

Research Article

Tualang honey improves memory and hippocampal changes in adult offspring of prenatally stressed rats

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INTRODUCTION: Prenatal stress has been shown to be associated with development of abnormal behaviour as well as disruption of learning and memory processing of spatial information in the offspring.

METHODS: This study investigated whether alteration of memory, changes in the hippocampus morphology, levels of malondialdehyde (MDA) and N-methyl-D-aspartate (NMDA) receptor in the hippocampus of adult rat offspring following prenatal stress could be prevented by administration of Tualang honey in the pregnant dams. Twenty-four pregnant rats were randomized into control (C), stress group (S) and stress group treated with Tualang honey (TH). Twenty-four adult offspring were sacrificed following Novel Object Recognition Test. Their brains were removed and histological changes, level of MDA and NMDA receptors in the hippocampus were determined.

RESULTS: The offspring from TH group showed significant increase in preference index ($p < 0.05$) and improved hippocampal morphology compared to S group. The group also demonstrated a significantly lower level of MDA and NMDA receptors ($P < 0.01$; $P < 0.05$ respectively) compared to S group. There were no differences in the parameters investigated between C and TH groups.

DISCUSSION AND CONCLUSION: The study has shown that Tualang honey administration was associated with improvement in memory and morphology, levels of MDA and NMDA receptors in the hippocampus in the adult offspring following prenatal stress. The results suggest the protective role of Tualang in prenatally stressed rat offspring.

Keywords: Prenatal stress, hippocampus, Tualang honey, malondialdehyde, NMDA receptor.

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20.06.2019

31.10.2019

Introduction

Studies have reported that prenatal stress might lead to development of abnormal behaviours in the adult offspring such as attention deficit hyperactivity disorder, schizophrenia and depression as well as disruption of learning and memory processing of spatial information in the offspring.¹⁻³ The mechanisms that are responsible for the behavioural abnormalities following prenatal stress might be related to higher maternal corticosterone levels and lower placental 11- β -hydroxysteroid dehydrogenase isozyme type 2 (11 β -HSD₂), enzyme that deactivates the maternal corticosterone.⁴ The changes in the hormone and enzyme will lead to higher level of corticosterone in the foetus. Prolonged exposure to the corticosterone will alter growth of foetal brain and lead to oxidative stress as shown by increased lipid peroxidation and reduced in enzymatic antioxidant activities in the brain.^{5,6}

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The oxidative stress may contribute to damage of the neurons in the hippocampus of offspring and impairment of memory function.⁷ Another report has shown that stress-induced elevation of N-methyl-D-aspartate (NMDA) receptors and corticosterone might mediate the reduced learning ability, impaired memory and other stress-induced neurologic disorders.⁸ Studies have demonstrated that the hippocampus of prenatally stressed animals e.g. rats and monkeys, was smaller compared to the non-stressed group and this suggests that prenatal stress is associated with reduced neurogenesis.^{9,10}

The reduced neurogenesis that occur following prenatal stress might be associated with oxidative stress in the brain with impairment of memory function.^{5,6,8} ~~Although direct administration of antioxidant e.g. Tualang honey has been reported to reduce oxidant level in stressed ovariectomized rats and improved memory function in the ageing rats, its role in improving memory function in the prenatally stressed rat offspring is not known.^{11,12}~~

Tualang honey is a wild rainforest [multifloral](#) honey produced by bees called *Apis Dorsata*. The honey can be collected from the hives which are built on branches of Tualang tree (*Koompassia excelsa*). ~~Tualang honey~~ contains fructose, glucose, maltose, amino acids, vitamins, minerals, enzymes, flavonoids and phenolic acids (Ahmed & Othman, 2013; Khalil et al 2011).^{13,14} The compositions will depend on the floral source and the environment surrounding the trees (Gheldof et al., 2002; Nurul et al., 2013).^{15,16} Tualang honey has been reported to have more antioxidant activity compared to Gelam and Manuka honey which are [available in Malaysia monofloral honey](#) (Khalil et al., 2011; Kishore et al., 2011).^{14,17}

~~Although direct administration of antioxidant e.g. Tualang honey has been reported to reduce oxidant level in stressed ovariectomized rats and improved memory function in the ageing rats, its role in improving memory function in the prenatally stressed rat offspring is not known.^{11,12}~~

Hence, this study investigated whether alteration of recognition memory, changes in the morphology, malondialdehyde (MDA) and NMDA receptor levels in the hippocampus of adult rat offspring following prenatal stress could be prevented by Tualang honey administration to the pregnant dams.

Materials and Methods

Twenty-four female and six male Sprague-Dawley rats, eight to ten weeks of age, were obtained from Animal Research and Service Centre (ARASC), Universiti Sains Malaysia. The rats were maintained on a 12 h light: 12-h dark cycle (light phase 7 am-7 pm) with adequate food and water available ad libitum with adaptation phase for 3-5 days in the physiology laboratory before the experiment. Experiments were done in ARASC at day time. After mating, vaginal smears from the female rats were assessed in the morning between 0900 and 1000, if sperms were detected, the day was labelled as day 0 of pregnancy.¹⁸ The rats were randomised into three groups (n=8 per group); control, stress, stress treated with honey (Tualang honey). The stress was given in a form of repeated restraint stress in a cylindrical restrainer measuring 23 cm x 6 cm. The stress was applied to the pregnant dams for three times daily: 30 min each at 0800, 1200 and 1600.¹⁸ Federal Agricultural Marketing Authority (FAMA) has supplied Tualang honey used in the present study. It was administered orally by gavaging to the pregnant rats (stress treated group) throughout pregnancy until delivery. The dosage used was 1.2 g/kg body weight/day and it was in a form of undiluted honey.¹⁸ Each pregnant dam was kept in an individual cage until delivery. At least one male

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offspring from each pregnant dam was included in the study. A total of twenty-four male offspring (8 to 10 weeks old) weighing 200 g to 250 g were investigated in the study.

Novel Object Recognition Test (NORT)

Each rat was adapted to an empty open field (35 cm x 60 cm) for 10 min/day for two consecutive days. The open field was used for training and retention sessions. At training session, two objects were placed in the field and each rat was permitted to explore freely for 10 minutes. The rats' behaviour was recorded using a video camera and the time used to explore was assessed from the recorded video. Exploration was defined as the orientation of animal's snout toward the object, sniffing or touching with snout.¹⁹

Retention test was performed a day after training session. One of the objects used in the training was substituted by a different object (novel object) and each rat was permitted to explore for 5 minutes.²⁰ The objects which vary in shape and colour and made of plastic were fixed to the floor. The objects were cleaned before each test to ensure lack of olfactory cues. The present study looked at the exploratory preference, a ratio of time spent exploring any one of the two objects (training) or the novel one (retention) over the total time spent exploring both objects.²¹ The preference index (PI) used was an indicator of recognition memory and Hammond et al has suggested that PI above 50% indicates novel object preference, below 50% familiar object preference, and 50% no preference.²²

Morphology of hippocampus

Hippocampus was quickly identified and isolated. Ten percent of formalin was used to fix the samples. The samples were then dehydrated in an automated tissue processor machine, blocked with paraffin wax and kept at 0°C for three hours. The tissues were cut using a microtome so that each section was about 5 µm thick. The tissues were then placed on glass slides, dried on a hot plate at 50-55°C for 30 min and kept at 37°C. The slides were then stained using Nissl staining. After completely dried from xylene, the slides were air-dried for 30 min, mounted in Cytoseal XYL mounting medium and covered with cover slips. Light microscope was used to observe the histology of the tissues and images were captured to assess the neuronal shape and arrangement.

Preparation of brain homogenate and MDA measurement

The hippocampus from each group was quickly removed from the brain. The isolated hippocampus was weighed and homogenate (10% w/v) were prepared in ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) by hand or grinders until no visible particles remain. The homogenates were centrifuged (10,000 x g) for 10 min. and the samples were stored at -80°C until assay. MDA level was analysed in the hippocampus using commercially available kits (USCNK, Wuhan).

Assay procedures for NMDA receptors

The isolated hippocampus was homogenized and the sample was centrifuged at the speed of 2000-3000 rpm for 20 minutes. Supernatant was taken and kept at -80°C until assay. The assay was done using reagent kit bought from USCNK (Qayee-Bio, Shanghai, China). NMDA receptor level in the sample was determined using a double antibody sandwich enzyme-linked immunosorbent one-step process.

Statistical analysis

The results were analysed using SPSS version 22 software. One-way ANOVA was used to analyse differences of preference index, number of Nissl-stained-positive neurons, MDA and NMDA receptor levels between the groups. The data in the present study were expressed as mean ± standard error mean (SEM). The differences were considered to be significant when p value is less than 0.05 (p<0.05).

Results

Effect on NORT in prenatally stressed male rats offspring

During training session of the NORT, there were no significant differences in the preference index ($P=0.787$) when compared between the three groups. The level of preference index for the novel object in stress group was significantly lower [$F(2,30)=0.007$, $P<0.01$] compared to other groups (figure 1) at retention session. The TH group has significantly longer time in exploring novel object than stress group [$P<0.05$], however the difference between TH and control groups was not statistically significant.

Effect on MDA level in prenatally stressed male rats offspring

There was a significant difference of MDA level when compared between the groups as determined by one-way ANOVA [$F(2,21) = 18.53$, $P=0.001$]. The level of MDA in stress group (377.55 ± 9.28 pmol/ml) was significantly higher ($P<0.01$) compared to control (327.55 ± 9.24 pmol/ml) and TH (297.75 ± 9.61 pmol/ml) groups when analysed using Bonferroni post hoc test. There was no statistically difference ($P=0.116$) between control and TH groups (figure 2).

Effect on NMDA receptor level in prenatally stressed male rats offspring

There was a significant difference of NMDA receptor level when compared between the groups as determined by one-way ANOVA [$F(2,21) = 7.039$, $P=0.05$]. The level of NMDA receptor was significantly higher in stress group (20764.34 ± 788.10 ng/ml) ($P<0.05$) compared to control (18003.45 ± 561.83 ng/ml) and TH (16999.95 ± 826.28 ng/ml) groups (figure 3), as analysed using Bonferroni post hoc test. There was no statistically difference ($P=1.000$) between control and TH groups.

Effect on Nissl-positive neurons in the hippocampus of prenatally stressed male rats offspring

There was a significant difference of Nissl-positive neurons when compared between the groups as determined by one-way ANOVA [$F(2,21) = 5.136$, $P<0.05$]. A Bonferroni post hoc test revealed that the number of Nissl-positive neuron in stress group (29.66 ± 1.24 mm²) was significantly lower ($P<0.05$) compared to TH (36.67 ± 1.67 mm²) group (figure 4). However, there was no significant difference in control compared to stress ($P=1.000$) and TH ($P=0.127$) groups. Meanwhile, the normal morphology of hippocampus was observed in the control group with abundant of healthy neurons. The architecture was maintained and Nissl substances were clearly visualised in the cytoplasm. In contrast, the density and the intensity of cytoplasmic staining of hippocampus in the stress group were reduced with altered architecture compared to the control group. In TH group the architecture was preserved with increased number of neurons (figure 5).

Discussion

[Recognition memory plays an important role to discriminate familiar from novel stimuli.](#)²⁰ In the present study the Novel Object Recognition Test, there was no difference of the preference index during training session of the NORT, however, 24 hours later the index was reduced significantly in the stress group compared to control and treated stress groups. [Recognition memory plays an important role to discriminate familiar from novel stimuli.](#)²⁰

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The reduced [preference index indicating reduced](#) recognition memory most probably is contributed by structural changes in the hippocampus.²¹ Although the [amount-number](#) of Nissl-positive neurons was not significantly different between stress and control group but there was altered characteristics of the [neuronal](#) cells. -Prenatal stress has been shown to induce histological changes in the brain of rat offspring e.g. amygdala, corpus callosum cerebral cortex, hippocampus.²³ [The present study also demonstrated altered architecture in the CA2 of hippocampus of the offspring in the stress group and the architecture was preserved with increased number of cells in Tualang honey group.](#)

In the present study, the number of Nissl-[stained-positive](#) neurons in the stress group was not significantly different, however, the morphology of CA2 of the hippocampus was altered. The altered morphology in the hippocampus might be contributed by oxidative stress as shown by increased in MDA level. Neuronal death due to oxidative stress has been shown to occur in the hippocampus in rat model of status epilepticus and Alzheimer's disease.^{24,25} Exposure to prenatal stress will activate hypothalamic-pituitary-adrenal axis leading to an increase in glucocorticoid level. There are abundant glucocorticoid receptors in the hippocampus and the hormone is able to modify neuronal structure, neuronal metabolism and may lead to oxidative stress in the brain of the offspring.²⁶ Furthermore, increased fetal glucocorticoid may increase activation of excitatory amino acid receptors such as NMDA receptors that up-regulate increase in intracellular calcium concentration that contribute to accumulation of oxidants.⁸

The altered morphology of hippocampal cells may influence learning and memory in the offspring as shown in the present study. Previous studies have shown that Tualang honey administration improved number and histological features of neurons in the hippocampus of rats exposed to various types of stress.^{27,28} The increased number of neurons was also seen in the spinal cord of the offspring, following Tualang honey administration during prenatal stress.²⁹ Luteolin, [which is](#) one of the flavonoids in Tualang honey, has been shown to stimulate neurogenesis in the hippocampus of mice model of Down's syndrome ([Khalil et al., 2011; Zhou et al., 2019](#)).^{16,30} The increased neurogenesis was associated with improved [memory-learning](#) and [memory learning](#) behaviour ([Zhou et al \(2019\)](#)).³⁰ The increased number of [hippocampal neuronal cells/neurons in the offspring](#) following Tualang honey administration in the pregnant dams suggests increased neurogenesis in the rats' offspring which is associated with improved [preference index/recognition memory](#).

Quercetin, another flavonoid available in Tualang honey, has been reported to suppress mRNA expression of corticotrophine releasing hormone, and reduce the level of adrenococorticotrophic hormone and corticosterone.³¹ A lower level of corticosterone plus the antioxidant activity of Tualang honey would reduce formation of [reactive oxygen species/ROS](#) and antioxidant utilisation in the brain of the offspring that may protect neuronal function.^{29,32} Apart from quercetin and luteolin, Tualang honey [has contains](#) other substances such as caffeic acid and vitamin C ([Khalil et al., 2011; Kishore et al., 2011](#)).^{16,17} [Koga et al \(2017\)](#) has reported that administration of caffeic acid in a group of mice lead to reduced oxidative stress and less microglial activation in the hippocampus.³³ Oxidative stress and microglial activation have been linked with various neurological and psychiatric disorders ([Koga et al., 2019](#)).³³ Vitamin C has also been shown to improve oxidative stress and neurogenesis in the hippocampus of rat model of ageing ([Khalil et al., 2011; Nam et al., 2019](#)).^{16,34} All the reports suggest that the substances present in Tualang honey have beneficial effects on neurogenesis and have the potential to [inhibit/mitigate](#) oxidative stress.

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Conclusion

In conclusion, the present study has shown that prenatal stress was associated with memory impairment probably contributed by ~~changes in~~altered hippocampal histology, increased levels of MDA and NMDA receptors in the hippocampus. Administration of Tualang honey was associated with improvement in the parameters investigated.

Study limitation

The present study was conducted on male offspring population and has excluded female population to avoid the influence of ovarian hormones on memory performance. In addition NMDA receptor subtype such as NR1 was not assessed in this study because of financial limitation. ~~Hence, it is recommended for future studies to~~Further study should look investigate ~~the~~ effects of Tualang honey on different subtype of NMDA receptor and different type of genes responsible ~~in~~for memory performance.

Acknowledgment

This study was supported by Bridging Grant (304/PPSP/6316130) awarded by Universiti Sains Malaysia.

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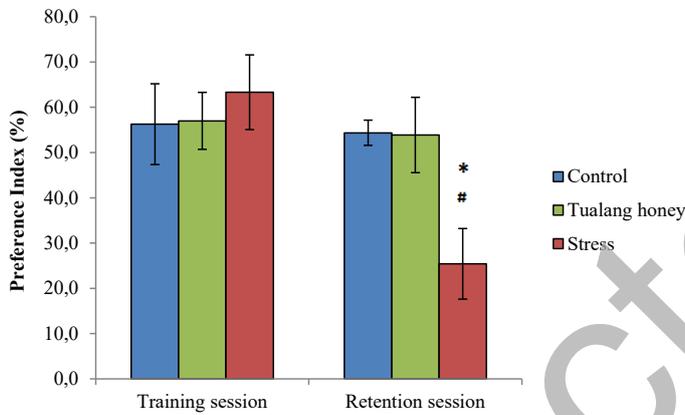


Figure 1 Preference index during training and retention sessions in the offspring from control, stress and Tualang honey (TH) groups. Preference index (%) = any one of object / (familiar object 1 + familiar object 2) × 100 (%) in the training session or novel object / (familiar object + novel object) × 100 (%) in the retention session. *P<0.05 shows a significant difference between control and stress; #P<0.05 shows a significant difference between TH and stress. Data were analysed using one-way ANOVA followed by Bonferroni test. Data are displayed as mean ± SEM for 8 rats in each group.

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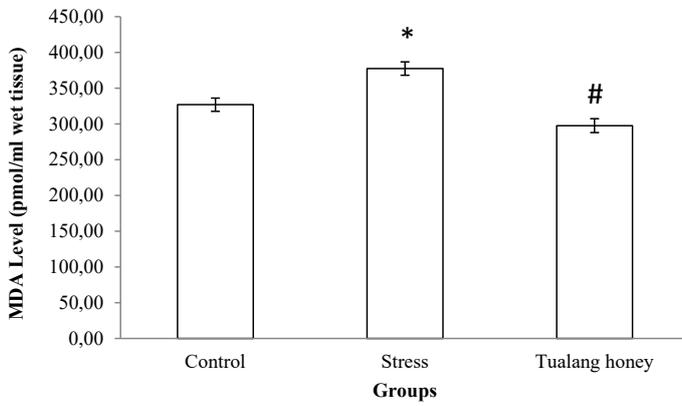


Figure 2. Level of MDA in the hippocampus's offspring from control, stress and Tualang honey (TH) groups. * $P < 0.01$ comparison between control and stress groups and # $P < 0.01$ comparison between stress treated with TH and stress groups. Data were analysed using one-way ANOVA followed by Bonferroni test. Data are represented as mean \pm SEM for 8 rats in each group.

Biçimlendirilmiş: Sola, Aralık Sonra: 0 nk, Satır aralığı: tek

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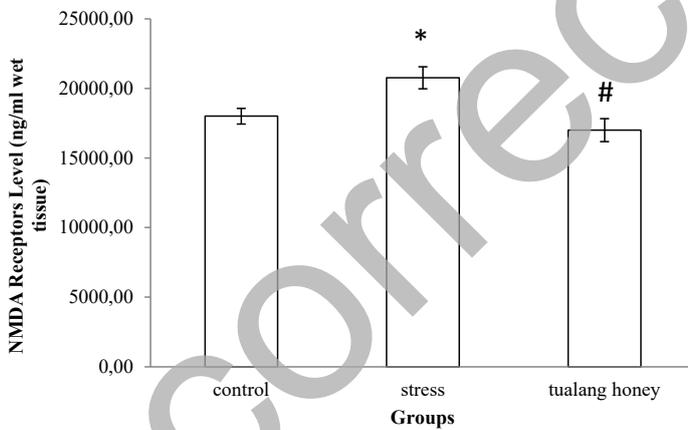


Figure 3. Level of NMDA receptors in the hippocampus's offspring from control, stress and Tualang honey (TH) groups. * $P < 0.01$ comparison between control and stress groups and # $P < 0.01$ comparison between stress treated with TH and stress groups. Data were analysed using one-way ANOVA followed by Bonferroni test. Data are represented as mean \pm SEM for 8 rats in each group.

Biçimlendirilmiş: Sola, Satır aralığı: tek

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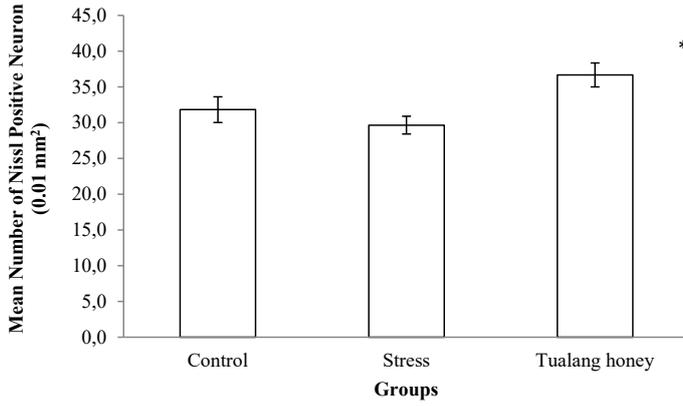
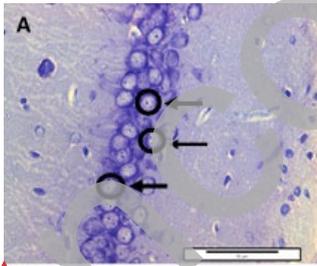


Figure 4. Mean number of Nissl-positive neurons in the hippocampus's offspring from control, stress and **Tualang honey (TH)** groups. * $P < 0.05$ comparison between stress and TH groups. Data were analysed using one-way ANOVA followed by Bonferroni test. Data are represented as mean \pm SEM for 8 rats in each group.



Biçimlendirilmiş: Sola, Satır aralığı: tek

Biçimlendirilmiş: Aralık Sonra: 0 nk, Satır aralığı: tek

Biçimlendirilmiş: Sola, Girinti: Sol: 0 cm, İlk satır: 0 cm, Aralık Sonra: 0 nk, Satır aralığı: tek

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Biçimlendirilmiş: Sola, Girinti: Sol: 0 cm, İlk satır: 0 cm

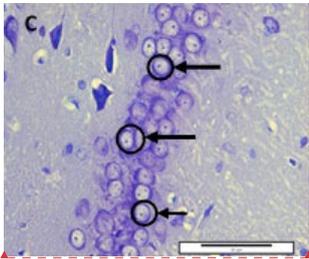
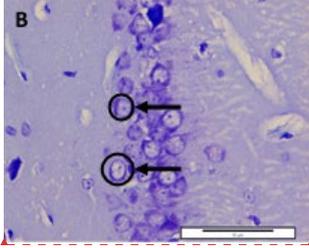


Figure 5. Neurons arrangement in the CA1 and CA2 (left side) of hippocampus section of the offspring from (A) control, (B) stress and (C) stress treated with Tualang honey (TH) groups. The arrows indicate the cells of interest (Nissl staining $\times 400$, scale bar: $50\mu\text{m}$). Note the normal architecture with layers of pyramidal cells and vesicular nuclei in (A). The architecture was altered and there was reduced intensity of cytoplasmic staining in (B). The architecture was preserved with increased number of cells in (C).

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Biçimlendirildi: Yazı tipi: Times New Roman, 12 nk, Yazı tipi rengi: Metin 1

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Biçimlendirilmiş: Sola

Biçimlendirildi: Yazı tipi: (Varsayılan) Times New Roman, Yazı tipi rengi: Metin 1

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