

TUALANG HONEY MODULATED NOCICEPTIVE RESPONSES IN THE THALAMUS OF REM SLEEP DEPRIVATION RAT MODEL

ANIS SYAHIRAH MOHD SHAFIE¹, CHE BADARIAH ABD AZIZ^{1*}, LIZA NOORDIN¹, IDRIS LONG², ANANI AILA MAT ZIN³ and ROSFAIIZAH SIRAN⁴

¹Department of Physiology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

²School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

³Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

⁴Neuroscience Research Group, Faculty of Medicine, Universiti Teknologi MARA, 47000 Sungai Buloh, Selangor, Malaysia

*E-mail: badariah@usm.my

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ABSTRACT

Sleep deprivation has been shown to alter pain responses in humans and animals. The present study investigated whether the administration of Tualang honey in the rapid eye movement (REM) sleep deprivation rat model would modulate nociceptive responses with associated changes in the thalamus. Forty-eight Sprague Dawley male rats were randomised into four groups (n=12 for each group): control group (FMC), REM sleep deprivation (REMSd), REM sleep deprivation pretreated with Tualang Honey for 1 month (REMSdH) and tank control (TC). Following sleep deprivation for 72 hours, a formalin test was conducted and pain behaviour was recorded and analysed. The rats were sacrificed, and the brains were removed for histological examination and assessment of N-methyl-D-aspartate receptor (NMDA R2) level in the thalamus. REMsdH group showed a significantly lower level of pain behaviour score and NMDA R2 compared to the REMsd group ($p < 0.05$). In addition, REMsdH also demonstrated a significantly higher number of Nissl stained neurons in comparison with the REMsd group ($p < 0.05$). Furthermore, dark neurons, suggestive of neuronal damage, were observed in the REMsd group. In conclusion, administration of Tualang honey before REM sleep deprivation modulated nociceptive responses and prevent changes in thalamic neurons and NMDA R2 level.

Key words: NMDA receptor, pain, REM sleep deprivation, thalamus, Tualang honey.

INTRODUCTION

Sleep is an important activity for man and sleep deprivation is linked to multiple diseases such as hypertension (Tochikubo *et al.*, 1996), diabetes (Gottlieb *et al.*, 2005) and cardiovascular diseases (Tengku Adnan *et al.*, 2017). In addition, increased pain perception was shown in human studies following sleep deprivation (Iacovides *et al.*, 2017; Schrimpf *et al.*, 2015). Patients with chronic pain syndromes often suffer from insomnia (Morin *et al.*, 1998) and a report has demonstrated that healthy individuals with sleep deprivation had increased pain intensity (Staffe *et al.*, 2019). Meanwhile, other

reports have shown that the severity of sleep deprivation was correlated with the degree of pain (Zelman *et al.*, 2006; Ajao *et al.*, 2011).

Studies have shown that rapid eye movement (REM) sleep deprivation is associated with hyperalgesia (Ajao *et al.*, 2003), elevated c-fos (Terao *et al.*, 2003) and nuclear Down regulatory antagonist modulator (DREAM) protein expression in the thalamus (Siran *et al.*, 2014). Thalamus is an important structure in the pain pathway and has been shown to modulate acute and chronic pain (Abd Aziz & Ahmad, 2006). Imaging studies have reported that REM sleep deprivation also leads to alteration in thalamocortical functional connectivity and reduced thalamic gray matter volume (Liu *et al.*, 2014; Shao *et al.*, 2014). In addition, N-methyl-D-aspartate

* To whom correspondence should be addressed.

(NMDA) receptors in the thalamus were reported to modulate hyperalgesia in carrageenan and formalin-induced inflammatory pain (Hasim *et al.*, 2020; Kolhekar *et al.*, 1997).

The anti-inflammatory property of Tualang honey has been shown in animal models with inflammation induced by chemicals (Bashkaran *et al.*, 2011; Ismail *et al.*, 2017). The high level of flavonoids and phenols in Tualang honey might contribute to its anti-inflammatory property (Kishore *et al.*, 2011; Khalil *et al.*, 2011). The honey has also beneficial effects in various conditions such as diabetes mellitus (Erejuwa *et al.*, 2010), prenatal stress (Abd Aziz *et al.*, 2019) and chronic noise exposure (Azman *et al.*, 2015). However, the effects of Tualang honey on pain behaviour responses in the rat model of sleep deprivation are not known. One of the pain behaviour responses that can be assessed is the pain behaviour score in the formalin test. The test is commonly used in an animal model of pain to determine the associated mechanism of pain or the effectiveness of a substance to inhibit pain. This preliminary study aimed to determine whether Tualang honey modulates nociceptive responses and related changes in the thalamus following REM sleep deprivation.

MATERIALS AND METHODS

Animal

This study has been approved by Universiti Sains Malaysia Institutional Animal Care and Use Committee [USM/IACUC/2019/(116)(964)]. Animal Research and Services Centre in University Sains Malaysia has provided the Sprague Dawley male rats (n=48) used in the study. The rats were around 180 to 200 g in weight and their age was about 8 to 10 weeks old. They were placed in a lightproof dry cage with 12:12 hour light/dark and controlled temperature. Standard laboratory food and water were given to them. The rats were divided equally into four groups: control group (FMC), REM sleep deprivation (REMSd), REM sleep deprivation pretreated with Tualang Honey (REMSdH) and tank control (TC). Tualang honey was administered daily at 1.2 g/kg body weight for four weeks by oral gavage before the adaptation period. Tualang honey was not given during adaptation and REM sleep deprivation. FMC is a negative control group and the rats in this group were placed in ordinary cages and were not exposed to the aquatic environment. Meanwhile, TC is a positive controlled group and they were placed in an aquatic environment (glass tank filled with water). The rats in the TC group were provided with two platforms that were larger in diameter compared to REMSd and REMSdH groups.

Period of adaptation

The adaptation phase was for 72 hours and in this period, all rats except the FMC group were placed individually in a dry tank before the experiment. Two glass tanks were positioned side by side to reduce the degree of social isolation. For REMSd and REMSdH groups, two platforms with 6.5 cm diameter were placed in the dry tank measuring 30 cm height, 30 cm length and 60 cm width. Meanwhile, for the TC group, two platforms with 13.5 cm diameter were placed in a similar size of a glass tank. The adaptation period was to ensure that the rats were adapted to the environment in the glass tank. During the adaptation period, each rat from FMC was placed individually in a dry cage (22 cm in height, 56 cm in length and 38 cm in width). The negative control group (FMC) was allowed to be in their usual cages to avoid any form of environmental stress.

REM sleep deprivation model

After adaptation, REM sleep deprivation was induced for 72 hours. The inverted flowerpot technique (Tengku Adnan *et al.*, 2017; Siran *et al.*, 2014) was used to accomplish REM sleep deprivation. The glass tanks and the platforms used in this phase were of similar sizes as during the adaptation period. In this phase, water was filled up to 1 cm from the top of the platforms in the glass tanks. Each rat in REMSd and REMSdH groups was placed in the glass tank. During REM sleep, the muscle became atonia and caused them to fall into the water, thus awakening the rat from sleep. Other studies such as electroencephalogram had shown that the inverted flowerpot technique was able to cause REM sleep deprivation (Mendelson *et al.*, 1974; Maloney *et al.*, 1999). Food and drink were available throughout the adaptation and experimental phases and the temperature of the water was kept at 30°C.

Control groups

All rats in the FMC group were maintained in normal dry lightproof cages individually. Two cages were placed side by side to minimize social isolation stress. For the TC group, the experimental design was similar to REMSd and REMSdH group but used larger platforms (13.5 cm diameter). Two platforms were placed in the glass tank. Rats in the TC group undergo similar adaptation and experimental phases. Rats in the TC group were used to examine the effect of exposure to the aquatic environment and the larger platforms allowed the rats to undergo non-REM sleep and REM sleep (Mendelson *et al.*, 1974).

Body weight gain and food consumption

The measurement of body weight gain was taken by subtracting the body weight of each rat on day

4 (BW4) with day 1 (BW1) for the adaptation phase (BW4 – BW1) and day 7 with day 4 (BW7 – BW4) for experimental phase. Meanwhile, the food consumption was calculated by subtracting the remaining food on day 4 (RF4) with the amount of food given on day 1 (AF1) in the adaptation phase (AF1 – RF4) and day 4 to 7 in the experimental phase (AF4 – RF7). The food consumption was normalised as grams per day per kilogram of body weight taken to the 0.67 power to compensate for variation in metabolic rate as a function of body mass (Siran *et al.*, 2014).

Formalin test

Before the formalin test was done, each rat was placed in a test chamber (26 cm × 20 cm × 20 cm) for around 30 minutes for acclimatisation. Subcutaneous injection of 1% of formalin (50 µL) was given at the right hind paw of the rat. Following the injection, the rat was immediately placed in the test chamber (Hayati *et al.*, 2006) and observed for 1 hour. The behaviour was analysed for every minute and averaged at 5 min by two observers (Hayati *et al.*, 2006). Scores were given based on nociceptive behaviour categories; score 0: The hind paw was placed normally on the floor; score 1: The hind paw rested lightly on floor / with limping gait, score 2: The hind paw was elevated completely, score 3: The hind paw was licked, shaken and bitten. The rats were sacrificed two hours after formalin injection with an intraperitoneal injection of sodium pentobarbitone (Hayati *et al.*, 2006) followed by decapitation using the guillotine. The brain was then removed for histological test and quantification of NMDA R2.

Histology of thalamus

The brain samples were collected and weighed using a digital analytical balance. The samples were fixed in a 10% formalin solution. Following the fixation, the brain samples were processed in an automated tissue processor, blocked with paraffin wax and stored at 0°C for three hours. The tissues were cut at 5 µm and mounted on glass slides before air-dried for 30 minutes and kept at 37°C. After dewaxing the wax on the glass slides, Nissl staining was done following the standard guideline. After xylene completely dried on the slide, the coverslip was mounted on with Cytoseal XYL mounting medium. The left thalamus was observed and the ventral posterolateral nucleus (VPL) was identified under a light microscope and images were captured for visualisation and quantification of the Nissl stained neurons. The left VPL was chosen because the receptive field for the right hind paw is located in this area (Abd Aziz *et al.*, 2005). Three sections of each brain sample were taken from each rat and the mean number of Nissl stained neurons was recorded. The thalamus sections were taken from

bregma -4.44 mm to -2.16 mm (Paxinos *et al.*, 2014) at a regular space interval of 50 µm.

Quantification of NMDA R2 level

Acrylic brain matrix (Ted Pella Inc, USA) was used to cut coronal sections at 3 mm thickness of each brain sample (Siran *et al.*, 2014). The thalamus region was identified using rat brain atlas (Paxinos & Watsons, 2014). Thalamus tissue homogenate (10% w/v) was made from ipsilateral and contralateral sides of the thalamus in ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4). The homogenate was centrifuged at 10 000 ×g for 10 minutes and kept at -80°C until assay. The level of NMDA R2 was assessed in the thalamus using enzyme-linked immunosorbent one-step process assay kits (Qayee Bio, China).

Statistical analysis

The data were analysed using Statistical Package for Social Science (SPSS, version 26, Chicago, US). Before statistical analysis, a normality test was done to ensure the distribution of the data. Food consumption, body weight gain, number of Nissl positive neurons and NMDA R2 level in the thalamus were analysed using one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test. Repeated measurement ANOVA was used to analyse pain behaviour score followed by post-hoc analysis. The differences in the results were considered statistically significant if $p < 0.05$. The data in this study are expressed as mean ± standard error of mean (SEM).

RESULTS

Food consumption (Fc) and body weight gain (BWg)

The Fc and BWg were not significantly different during adaptation when compared between all groups. During REM sleep deprivation, the differences in the Fc were significant when compared between the groups ($F(3, 36) = 10.17, p < 0.001$). The food consumption was significantly higher in REMsd ($p < 0.001$) and REMsdH ($p < 0.05$) groups compared to FMC and TC groups as shown in Figure 1. During the experiment, the differences in BWg were also significant among the groups ($F(3, 38) = 68.95, p < 0.001$). The BWg was significantly lower in REMsd ($p < 0.001$) and REMsdH ($p < 0.05$) groups when compared with FMC and TC groups (Figure 2).

Formalin test

The formalin injection resulted in two phases of nociceptive responses. Phase 1 was shown by an increased nociceptive response in the first five minutes following formalin injection. It was followed by the decreased nociceptive response for 5 minutes

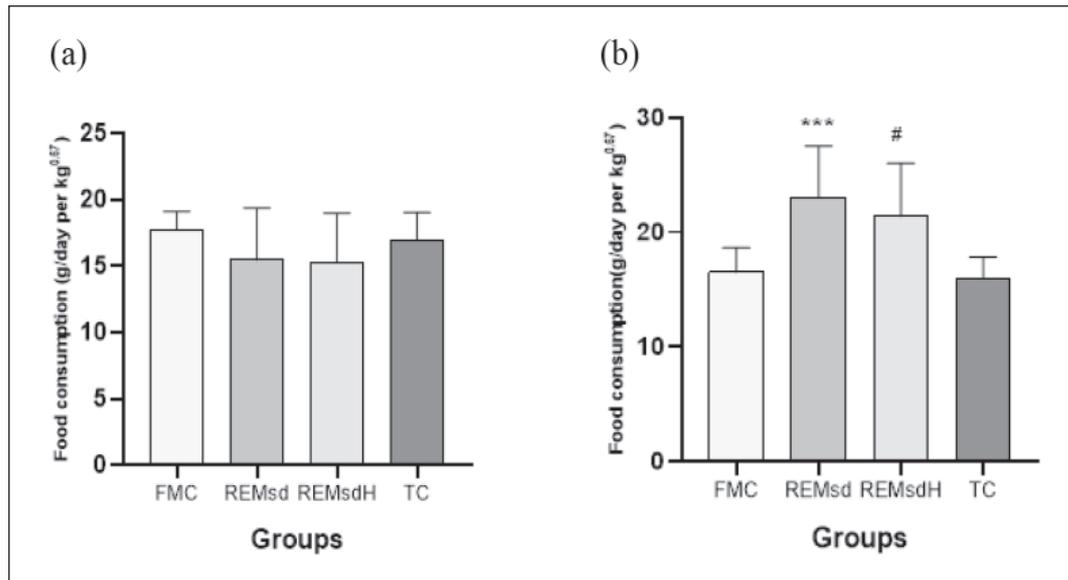


Fig. 1. Food consumption (g/day/kg^{0.67}) in all groups during (a) adaptation and (b) experiment. ***p<0.001, statistical comparison between REMsd group with FMC and TC. #p<0.05, statistical comparison between REMsdH group with FMC and TC.

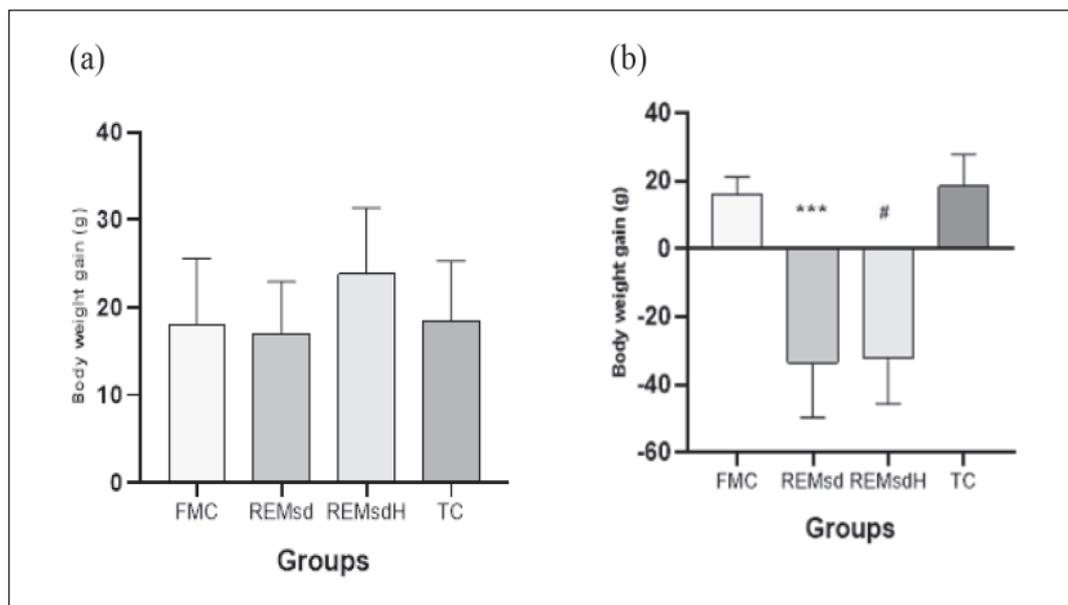


Fig. 2. Body weight gain (g) in all groups during (a) adaptation and (b) experiment. ***p<0.001, statistical comparison between REMsd group with FMC and TC groups. #p<0.05, statistical comparison between REMsdH group with FMC and TC groups.

(between minute 5 to 10). Phase 2 of nociceptive response was seen after ten minutes. The score gradually decreased in later part of phase 2 (minute 45 to 60) (Figure 2). There was a significant difference in time effect [$F(12, 25) = 479.80, p < 0.001$], group effect [$F(3, 36) = 18.974, p < 0.001$] and interaction between time and group effects [$F(36, 74) = 3.180, p < 0.001$]. The pain behaviour score from the REMsdH group was

significantly lower compared to the REMsd group at min 20, 30, 35, 45, 50, 55 and 60 (Figure 2). The differences in the scores were also significant when compared between the REMsdH and control groups (FMC and TC) at min 20 ($p < 0.05$) and min 45 ($p < 0.001$). The REMsd group also showed a significantly higher score compared to FC and TC groups at min 30, 35, 50, 55 and 60 (Figure 3).

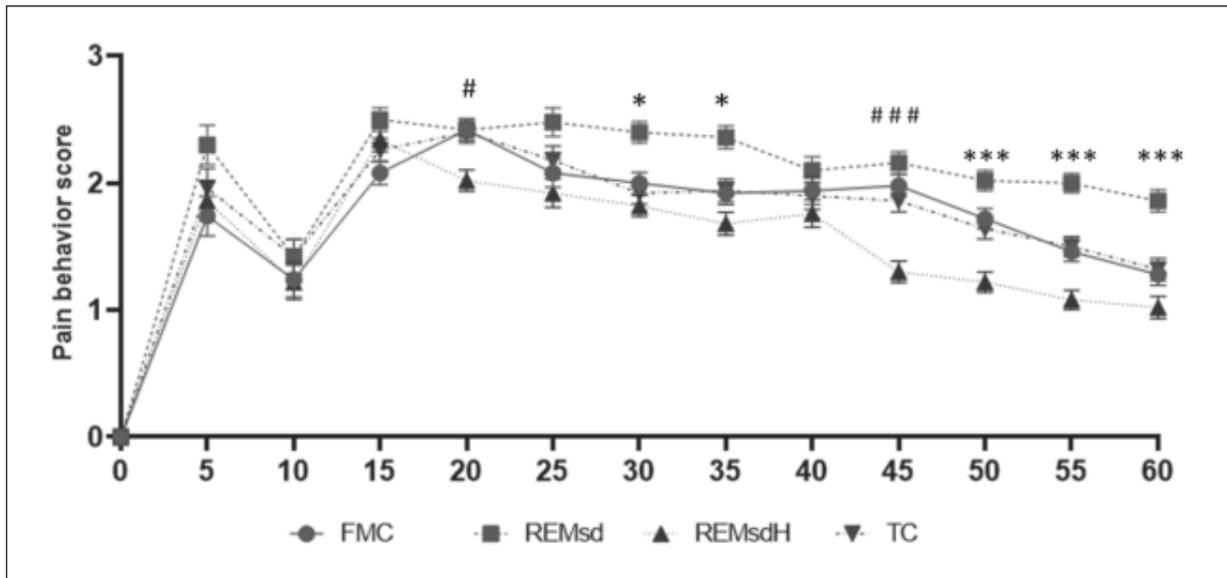


Fig. 3. Pain behaviour score in all groups including FMC, REMsd, REMsdH and TC. # $p<0.05$, statistical comparison between REMsdH with other groups. ### $p<0.001$, statistical comparison between REMsdH with other groups. * $p<0.05$, statistical comparison between REMsd with other groups. *** $p<0.001$, statistical comparison between REMsd with other groups.

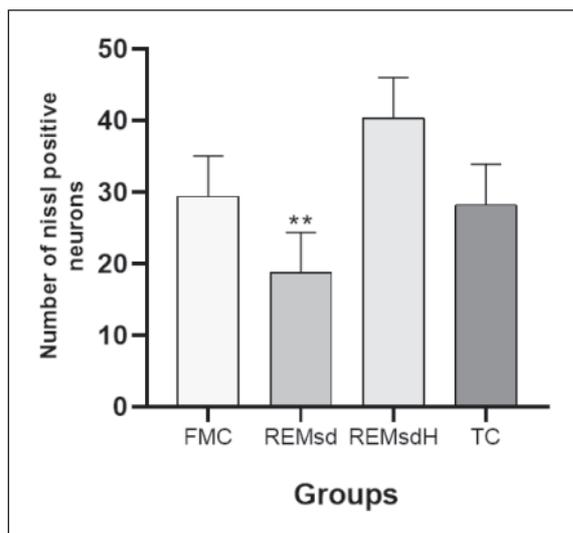


Fig. 4. Number of Nissl positive neurons in all groups including FMC, REMsd, REMsdH and TC. ** $p<0.01$, statistical comparison between REMsd and other groups.

Quantification of Nissl positive neurons in the thalamus

The number of Nissl positive neurons was significantly different when compared between the groups ($F(3,20)=6.169$, $p<0.01$). The quantity was significantly lower in the REMsd group compared to REMsdH, FMC and TC groups ($p<0.01$) as shown in Figure 4.

Histology of ventral posterolateral thalamic (VPL) nucleus

The FMC group showed normal histology of the VPL nucleus. The architecture was preserved with abundant healthy neurons. The Nissl substances in the cytoplasm were visible in Figure 5. Similar features were observed in REMsdH and TC. In the REMsd group, the density of neurons in VPL was reduced. The neurons were smaller in size and there was reduced intensity of cytoplasmic staining (Figure 5). There was a scanty number of neurons with the presence of dark neurons that suggests neuronal injury in the REMsd group.

Quantification of NMDA R2

There was a significant difference in the NMDA R2 level when compared between the groups (Figure 6) [$F(3,14)=14.241$, $p<0.01$]. Bonferroni post-hoc test showed NMDA R2 level in REMsd group was significantly higher compared to FMC, REMsdH and TC groups ($p<0.01$).

DISCUSSION

The current work investigated whether Tualang honey modulated pain behaviour, histological changes and NMDA R2 level in the thalamus of the REM sleep-deprived rat model. The work confirmed REM sleep deprivation by demonstrating the

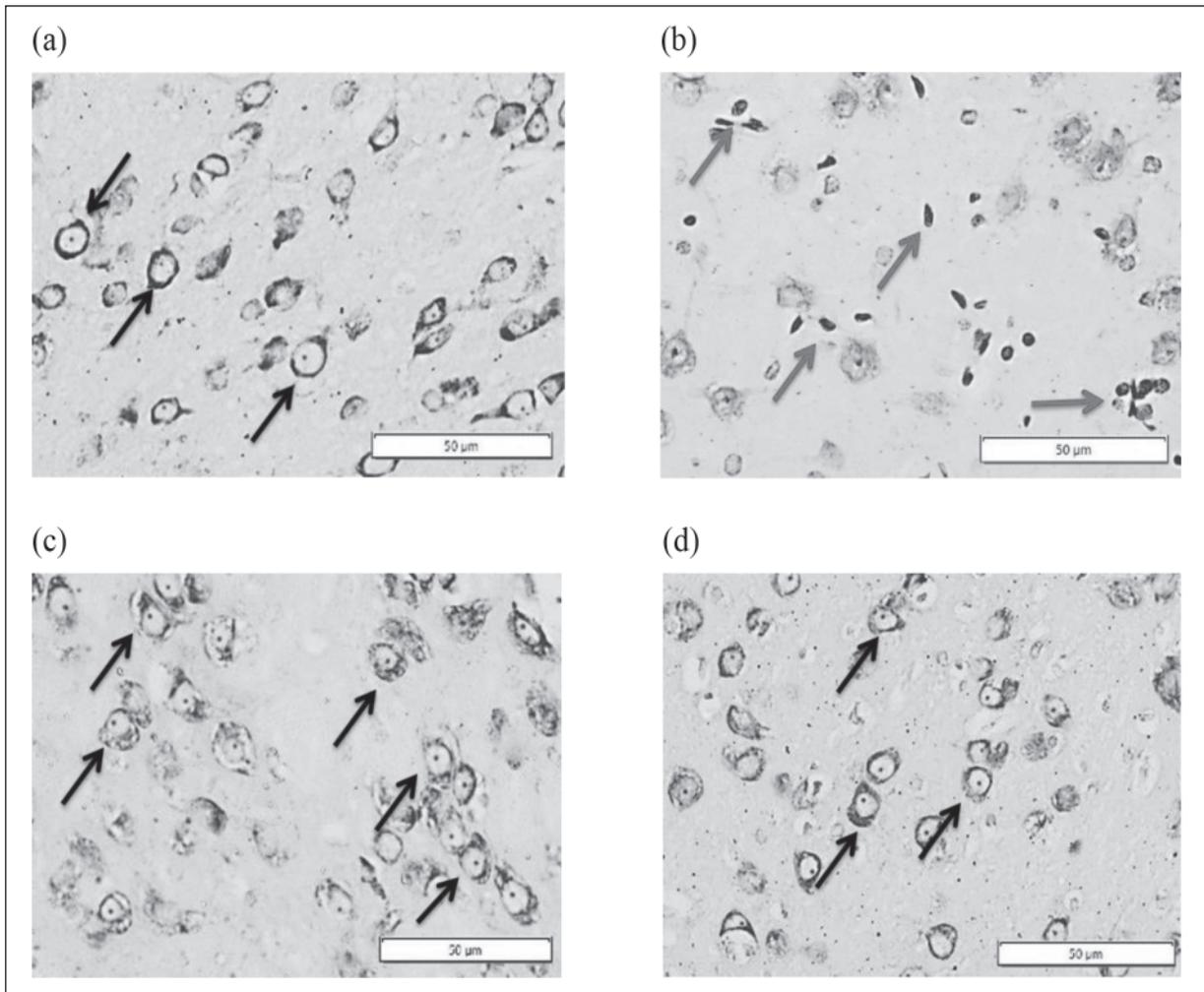


Fig. 5. Histology of the left ventral posterolateral nucleus in the thalamus sections from (a) FMC (b) REMsd (c) REMsdH and (d) TC groups. The arrows in (a), (c) and (d) indicate normal Nissl staining neurons. (b) The dark neurons which represent damaged neurons are shown by the gray arrows. The density of nissl stained neurons was also reduced compared to (a), (c) and (d) (Nissl stain, magnification of $\times 200$, scale bar: $50 \mu\text{m}$).

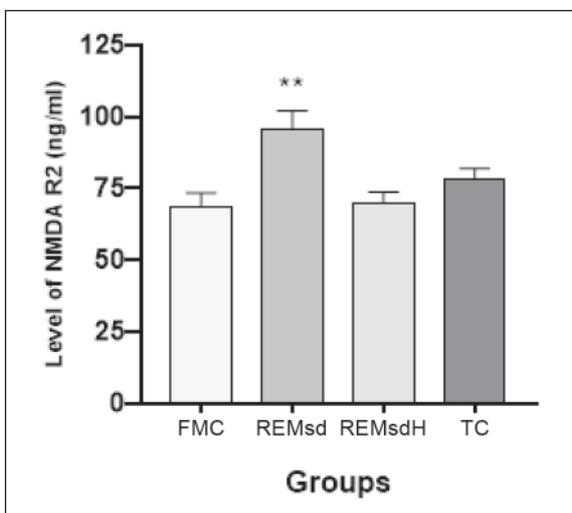


Fig. 6. Level of NMDA R2 in all groups including FMC, REMsd, REMsdH and TC. Data are presented as mean \pm SEM ($n = 12$ rats in each group). ** $p < 0.01$, statistical comparison between REMsd and other groups.

presence of hyperphagia associated with bodyweight loss in the rat model (Siran *et al.*, 2014). During sleep deprivation, increased levels of leptin, and changes in the hypothalamic immunoreactive neuropeptides that regulate food intake can lead to hyperphagia in the rats. The weight of REM sleep-deprived rats was lower compared to control groups despite increased food intake (Koban *et al.*, 2008, Siran *et al.*, 2014). The calorie intake did not match with calories output during the REM sleep deprivation. The output was contributed by substantial mobilisation of liver and skeletal muscle glycogen that leads to weight loss (Koban *et al.*, 2008). Following sleep deprivation, there was increased pain behaviour score in the REMsd group, however, the increased scores were not seen in the treated group (REMsDH). The lower scores in the REMsdH group at min 20 and 45 compared to FMC and TC groups are expected as Tualang honey administration is associated with reduced nociceptive responses compared to the

control group in other studies (Hasim *et al.*, 2020; Ismail *et al.*, 2017). The enhanced pain responses have been shown in other reports following 48 hours and 96 hours of REM sleep deprivation (Kim *et al.*, 2019; Onen *et al.*, 2001). The present study has utilised 72 hours of REM sleep deprivation based on the preliminary study done in our laboratory that showed the period was adequate to cause cardiovascular changes in the rat model of sleep deprivation (Tengku Adnan *et al.*, 2017).

The associated mechanisms that modulate pain following sleep disturbances are not fully understood. However, structures such as periaqueductal gray (PAG) and raphe nuclei (RN) are parts of the descending pain control system (Foo *et al.*, 2003) and are also involved in the regulation of sleep (Lu *et al.*, 2006). In addition, sleep deprivation was associated with increased Fos expression in orexin neurons in the hypothalamus (Estabrooke *et al.*, 2001) and Downregulatory antagonist modulator (DREAM) protein expression in the thalamus (Siran *et al.*, 2014). Hypothalamus and thalamus are important structures involved in the modulation of pain. Increased pain responses in phase 2 of formalin-induced pain, could be contributed by alteration of descending serotonergic neurons, GABAergic neurons or changes in the thalamic neurons.

The results of the present study demonstrated a significantly lower number of positive Nissl stained neurons in the REMsd group compared to other groups. Sleep deprivation is one of the factors that can lead to neurodegenerative changes in the brain (Li *et al.*, 2020). Thalamus is part of the brain that is involved in sleep and wake regulation as well as modulation of pain (Hasim *et al.*, 2020). Due to close associations of thalamic neurons with the hypothalamus and brainstem, sleep disturbance may contribute to the alteration of pain perception (Gent *et al.*, 2018). Sleep deprivation may alter level of neurotrophic factors that have an important role in neuron growth, synaptic formation and maturation and neuronal survival (Li *et al.*, 2020). These factors may contribute to neuronal damage and decreased number of Nissl stained neurons in the thalamus.

Apart from that, reports have demonstrated that sleep deprivation was associated with impairment in NMDAR-mediated synaptic transmission and increased NMDA receptor expression in the hippocampus (Kopp *et al.*, 2006; McDermott *et al.*, 2006). The altered NMDA receptor level is most probably a compensatory mechanism to overcome the impaired synaptic transmission. The upregulation of the N-methyl D-aspartate (NMDA) receptor can lead to oxidative stress and disrupts the blood-brain barrier (BBB). A massive influx of calcium with the activation of NMDA receptors can also contribute

to cell damage and apoptosis (Betzen *et al.*, 2009). NMDA R2 was chosen in the study as it is involved in nociception. There are also other subtypes of NMDA including NR1, NR2 comprises of NR2A, NR2B, NR2C, NR2D and NR3 (Petrenko *et al.*, 2003).

The present study showed decreased pain behaviour score, increased number of Nissl stained neurons and decreased NMDA receptor level in the REMsdH group compared to the REMsd group. The anti-inflammatory and antioxidant properties of Tualang honey (Bashkaran *et al.*, 2011; Ismail *et al.*, 2017) may have contributed to the antinociceptive effects in the REM sleep deprived rat model. Sleep deprivation may lead to oxidative stress (Mathangi *et al.*, 2012) which might contribute to neuronal damage in the thalamus. Although Tualang honey was not administered during the REM sleep deprivation procedure, the honey given prior to the sleep deprivation may have protective effects on the thalamus by modifying certain gene expressions that may reduce oxidative stress. A recent report has shown that candidate plasticity-related gene 15 (cpg15), a neurotrophic gene, protected the brain from oxidative stress (Li *et al.*, 2020) following sleep deprivation. If Tualang honey has positive effects on the gene, the gene may contribute to reduce oxidative stress and protect the neurons in the thalamus. Further study should examine the effects of Tualang honey on the gene and oxidative stress in the rat model of sleep deprivation.

In conclusion, administration of Tualang honey for one month before REM sleep deprivation modulated the nociceptive responses and associated changes in the thalamus of the REM sleep deprivation rat model.

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