydrated in an automated tissue processing machine, blocked with paraffin wax and kept at 0 °C for 3 h. The tissues were sectioned to 5 μm using a microtome. The tissues were then mounted on glass slides which were placed on a hot plate at 50–55 °C for 10 min. After dewaxing and rehydrating, the spinal cord tissue sections were immersed in 0.5% cresyl violet for 2 min, rinsed with double distilled water, dehydrated in ethanol solutions with increasing concentrations and cleared in xylene. Then, the sections were mounted with Cytoseal XYL mounting medium, covered with cover slips, and observed under a light microscope. Damaged neurons were identified by loss of Nissl substance, cavitation around the nuclei and presence of pyknotic homogenous nuclei, whereas normal neurons contained Nissl substance in the cytoplasm, loose chromatin and prominent nucleoli [21].

2.7. Spinal cord oxidative stress markers

Evaluation of oxidative stress markers in the spinal cord homogenates was performed by measuring the levels of plasma glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) using reagent kits supplied by USCNK (USCN Life Science, Wuhan, China).

2.8. Statistical analysis

Statistical Package for the Social Sciences (SPSS, Version 21, Chicago, USA) was used for data analysis. Nociceptive behaviour scores were analysed using repeated measures analysis of variance (ANOVA) with the within-subject factors TIME (13 levels) and TREATMENT (3 levels). The Tukey test was used for posthoc analy-

sis. One-way ANOVA was used to compare the number of Nissl-positive cells and oxidative stress parameters in the lumbar region of spinal cord. Significance level for all data was accepted at $P < 0.05$.

3. Results

3.1. Formalin test

Formalin produced the typical biphasic response in the offspring of all experimental groups. The first phase, shown by a peak in response lasting about 5 min, was followed by a 5–10 min of reduced nociceptive response. The second phase of the nociceptive response was present from 15 min onwards and lasted for at least 60 min after the formalin injection. There was a significant main effect of time ($F_{4.941,\ 177.869} = 45.548$; $P < 0.001$) meaning that there were differences in the pain behaviour scores among the different time points. There was also a significant main effect of treatment ($F_{9.882,\ 177.869} = 1.946$; $P < 0.05$). Offspring of rats that underwent stress and were treated with Tualang honey displayed significantly reduced nociceptive behaviour score compared to offspring of the control and stress groups (Fig. 1).

3.2. Number of Nissl-positive neurons in lumbar region of spinal cord

There was significant difference in the number of Nissl-positive neurons among the three groups, as determined by one-way ANOVA ($F_{2,15} = 8.898$; $P = 0.003$). Tukey posthoc test revealed that the number of Nissl-positive cells in the stress group (29.8 ± 2.7) was significantly lower than those of the stress treated with honey group (36.0 ± 2.9; $P = 0.019$) and the control group (37.8 ± 4.4; $P = 0.003$). There was no significant difference between the control and stress treated with honey groups ($P = 0.635$; Fig. 2). Morphology results of the spinal cord for the three groups are shown in Fig. 3.

3.3. Oxidative stress parameters

One-way ANOVA revealed a significant main effect of treatment on the levels of GSH ($F_{2,\ 24} = 8.818$; $P < 0.001$), CAT ($F_{2,15} = 121.146$; $P < 0.001$), SOD ($F_{2,15} = 5.146$; $P < 0.019$) and MDA ($F_{2,\ 24} = 7.036$; $P < 0.004$). The stress group showed significantly lower levels of GSH, CAT and SOD, and a higher level of MDA when compared to the control group (Table 1). GSH activity was significantly higher ($P < 0.05$) and MDA was significantly lower in the stress treated with honey group when compared to the stress group. Although there was a trend of higher CAT levels in the stress treated with honey group relative to the stress group, it did not reach significance. SOD level in the stress treated with honey group was not significantly different when compared with the stress group.

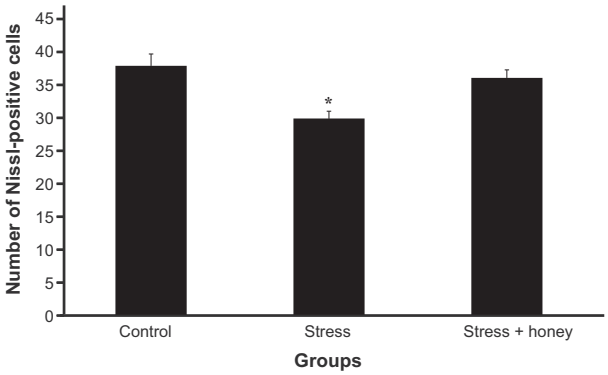

Fig. 2. Number of Nissl-positive cells in lamina I of spinal cord ($n = 9$ rats in each group). Data are presented as mean ± standard error of mean. $^{*}P < 0.05$, statistical comparison between stress group and the other two groups.

4. Discussion

4.1. Formalin test

In this test, the first phase (0–5 min) of nociceptive behaviour is the direct effect of formalin on the peripheral nociceptors [22]. The second phase (15–60 min) is related to persistent peripheral inflammation secondary to formalin injection and *N*-methyl-D-aspartate (NMDA) receptor activation in the central nervous system [23]. In the present study, the rat offspring from the stress group showed higher nociceptive behaviour scores in both phases compared to the control and the Tualang honey-treated stress groups.

The elevated response in the first phase might be due to alteration of nociceptor responses in offspring of the prenatally stressed male rats [24]. Butkevich et al. [18] did not find a difference in nociceptive behaviour score in the first phase of offspring of the prenatally stressed rats compared to the control, most probably because of the different method used to score the behaviour and difference in the age of the offspring. The increased pain behaviour in the second phase might be contributed to reduced descending serotonergic neurons, reduced local spinal cord γ-aminobutyric acidergic (GABAergic) inhibitory neurons and increased NMDA

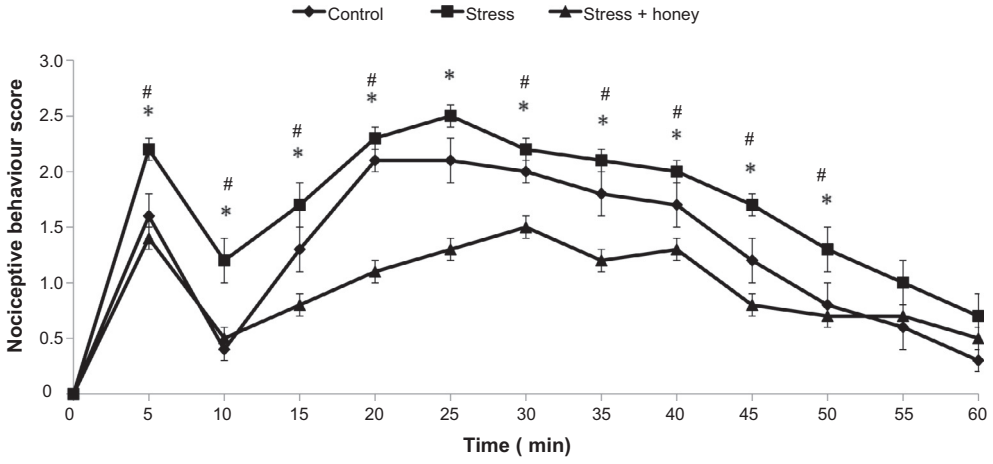

Fig. 1. Nociceptive behaviour score in the offspring of control, stress and stress + honey groups. Data are presented as mean ± standard error of mean ($n = 9$ rats in each group). $^{*}P < 0.05$, statistical comparison between stress and stress + honey groups; $^{\#}P < 0.05$, statistical comparison between stress and control groups.

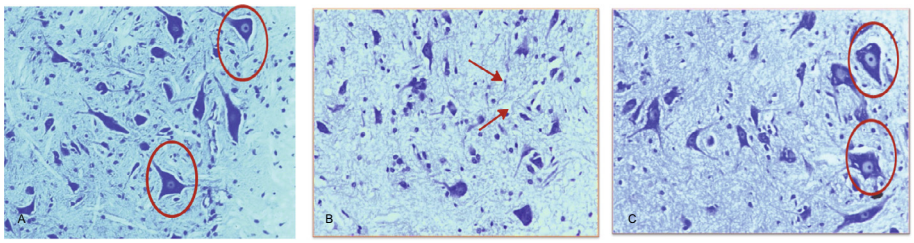

Fig. 3. Light microscope photography of spinal cord morphology (×20). (A) Control group, circles indicate clearly visible large multipolar neurons with big nuclei; (B) stress group, reduction in the number of normal neurons and arrows show marked vacuolisation; (C) stress + honey group, circles show the normal multipolar neurons with clear cell boundaries and regular nucleus shape.

Table 1
Level of oxidative stress markers in spinal cord in different groups.

Group	Glutathione (μg/mL)	Catalase (ng/mL)	Superoxide dismutase (ng/mL)	Malondialdehyde (ng/mL)
Control group	6.112 ± 0.317	12.317 ± 0.378	40.618 ± 1.530	5.026 ± 0.077
Stress group	4.753 ± 0.205*	8.550 ± 0.815*	36.069 ± 0.577*	5.466 ± 0.073**
Stress treated with honey group	5.705 ± 0.152$^{\#}$	10.300 ± 0.267	37.608 ± 1.127	5.184 ± 0.059$^{\#}$

Data are presented as mean ± standard error of mean (n = 9 rats in each group). $^{*}P < 0.05$, $^{**}P < 0.01$, comparison between stress group and control group; $^{\#}P < 0.05$, comparison between stress group and stress treated with honey group.

receptor activity in the adult offspring of prenatally stressed mothers [25–27]. The serotonergic and GABAergic neurons have modulatory roles and are capable of inhibiting transmission of pain impulses [28]. The mechanism for the increased release of SOD-mediated oxidative stress markers is partly the result of NMDA receptor hyperactivity, which leads to neurotoxicity and cell death [29,30]. In addition, oxidative stress is able to upregulate NMDA receptors in the central nervous system and increase pain responses in the offspring [31].

Tualang honey contains flavonoid and other polyphenolic acids [14]. Flavonoid administration was associated with reduction in nociceptive behaviour scores in diabetic rats and in the rat model of neuropathic pain [32,33]. Dietary flavonoid from the pregnant dams can be transferred across the placenta and stored in foetal brain and other tissues [34]. There is a possibility that flavonoid prevented alteration of nociceptive responses by modulating signalling cascades and gene expression involved in nociceptors' transduction, preventing damage to serotonergic and GABAergic inhibitory neurons, and decreasing upregulation of NMDA receptor in the central nervous system of the rat offspring.

4.2. Number of Nissl-positive neurons and oxidative stress parameters

Our study found reduced number of Nissl-positive neurons in the spinal cord of offspring of the prenatally stressed rats. The number of neurons was significantly higher with administration of Tualang honey to the pregnant dams. Other reports have shown that neuronal damage in the brain of offspring was associated with increased glucocorticoids and oxidative stress [35–37]. Glucocorticoid has an important role in intrauterine programming and it exerts its effects by altering the expression of various proteins including receptors, enzymes, ion channels, transporters and growth factors [38]. The hormone is required to initiate the terminal neuronal maturation and the remodelling of axons and dendrites during development of the central nervous system. However, excess glucocorticoid may lead to behavioural abnormalities in the adult offspring [39,40]. In addition, excess glucocorticoid might cause neuronal damage in the spinal cord by altering expression of genes involved in reactive oxygen species (ROS) generation [41] and reducing transcription of the mitochondrial-respiratory chain enzymes that might lead to mitochondrial dysfunction and oxidative stress [42].

The present study demonstrated increased MDA and lower antioxidant (CAT, SOD and GSH) levels in the spinal cord of the adult offspring, following formalin injection. The altered levels of antioxidants and MDA in this group most probably contributed to the effects of prenatal stress, as the formalin used in the present study was at a low dose and insufficient to cause oxidative stress [43]. Increased MDA level has also been shown in patients with preeclampsia and in the offspring of rats exposed to prenatal stress induced by low-protein diet and lipopolysaccharide administration [44–46].

The decrease in the spinal cord GSH found here is similar to a study done by Sahu et al. [11], which demonstrated a decrease in brain GSH of the offspring, following prenatal stress. The decrease in GSH was probably due to conversion of GSH to its oxidised form, glutathione disulphide. In addition, a decrease in glutathione reductase, an enzyme that is required for production of GSH, may play a role in reducing the GSH level [11]. GSH has an important role in cellular detoxification of ROS and prevention of the cell damage induced by oxidative stress [47–48].

Offspring from the Tualang honey group showed improvement in MDA, GSH and CAT activity. The results are partly contributed to suppressing the mRNA expression of corticotropin-releasing factor (CRF), thus reducing the release of CRF and glucocorticoid [34,49,50]. The reduced level of glucocorticoid, together with Tualang honey antioxidant activity, would further reduce ROS formation and utilisation of antioxidants. Reduced oxidative stress would benefit the pain-modulating structures in the central nervous system, including the spinal cord. In the present study, however, Tualang honey administration did not significantly alter the SOD level in the offspring, although other animal studies showed significant improvement in the SOD level, following direct administration of Tualang honey to adult rats [15,16]. Further studies are needed to address this inconsistency.

5. Conclusion

Our study provides evidence that prenatal stress is associated with morphological changes and alteration in oxidative stress markers at the level of the spinal cord, and these changes may contribute to modulation of pain behaviour seen in the rat offspring exposed to prenatal stress. Tualang honey administration is seen to offer protection against these changes. Further investigation

looking at the molecular level of the mechanism will give further light in the protective effect of Tualang honey in similar offspring.

Acknowledgements

This project was supported by the Fundamental Research Grant Scheme (FRGS No. 203/PPSP/6171162).

Competing interests

The authors declare that they have no competing interests.

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