Morphometric Study of Hippocampal CA1 Pyramidal Neurons after Tualang Honey Administration

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Abstract

Tualang honey can be collected from the hives of *Apis dorsata* bee species on Tualang trees. Its various nutritional and curative properties could probably be due to its antioxidant effects. Subsequent to previous studies demonstrating its positive effects on spatial memory performance and hippocampal neuronal count, the current study investigated whether it has morphometric effects on the hippocampal cornu ammonis 1 (CA1) pyramidal neurons. It is important to evaluate the characteristics of hippocampal constituent neurons since this brain structure, which is primarily involved in memory processing, is most vulnerable towards oxidative stress. Male *Sprague Dawley* rats were force-fed five days a week for 12 consecutive weeks with 1.0ml/100g body weight of 70% Tualang honey (HON) or with 0.9% saline (SAL) as control. Nissl's stained dorsal transverse hippocampal sections (8µm thick) of both groups were visualized under Olympus BX51 light microscope. Images were captured using Analyzer Life Science software and morphometric analysis was conducted using Image-Pro Premier 9.1 64-bit software. Only neuronal somas with clear nucleus and nucleolus were included in the morphometric analysis. Significant differences were observed between the groups for all five parameters selected (somatic area [SA], somatic perimeter [SP], somatic aspect ratio [SAR], somatic circularity index [SCI], and somatic roundness [SRo]). Values of SA and SP of HON group indicated significantly bigger sized CA1 neurons. Values of SAR, SCI and SRo, which indicated the shape of the neuronal somas, are biased towards less rounded shape. These values demonstrated HON has effects at the neuronal morphometric level.

Keywords: Morphometric, hippocampal neurons, Tualang honey.

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Introduction

Honey has been long-used and consumed for its nutritional and curative properties. Tualang honey is a multifloral jungle honey, collected from the hives of the wild Asian rock bee species (*Apis dorsata*) on Tualang trees in Malaysia [1]. The name of Tualang honey is derived from the Tualang trees (*Koompassia excelsa*), which are numerous in the tropical rainforests of Sabah, Sarawak and few states of Peninsular Malaysia [2]. It has a dark brown appearance and a low pH which makes it more acidic than other local Malaysian honey, thus, more effective against pathogenic microorganisms. The potential health benefits of Tualang honey is increasingly being recognized, proven by the increment of medical studies on it [3]. Previous studies have demonstrated the positive effects of Tualang honey in male reproductive physiology [4], cardiology [5], osteology [6], oncology [7], wound healing [8], and neuroscience [9].

Free radicals and reactive oxygen species (ROS) such as superoxide and hydroxyl radicals have been implicated in the process of cellular dysfunction and the pathogenesis of numerous diseases, including neurodegenerative diseases [10]. Therefore, the role of antioxidants is considered significant as a therapeutic approach and Tualang honey is well-acknowledged as an effective antioxidant since it contains approximately 14-18 compounds that helps improve the body defense mechanism against oxidative stress [11]. Phenolic acids [1] and flavonoids [12] are the primary contents of Tualang honey that act as antioxidants, besides organic acids and vitamins [13]. Other constituents of Tualang honey include amino acids, proteins and minerals [14], enzymes and carbohydrates [15].

Hippocampus is the main structure of the brain involved in learning and memory processes; thus, it is also involved in the behavioural performance. Morphologically identifiable pyramidal cells constitute the main population of hippocampal neurons. Diseases related to the central nervous system (CNS) are mostly accompanied by damage to the hippocampus. Alzheimer’s disease and Parkinson’s disease are known to be partially caused by the injury of hippocampal neurons [16]. As a follow up to previous studies which showed the enhancing effects of Tualang honey on the hippocampal pyramidal count and spatial memory performance [17], the current study focused on its morphometric effect on *cornu ammonis* 1 (CA1) pyramidal neurons of the hippocampus.

Materials and methods

Sample preparation

Honey used in this study was Natural Tualang honey (AgroMas) from the Federal Agricultural Marketing Authority (FAMA), Malaysia. Natural Tualang honey (AgroMas) is honey collected from the Tualang trees in Kedah. The honey was kept at room temperature away from direct sunlight.

Animals

Twelve male *Sprague Dawley* rats aged between 7-9 weeks were randomly allotted into two groups (six rats per group): (a) 70% tualang honey (HON) (1ml/100g body weight) prepared using 0.9% saline, and (b) 0.9% saline (SAL) (1ml/100g body weight) as control. Rats were force-fed for five days a week for 12 consecutive weeks. All protocols used were approved by the Institutional Animal Care and Use Committee (IACUC), University of Malaya [ISB/20/04/2012/DSHA (R)]. Rats were deeply anesthetized before intracardially perfused. Brains were dissected and fixed immediately in 10% formalin. Once fixed, the tissues were processed, embedded in paraffin, and finally cut into 8μm-thick transverse sections.

Microscopic analysis

For microscopic analysis, tissues were stained with Nissl’s staining using cresyl violet dye. Slides were observed under light microscope (Olympus BX51) and photographed using analysis Life Science Soft Imaging System software (Olympus, Germany). A total of six slides from each animal with sections of both right and left brain hemispheres were considered for morphometric analysis.

Morphometric analysis

Morphometric analysis was conducted using Image-Pro Premier 9.1 64-bit software. Five morphological variables selected for this study were in accordance with the quantitative criteria outlined by Tsiola et al. [18]: (a) Somatic area (μm²): area inside the soma; (b) Somatic perimeter (μm): length of the soma's boundary; (c) Somatic aspect ratio: ratio between major axis of the soma to its minor perpendicular axis; (d) Somatic circularity index, $(4\times\pi\times\text{area})/(\text{perimeter})^2$: circularity of the soma; (e) Somatic roundness, $(4\times\text{area})/(\pi\times\text{maximum axis})^2$: circularity of the soma but more specific; could meticulously distinguish differences in the cell outlines. Only neurons with clear somatic nuclei and nucleolus were counted (Figure 1).

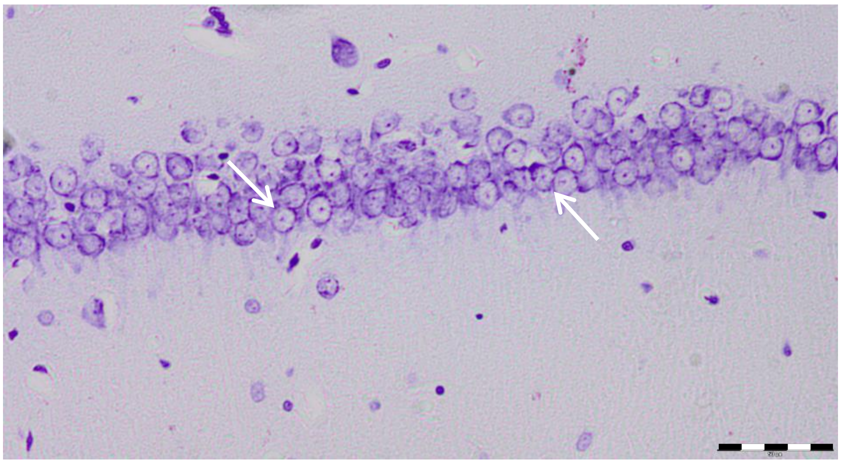

Figure 1: Hippocampal CA1 pyramidal neurons with each having whole visible nucleus and nucleolus (arrows) considered in the study. (200× magnification, 50 μm calibration)

Statistical analysis

Statistical analyses were done using two-tailed independent sample t-test. The results obtained were presented as mean±standard deviation (SD) and differences between groups were considered significant when $p<0.05$.

Results and discussion

There were significant differences of hippocampal CA1 neuronal morphology between the HON and SAL groups (Table 2, Figure 2).

Table 2: Measurements of the morphological size and shape of CA1 pyramidal neurons

Group	Somatic parameters				
	Size		Shape		
	Somatic Area/SA (μm²)	Somatic Perimeter/SP (μm)	Somatic Aspect Ratio/SAR	Somatic Circularity Index/SCI	Somatic Roundness/ SRo
HON	126.29 ± 19.37*	40.33 ± 3.02*	1.20 ± 0.12	0.79 ± 0.07*	1.06 ± 0.03*
SAL	114.34 ± 17.73	38.43 ± 3.00	1.23 ± 0.15*	0.80 ± 0.11	1.04 ± 0.09

* Differences between groups considered significant when $p<0.05$

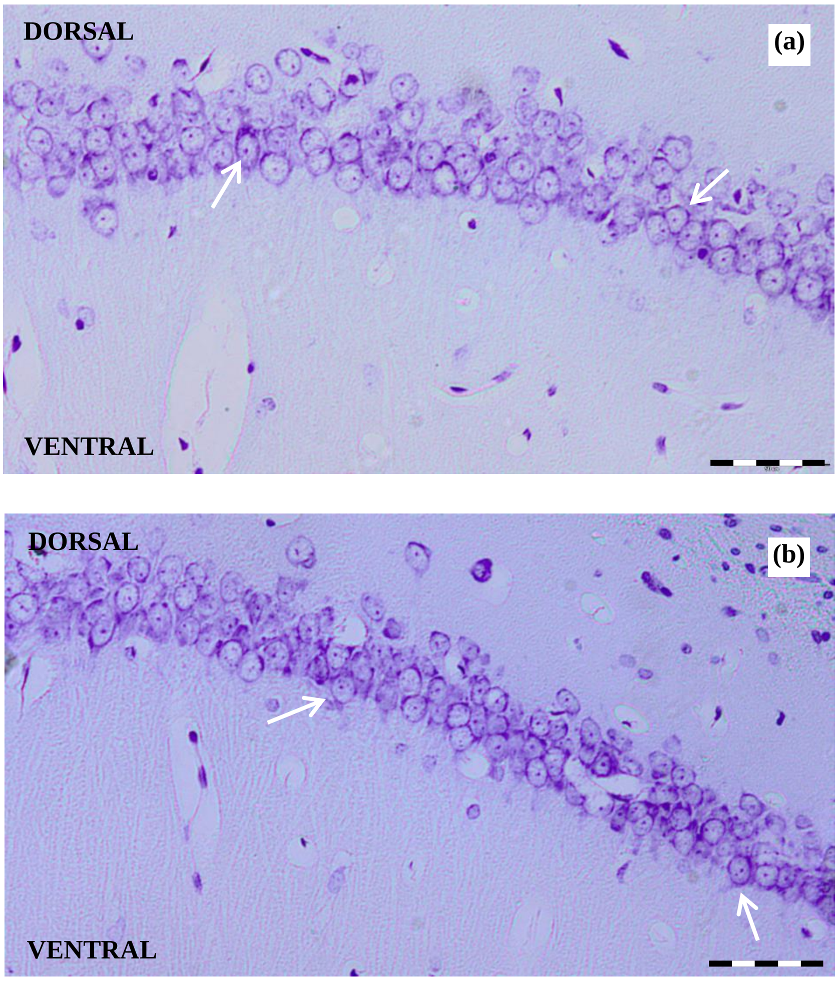

Figure 2: Morphological features of hippocampal CA1 pyramidal neurons (arrows): (a) HON group, and (b) SAL group. (200× magnification, 50 μm calibration)

Most of the neuronal morphological studies focused on somatic, dendritic, axon and spine areas of neurons. This study focused on the neuronal soma or the neuronal cell body. Soma is the central structure of neurons, and also the location of the nucleus and subcellular organelles that are crucial for information processing. The features highlighted were the shape and size of the neuronal cell body. Size is the primary characterization commonly used in somatic morphological studies [19]. Besides size, shape is another relevant quantitative measurement in defining neuronal morphological aspects. The various neuronal shapes include sphere, elliptic, pyramid, rhomboid and elongated [20].

Somatic size could be analyzed based on the somatic area (SA) and somatic perimeter (SP) [21]. For both parameters, neurons in HON group exhibited significantly larger values compared to neurons in SAL group. SA of neurons in HON group was 126.29 ± 19.37 μm^2 while it was 114.34 ± 17.73 μm^2 for SAL group. SP of HON group was 40.33 ± 3.02 μm, whereas it was 38.43 ± 3.00 μm for SAL group. Previous studies on *Nigella sativa* oil also demonstrated bigger somatic size of hippocampal CA1 neurons, as compared to its control group [22]. However, in this study Tualang honey was shown to have more significant effects on the neuronal soma size as compared to *Nigella sativa* oil. Since neuronal soma acts as the integrator of incoming information, larger somas could be interpreted as having better cellular preservation and better ability to retain cellular information, thus might be reflective of enhanced memory and learning ability [23]. A larger soma may possibly have larger cellular and metabolic systems, which are needed to serve a larger neuronal dendritic tree, more synaptic connections and higher neuronal activities. All three properties correspond to larger memory ability [23].

The morphological shape of cell bodies was analyzed based on the somatic aspect ratio (SAR), somatic circularity index (SCI), and somatic roundness (SRo). HON group exhibited significantly lower SAR (1.20 ± 0.12) than SAL group (1.23 ± 0.15). SCI and SRo emphasize on the roundness of the soma, with a value further from 1.00 indicates a less circular soma. However, the value for SRo could distinguish pyramidal soma from round ones, while SCI only distinguishes circular-shaped neurons from irregular shaped ones [18]. The results demonstrated lesser roundness of HON group, hence, indicating the shape of CA1 neuronal somas that were not so rounded; thus, possibly reflecting a better retention of pyramidal shape. The HON group showed a significantly lower SCI (0.79 ± 0.07), further away from the value 1.00 compared to SAL group (0.80 ± 0.11). In comparison to the value of SRo of SAL group (1.04 ± 0.09), the value for HON group (1.06 ± 0.03) was significantly higher and further away from 1.00. Therefore, findings from the selected measurements of the CA1 neuronal soma morphological characteristics indicated the potential neuroprotective effect of honey.

Previous studies have manifested the positive effects of Tualang honey in animals' memory performance [9, 17], gross morphology of brain [17], and neuronal count of hippocampus pyramidal cells [17]. However, till date, no studies focusing on the effects of Tualang honey on the morphometric size and shape of neurons has been done. Findings by Jafari *et al.* [24] elucidated on the capability of natural honey in maintaining the viability, structure and function of hippocampal neurons. Besides that, the morphology of memory-related brain regions was also significantly improved in animals given Tualang honey supplementation [2].

Multivitamins and antioxidants act as neuroprotective agents that reduce cell damage [25]. Clinical studies have also shown that intake of diet and supplements containing natural antioxidants may prevent neurodegeneration [25]. The possible mechanism of honey on the observed significant development of the somatic size and shape of the hippocampal CA1 neurons is most likely due to its antioxidant properties. Oxidative stress leads to ROS accumulation, which disrupts nerve cell membrane and disturbs mitochondrial membrane permeability. Honey has the capability to protect the cell membrane from destruction by neutralizing these free radicals [9]. Tualang honey may have neuroprotection mechanisms, improve memory performance, and reduce hippocampus damage due to several factors such as its antioxidant properties, its capability to regulate the brain-derived neurotrophic factor (BDNF), increase the choline acetyltransferase and acetylcholinesterase activities in certain brain regions, or all of the mechanisms mentioned. Further studies are required to determine the specific neuroprotective mechanisms of Tualang honey [9].

Tualang honey has been proven to be one of the local honey types in Malaysia that contains high antioxidant contents. As mentioned earlier, its phenolic content contributes the most to its antioxidant property followed by other non-phenolic compounds such as protein, gluconic acid, ascorbic acid, peroxides and hydroxymethylfuraldehyde. The antioxidant capacity of the Tualang honey may be due to the interactions and activities between its various constituents [26]. Six phenolic acids (gallic, syringic, benzoic, trans-cinnamic, p-coumaric, and caffeic acid) and five flavonoids (catechins, kaempferol, naringenin, luteolin, and apigenin) have been identified in Tualang honey. However, vitamins, enzymes, amino acids, and trace elements in Tualang honey have yet to be identified [27]. Therefore, extensive studies in identifying the other active biological components of Tualang honey is required, to further identify which components play significant roles in the mechanism of action of Tualang honey.

Conclusion

Morphometric analysis of the hippocampal neuronal somas provides a valuable insight to monitor the effects of Tualang honey on the somatic structure and function, and a better understanding of morphology and the changes taking place in this brain region. The findings suggested that the specific effects of Tualang honey on the morphological somatic characteristics of hippocampal CA1 neurons were possibly due to the type and quantity of the various honey biochemical constituents and their distinct neurological effects. However, further studies need to be conducted to elucidate the specific mechanisms at work.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosures to declare.

Compliance with ethical standards

The work is compliant with ethical standards.

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