

Tualang Honey Consumption Enhanced Hippocampal Pyramidal Count and Spatial Memory Performance of Adult Male Rats

(Pengambilan Madu Tualang Meningkatkan Bilangan Sel Piramid Hipokampus dan Prestasi Ingatan Reruang Tikus Jantan Dewasa)

MOHD AMIRUDDIN KAMARULZAIDI, M.Y. ZULKIFLI MOHD. YUSOFF, ABDUL MAJID MOHAMED & DURRIYYAH SHARIFAH HASAN ADLI*

ABSTRACT

As a natural anti-oxidant source, Tualang honey, produced by wild bees nesting on the Tualang tree (Koompassia excelsa) is expected to have positive influence on health, including memory. This study investigated the effect of Tualang honey on the cell count of memory formation related hippocampal pyramidal neuron and on spatial memory performance (SMP) of rats using the radial arm maze (RAM). Sprague Dawley male rats (n=24), 7-8 weeks old were divided into two groups; experimental group group force-fed 1 mL/100 g body weight with 70% honey (HG); and the control group with 0.9% saline (CG) for 12 weeks. Nissl staining technique (with cresyl violet) was employed for neurohistological analysis of the hippocampal tissue. Six randomly selected rats from each group were used for the neuronal soma counting of pyramidal cell layer CA1, CA3a and CA3c regions. Two-way ANOVA analysis showed positively significant differences between treatment and control groups for SMP comparison of working memory and reference memory components, as well as the number of pyramidal neurons. Hence, this positive effects of Tualang honey, as demonstrated behaviorally and neurohistologically, supported report that Tualang honey could improve memory and deter hippocampal morphological impairments; possibly due to its high anti-oxidant properties.

Keywords: Radial arm maze; reference memory; Tualang honey; working memory

ABSTRAK

Sebagai sumber antioksidan semula jadi, madu Tualang yang dihasilkan oleh lebah liar yang bersarang di pokok Tualang (Koompassia excelsa) dijangka mempunyai pengaruh yang positif ke atas kesihatan, termasuk ingatan. Penyelidikan ini mengkaji kesan madu Tualang ke atas bilangan neuron piramid hipokampus yang berkaitan dengan pembentukan ingatan dan prestasi ingatan reruang (SMP) tikus menggunakan lorongan keliru lengan radial (RAM). Tikus Sprague Dawley jantan (n=24), 7-8 minggu telah dibahagikan kepada dua kumpulan; kumpulan eksperimen disuap-paksa 1 mL/100 g berat badan dengan 70% madu (HG) dan kumpulan kawalan dengan 0.9% salin (CG) untuk 12 minggu. Teknik pewarnaan Nissl (dengan ungu kresil) telah digunakan untuk analisis neurohistologi tisu hipokampus. Enam tikus dipilih secara rawak daripada setiap kumpulan dan telah digunakan untuk pengiraan lapisan sel soma neuron piramid daripada kawasan CA1, CA3a dan CA3c. Analisis ANOVA dua hala menunjukkan perbezaan positif yang signifikan antara kumpulan HG dan CG untuk perbandingan SMP antara komponen ingatan kerja dan ingatan rujukan, serta bilangan neuron piramid. Oleh itu, kesan positif Tualang madu, seperti yang ditunjukkan daripada aspek tingkah laku dan neurohistologi, menyokong laporan bahawa madu Tualang boleh meningkatkan ingatan dan menghalang gangguan terhadap morfologi hipokampus; berkemungkinan kerana sifat antioksidannya yang tinggi.

Kata kunci: Ingatan kerja; ingatan rujukan; lorongan keliru lengan radial; madu Tualang

INTRODUCTION

Honey, which is commonly used for health and medical purposes since time immemorial across various continents and civilizations, as recorded in the Sumerian tablet, the Bible and the Quran (Bogdanov et al. 2008) has been proven to have anti-oxidant properties. In addition to having sugars (e.g. glucose), it also has a variety of phytochemicals (e.g. phenol) and other materials (e.g. organic acids, vitamins and enzymes) (Ferreira et al. 2009). The *Tualang* honey collected from hives of bees (*Apis* sp.) built in the *Tualang* (*Koompassia excelsa*) trees (Tan et al.

2009) is also commonly used in food and health products (Che Ghazali 2009). Such practices agree well with *Tualang* honey having anti-oxidant, anti-inflammatory, anti-mutagenic, anti-tumor, anti-diabetic and wound-healing properties (Ahmed & Othman 2013).

Anti-oxidant has been proposed to be good for the various memory types. The spatial memory specifically identifies, encodes, stores and recalls spatial information or a particular arrangement of objects or specific routes (Kessels et al. 2001), including related spatial mental representation within the environment. Hence, it can be

used to manipulate, recall and navigate between spaces, either in the real or imaginative world (Jacobs 2003). Brain hippocampus is a structure involved in memory formation and it consists of six specific structures: Dentatus gyrus, hippocampus proprius (Cornu ammonis, CA1, CA2 and CA3), proprium subiculum, presubiculum, parasubiculum and entorhinalis area (El Falougy & Benuska 2006; Xavier et al. 1999).

Honey is one of the commonly recommended alternative foods to enhance memory (Chepulis et al. 2009), similar to *Ginkgo biloba* (Araujo et al. 2008), *Nigella sativa* (Sahak et al. 2013) and fruits rich in flavonoids (Spencer 2010). However, scientific research showing specific positive effect of honey, for example the *Tualang* honey, on spatial memory and hippocampus is still lacking. Therefore, the objectives of this study were to investigate the effects of the Malaysian *Tualang* honey on the memory of rats in relation to hippocampal pyramidal neurons and spatial memory performance (SMP) using behavioural approach.

MATERIALS AND METHODS

Natural *Tualang* honey (AgroMas), a product of Federal Agricultural Marketing Authority (FAMA), Malaysia, was used in this study. The honey was kept at room temperature and protected from direct sunlight and 70% honey concentration was prepared using 0.9% saline.

Sprague Dawley male rats aged 7-8 weeks ($n=24$ rats) were obtained from the Animal Unit, Faculty of Medicine, University of Malaya. Two rats per cage housed in the Animal House, Centre for Foundation Studies in Science, University of Malaya were kept under 12/12 light/dark cycle, fed with standard food pellets and given water *ad libitum*. Cages were cleaned and saw dusts were changed every three days to ensure clean environment. For 12 weeks, rats in the treatment group were force-fed with honey (HG), while those in the control group (CG) were given 0.9% saline for the first five days of each week ($n=12$ rats per group). Rats were deprived of food on the sixth day prior to the behavioural test on the seventh day and were fed as usual immediately after the test was completed. Rats were weighed every three days so that 1 mL/100 g body weight of 70% honey or 0.9% saline could be force-fed accordingly. The protocol used was approved by the Institutional Animal Care and Use Committee (IACUC), University of Malaya (ISB/20/04/2012/DSHA (R)).

BEHAVIOURAL TEST

A standard radial arm maze (RAM) was used to evaluate spatial memory performance (SMP) behaviour. It consisted of eight arms, each measuring 70 cm (length) \times 10 cm (width) \times 15 cm (height), extending from the middle octagon-shaped platform measuring 25 cm² in diameters. The white painted maze was placed at a fixed position for each test to reduce variability. Acclimatization of the rats before the behavioural testing was conducted according to

standard procedure (Levin 2001). The weekly behavioural test was conducted in the morning of the seventh day of each week for three months. With the motivation of finding food during the test session, the rats were expected to learn and remember the best strategy to cope with the situation (Tolman 1984). A food-filled container was placed at the end of each 1st, 3rd, 5th and 7th arms. A rat was placed at the maze centre facing the same direction every time at the beginning of the test session and was allowed to explore the entire maze for 3 min. It was considered to have entered a specific arm when all four paws were in the initial part of the arm. The maze was wiped cleaned with 70% alcohol after each test session of a rat to prevent odour cues. SMP was recorded and analyzed for both HG and CG. First entry into any arm without food was considered as reference memory error (RME). Re-entry into the arm that previously had food, but had been eaten, was considered as working memory error (WME). First entries and re-entries into arms that had no food made up the total error (TE). Quantitative analysis of SMP was based on four phases: Phase 1 (weeks 1-3), Phase 2 (weeks 4-6), Phase 3 (weeks 7-9) and Phase 4 (weeks 10-12). The data were subjected to two-way analyses of variance (ANOVA) using the Statistical Packages for the Social Sciences version 20.0 (SPSS Inc., Chicago, IL, 2011). The least-squares means for the interaction term were used to plot curves showing changes in treatment with respect to phase. A value of ($P<0.05$) was considered statistical significant.

NEUROHISTOLOGICAL STUDIES

For the purpose of examining the hippocampal tissue under the microscope, Nissl staining technique was used. Cell counting was done using microscope (Olympus BX51, Germany) at 200 \times magnification with the help of Life Science Software Analyzer software. A total of five slides from each animal with both right and left hemispheres in a brain section were considered. Soma neurons in a standard size hippocampal area involving CA1, CA3a, CA3c pyramidal cell layers were counted (Figure 1). Only neurons with clear somatic nuclei and nucleolus were counted.

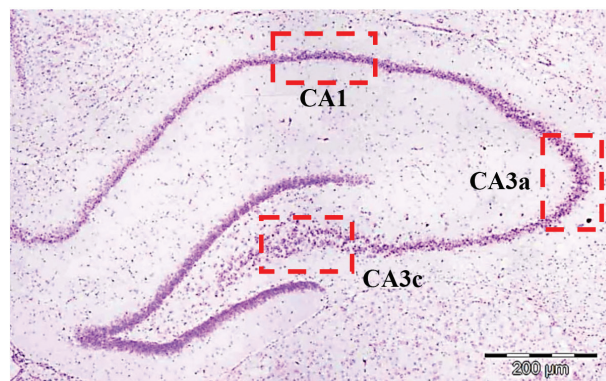


FIGURE 1. Hippocampal cross section showing the regions for cell counting

RESULTS

Pairwise comparisons for the effect of treatment on HG and CG groups by phase showed significant differences for TE, RME and WME (Table 1) in the third (weeks 7-9) and fourth (weeks 10-12) phases. The least-squares means from the ANOVA for the interaction effects were used to plot the curves for changes in treatment effect by phase

(Figure 2). Figure 2 shows that both groups tended to make less WME, compared to TE and RME. Fairly similar number of errors (TE, RME and WME) were committed by both groups ($p>0.05$) in the early phase 1 and phase 2. However, the HG rats committed significantly less ($p<0.05$) number of errors than the CG rats in phases 3 and 4.

TABLE 1. Pairwise comparisons (Mean \pm SEM) for treatment effects of (a) total error (TE), (b) reference memory error (RME) and (c) working memory error (WME) of spatial memory performance of rats treated with honey and saline for 4 phases (3 weeks per phase)

(a)				
Group	Phase 1	Phase 2	Phase 3	Phase 4
Honey	1.281 \pm 0.121	1.311 \pm 0.121	0.878 \pm 0.121*	1.085 \pm 0.121*
Saline	1.179 \pm 0.099	1.551 \pm 0.099	1.674 \pm 0.009*	1.802 \pm 0.009*

(b)				
Group	Phase 1	Phase 2	Phase 3	Phase 4
Honey	1.186 \pm 0.108	1.220 \pm 0.108	0.861 \pm 0.108*	1.072 \pm 0.108*
Saline	1.080 \pm 0.088	1.476 \pm 0.088	1.565 \pm 0.088*	1.690 \pm 0.088*

(c)				
Group	Phase 1	Phase 2	Phase 3	Phase 4
Honey	0.208 \pm 0.107	0.208 \pm 0.107	0.167 \pm 0.107*	0.333 \pm 0.107*
Saline	0.296 \pm 0.088	0.453 \pm 0.088	0.661 \pm 0.088*	0.631 \pm 0.088*

Asterisks denote significant differences ($p<0.05$)

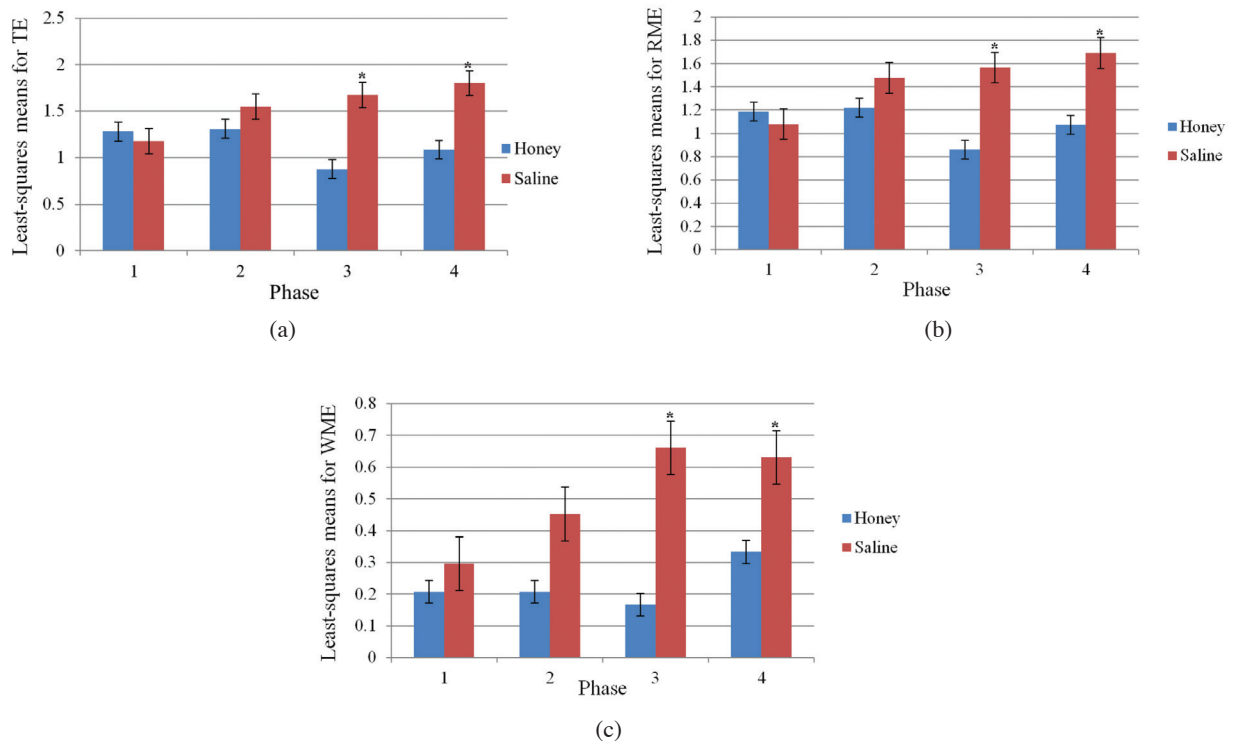


FIGURE 2. Least-squares means and standard errors for (a) total error (TE), (b) reference memory error (RME) and (c) working memory error (WME) of spatial memory performance of rats treated with honey and saline for 4 phases (3 weeks per phase). Asterisks denote significant differences ($p<0.05$)

As for the hippocampal histological results, pairwise comparisons for the effect of treatment on HG and CG groups showed numbers of neurons in hippocampal CA1, CA3a and CA3c layers (Table 2) were significantly different between groups ($p>0.05$). However, CA1 region for both groups showed the most number of neurons followed by CA3a and CA3c regions (Figure 3).

DISCUSSION

Partially baited RAM, which was used to evaluate the SMP in this study, allowed concurrent assessment of working memory (short term memory) and reference memory (long term memory) of the rats (Tarragon et al. 2012). As the experiment progressed, the rats were expected to remember previously-visited arms and arms that had no reward, aided by the environmental signals and the viewing period as cues (Mazmanian & Roberts 1983) since the position of all objects in the room were maintained throughout the experiment. As in other studies, the rats possibly depended on spatial cues or extra-maze signals in the environment to solve the spatial problem and not response strategies (Olton 1983) and/or intra-maze signal, like odour (Olton & Collison 1979). The odour signals in this study were eliminated when the maze was cleaned each time prior to a test.

The significant decreases ($P<0.05$) in TE, RME and WME in the last two phases committed by HG rats suggested that *Tualang* honey significantly enhanced learning and/or memory after six weeks of its consumption. This positive effect appeared to remain until the 12th week and was

more obvious for working memory. One previous study showed rats given honey had better spatial memory in the ninth and twelfth weeks compared to rats on a sugar-free or sucrose-based diet (Chepulis et al. 2009). Physiologically, the glucose in honey worked as an energy source for HG rats. A study involving a group of college students demonstrated glucose uptake improved memory, increasing their learning process (Korol & Gold 1998). This is further supported since glucose or a metabolite could augment acetylcholine (ACh) release in rat hippocampus. The rate of ACh released in the hippocampus was higher during a learning process involving spatial memory compared to when the rat sat quietly in the cage (Ragozzino et al. 1996). Hence, it is postulated that the glucose rich *Tualang* honey caused better regulation of brain-derived neurotrophic factor (BDNF) or increased activity of choline acetyltransferase and acetylcholinesterase in certain brain areas or a combination of these mechanisms (Al-Rahbi et al. 2013).

Oxidative stress, the lack of balance between free radicals generated and the anti-oxidant protection of the organism, has negative effects on cognitive function (Wattanathorn et al. 2014). It is caused by an increase in production and/or decrease elimination of reactive species by anti-oxidant defence (Erejuwa et al. 2012). Brain is very susceptible to the accumulation of oxidative stress-induced damage since oxidative damage to cellular components will damage the physiological functions (Schmitt-Schillig et al. 2005). Consuming foods rich in anti-oxidants can reverse age-related impairment in neuronal signal transduction and also deterioration of cognitive and motor functions (Lau et al. 2005). A review paper by

TABLE 2. Pairwise comparisons (Mean±SEM) for treatment effects on numbers of neurons of hippocampal CA1, CA3a and CA3c layers

Group	CA1	CA3a	CA3c/Hilus
Honey	64.66±0.65*	37.24±0.65*	52.21±0.65*
Saline	61.11±0.65*	33.46±0.65*	48.78±0.65*

Asterisks denote significant differences ($p<0.05$)

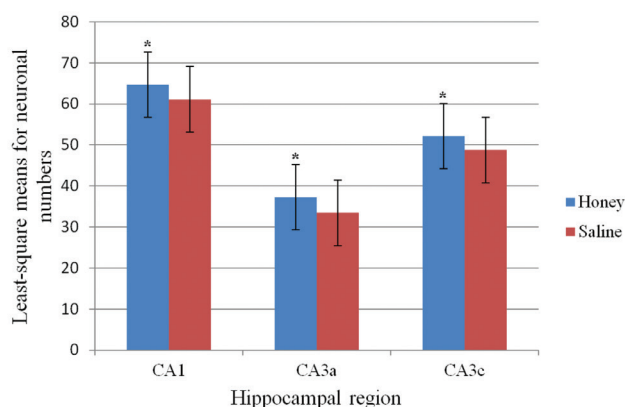


FIGURE 3. Least-squares means and standard errors for neuronal numbers in different hippocampal regions. Asterisks denote significant differences ($p<0.05$)

Rahman et al. (2014) postulated that the components of polyphenols in honey, e.g. apigenin, ferulic acid, catechin, luteolin and myricetin can overcome oxidative stress by limiting the generation of reactive species, strengthening the cellular anti-oxidant defence system, preventing neuronal cell death by weakening the neuroinflammation, apoptosis and modulating synaptic plasticity. The anti-oxidant effect of honey due to flavonoids, phenolic acids, ascorbic acid, catalase, peroxide, carotenoids and Maillard reaction products (Bogdanov et al. 2008) could possibly be responsible for reducing anxiety and improving spatial memory in rats (Chepulis et al. 2009).

The wide range of honey anti-oxidant activity also depends on the nectar source, the indirect source of its chemical components. *Tualang* honey, which has good colour intensity and contains phenolic compounds with good anti-oxidant activity, is comparable with different honey types reported such as Slovenian honey (e.g. Chestnut, Fir, Spruce, Multifloral and Forest honey) and Romanian honey types (e.g. Acacia, Lime, Sunflower, Chestnut and Honeydew honey) (Mohamed et al. 2010). *Tualang* honey biochemical properties are partly similar to the infamous Manuka honey, in addition to having higher phenolic content, flavonoids and hydroxymethylfurfural than Manuka honey and other local Malaysian honey (Ahmed & Othman 2013).

The SMP findings were supported by the hippocampal neurohistological results. The numbers of HG rats CA1 and CA3 hippocampal pyramidal cells were significantly different compared to CG, agreeing well with the demonstration that HG rats committed fewer errors during RAM test. Al-Rahbi et al. (2013) reported that neuronal counts in hippocampal CA1, CA2, CA3 layers and dentate gyrus area in stressed ovariectomised rats treated with *Tualang* honey were significantly different compared to the untreated rats reflecting improved memory performance observed.

It had also been demonstrated that chronic psychosocial stress-induced reactive oxygen species in the hippocampus caused morphological changes in the hippocampal CA3 region and deterioration of cognitive function (Palumbo et al. 2007; Shafin et al. 2014). The normal adult rats in the present study probably had less oxidative stress since free radical-mediated reactions are normally related to the deterioration associated with aging (Esposito et al. 2002). Oxidative damage causes behavioural dysfunction (Forster et al. 1996) and can cause neuronal and cognitive dysfunction in aging (Beckman & Ames 1998). Expectedly younger rodents showed better performance compared to older group (Kishore & Singh 2005), since the anti-oxidant protection system in the young is at its optimum, enabling it to reduce oxidative stress (Saleem et al. 2012).

CONCLUSION

This study suggested that consumption of *Tualang* honey could improve both working memory and reference memory in RAM evaluation in male adult rats. The findings

were supported by the neurohistological study. Hence, the anti-oxidant rich *Tualang* honey potential in facilitating spatial memory should be further explored.

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Mohd Amiruddin Kamarulzaidi
Institute of Graduate Studies
University of Malaya, 50603 Kuala Lumpur
Malaysia

M.Y. Zulkifli Mohd. Yusoff
Al-Qur'an and Hadith Department, Academy of Islamic Studies
University of Malaya, 50603 Kuala Lumpur
Malaysia

Abdul Majid Mohamed
Division of Biology, Center for Foundation Studies in Science
University of Malaya, 50603 Kuala Lumpur
Malaysia

Durriyyah Sharifah Hasan Adli*
Division of Biohealth Science, Institute of Biological Sciences
Faculty of Science, University of Malaya
50603 Kuala Lumpur
Malaysia

*Corresponding author; email: durriyyah@um.edu.my

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