Tualang honey ameliorates hypoxia-induced memory deficits by reducing neuronal damage in hippocampus of adult male Sprague-Dawley rats

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INTRODUCTION: A growing body of evidence indicates that hypoxia exposure causes learning and memory deficits. Effective natural therapeutic approach has, however, not been explored widely. Our previous studies found that Tualang honey administration protected learning and memory functions in ovariectomised rats. Therefore, the present study investigated its efficacy in ameliorating hypoxia-induced memory deficits in adult male Sprague-Dawley rats.

METHODS: The rats were divided into four groups; i) Normoxia treated with sucrose (n=12), ii) Normoxia treated with Tualang honey (n=12), iii) Hypoxia treated with sucrose (n=12), and iv) Hypoxia treated with Tualang honey (n=12). Tualang honey (0.2 g/kg/BW) and sucrose (1 mL of 7.9%) supplementations were administered orally to the rats daily for 14 days. Then, hypoxia groups were exposed to hypoxia (~11%) for 7 days while normoxia groups were kept in normal condition. Following exposure to hypoxia, rats memory were analyzed using novel object recognition task and T-maze test.

RESULTS: The data revealed that rats exposed to hypoxia showed significant impairment in short-term memory (STM), spatial memory (p<0.01) and long term memory (LTM) when compared to the normoxia group. Hypoxia rats treated with Tualang honey showed significant improvement in STM, LTM and spatial memory (p<0.05) as compared with hypoxia treated with sucrose (p<0.05). Tualang honey also reduced neuronal damage in hippocampus of adult male Sprague-Dawley rats exposed to hypoxia.

DISCUSSION AND CONCLUSION: Therefore, it is suggested that Tualang honey pre-treatment has protective effects against hypoxia-induced memory deficits, possibly through its antioxidant contents.

Keywords: Hypoxia, Tualang honey, sucrose, memory performance, novel object recognition task, T-maze
INTRODUCTION

High altitude is considered as one of the most adverse environment where hypoxia condition causes many physiological and psychological changes in human as well as animals. The challenge to regulate oxygen homeostasis in high altitude is important to the survival of all vertebrate species. Failure to maintain oxygen homeostasis leads to damage in the periphery as well as in the central nervous system (CNS). CNS is responsible for cognitive functions including learning and memory. Findings from previous studies suggest that the hippocampus, which is involved in spatial learning and memory, is also vulnerable to hypoxia. Exposure to hypoxia has been shown to affect the hippocampus that causes memory impairment. Although previous findings strongly reported that hypoxia exposure induced memory loss, there are few studies that evaluate protective effects of natural products and their compounds on memory loss following exposure to hypoxia.

Administration of exogenous antioxidants such as polyphenols and vitamin E has been reported to be a potential way to combat the adverse effects of oxidative stress-induced hypoxia. Antioxidants rich diets or treatments prevent memory and learning deficit in animal models and in patients with impaired cognition. Several approaches targeting oxidative stress have been used, including supplement such as blueberry extracts, melatonin, and vitamin E. Despite the promising results from both rodent and human studies, much is still being surveyed and studied regarding the benefit on specific nutritional supplements and their role in the treatment of various hypoxic conditions.

Numerous studies have demonstrated the beneficial effects of Tualang honey and its possible medicinal uses as anti-diabetic, anti-cancer, and anti-microbial agent as well as possessing wound healing properties. Besides, Tualang honey improves neuronal morphological changes and minimises neuronal damage in the hippocampus. Tualang honey has been analyzed and reported to contain antioxidant compounds such as quercetin, flavonoid and is rich in phenolic acid. A recent study reported that Tualang honey improved brain function through the cholinergic system. Our previous studies concluded that Tualang honey improved memory performance in stressed ovariectomised rats, rats exposed to noise stress, and postmenopausal women; elevated brain-derived neurotrophic factor (BDNF) level; and reduced depressive-like behavior in ovariectomised rats. Correspondingly, another study
reported that oxidative stress markers significantly reduced and increased anti-oxidative enzymes further supporting the protective effect of Tualang honey against brain oxidative stress. However, to our knowledge, no study has investigated the role of Tualang honey on learning and memory of rats in hypoxic condition. Thus, the present study aims to evaluate the efficacy of Tualang honey to ameliorate hypoxia-induced memory deficit in adult male Sprague-Dawley rats.

MATERIALS AND METHODS

Forty eight adult male Sprague-Dawley rats at approximately eight weeks old, with body weight of 200 ± 20 g, were obtained from the Laboratory Animal Research Unit, Universiti Sains Malaysia (USM). All rats were housed in polypropylene cages (32 × 24 × 16 cm), exposed to 12 hour light-dark cycles, maintained at a room temperature of 23°C, and had free access to food and water. The experimental protocol was approved by the Research and Ethics Committee of this university (USM/Animal Ethics Approval/2015/ (95) (609)), in accordance with the internationally accepted principles for laboratory animal use and care.

Experimental Animals
The rats were divided into four groups; i) Normoxia treated with sucrose (n=12), ii) Normoxia treated with Tualang honey (n=12), iii) Hypoxia treated with sucrose (n=12), iv) Hypoxia treated with Tualang honey (n=12). Tualang honey (1 mL of 0.2 g/kg body weight)23 and sucrose (1 mL of 7.9%)31 supplementations were freshly prepared and administered by oral gavage to the rats daily for 14 days. The Tualang honey and sucrose used in the present study was from a single batch honey supplied by Federal Agricultural Marketing Authorities (FAMA), Malaysia and Sigma-Aldrich, Inc., St. Louis, MO, USA, respectively.

Hypoxia exposure
The rats in the hypoxia groups were kept in an acrylic air-tight chamber with ~ 11% O2, constant temperature (~ 25°C) and humidity (~ 76%). The O2 content of ~ 11% was generated by the HCA HYPO-002 high-altitude simulation system. The rats in the
normoxia groups were kept in room condition. The rats’ food intake and body weight change were recorded weekly.

**Behavioural tests**

The behavioural tests were performed before and after exposure to hypoxia or normoxia for two weeks. The experiments were carried out in a ventilated, soundproof and dimly lit room. The room temperature was maintained at about 23 °C.

**Novel Object Recognition (NOR) Test**

All animals underwent a habituation session for three days during which they were placed in an empty open field (60 × 60 × 30 cm) and left to freely explore the field for 10 minutes. During training sessions, two identical objects (A1 and A2) were placed in the field, and each rat was allowed to explore freely for 10 minutes as described in previous studies.32,33 Time spent exploring each object was recorded manually. For test sessions, animals were tested for memory retention 2 hours after commencing the training session (short-term memory/retention, STM). In the STM test, the rats explored the open field for 10 minutes in the presence of one familiar (A1 or A2) and one novel (B) object. The location of the object was alternately shifted with each new animal; in 50% of the trials it was placed on the right side while in the other 50% on the left side of the field. The same test was repeated 24 hours after the training session and this was the long-term memory/retention, LTM.

All objects were made of plastic toys and had a height of about 5 cm. Objects were of similar textures, colors and sizes, but with distinctive shapes. The objects were positioned in two adjacent corners, 10 cm from the walls. Between tests, the objects were cleaned with a 10% ethanol solution to mask any olfactory cues. Exploration was defined by sniffing or touching the object with the nose. Sitting on the object was not considered exploration.34 Total exploration time (s) of the familiar and novel objects were recorded and used to calculate a discrimination index [time spent with novel object (B) - time spent with familiar object (A)] / [total time exploring both objects] for training and test sessions.35 This index was used to measure recognition memory.36 Increased exploration time of the novel object or preference for novel objects was interpreted as successful retention of memory for the familiar object. Decreased or absence of any variance in the exploration of the two objects was interpreted as memory deficit.37
T-maze

The T-maze apparatus consisted of three arms made of black perspex (start arm: length=60 cm, width=16.5 cm; goal arms: length: 50 cm, width=16.5 cm, maze height 30 cm). The maze was equipped with three doors. Rats were kept in the room for at least 30 min before the first trial. Training of each rat consisted of one single continuous session, which started with one forced-choice trial, followed by 14 free-choice trials. During the first trial (the forced trial), the doors were lowered to block the left or right goal arm. After the animal was released from the start arm, it was allowed to negotiate the maze, until it entered the open goal arm, and then returned to the start position. Then, the animal was confined for 5 seconds by lowering the door of the start arm. During the 14 free-choice trials, the animal could choose freely between the left and right goal arm. After opening the door of the start arm, the animal was free to choose between both goal arms. Once it entered one goal arm, the door of the other goal arm was lowered. Once the animal returned to the start arm, the next free-choice trial started after a 5 seconds detention in the start arm. A session was terminated and the animal was removed from the maze as soon as 14 free-choice trials had been performed or 30 min had elapsed, whichever event occurred first. During the session, the experimenter never handled the animals. If an animal failed to complete a trial within 2 min the door was used to gently direct the animal back to the start arm. In order to mask any olfactory cues, care was taken by removing droppings and cleaning the T maze with 10% ethanol before each new animal started its session.

Morphological Changes

Histological analysis

For tissue collection and processing, rats were euthanized with a mild anesthesia, sodium pentobarbital (0.27 ml) (Dorminal, Din 02333708, Alfasan, Woerden, Holand) and perfused intracardially with 0.1 M phosphate-buffered saline (PBS, pH0.7) for 2 minutes followed by 4% paraformaldehyde (PFA, pH 7.0) (Acros Organics, AC416780010, Fisher Scientific, USA) for pre-fixation of the tissues for 3 min. The brain was then carefully dissected out, post-fixed in 4% paraformaldehyde (Acros Organics, AC416780010, Fisher Scientific, USA) and kept in the refrigerator at 4°C.
Tissues were then processed for paraffin sectioning according to standard procedure.

**Cresyl violet staining**

Paraffin sections were deparaffinised and rehydrated with gradients of alcohol and then washed with tap water. Tissue sections were then stained with cresyl violet staining solution (Sigma, C5042, Sigma Aldrich, USA) and washed with tap water. After that the tissue sections were dehydrated in gradient of alcohol, cleared with xylene (HmbG, C0900-2190239, HmbG Inc., Germany) and mounted by using DPX mounting medium (BDH Chemicals, 360294H, BDH Chemicals, UK). Tissue sections were then covered with coverslip (HmbG, G0543, HmbG Inc. Germany) and observed under light microscope with an image analyzer (20 × objective lens power) (Olympus, BX-14-32PO2, Olympus Corporation, Japan) by 3 blinded investigators.

**Apoptosis detection by propidium iodide assay**

Propidium iodide staining assay was employed to identify cells that were undergoing apoptosis under fluorescent microscope (Olympus, BX-14-32PO2, Olympus Corporation, Japan). Briefly, paraffin sections were dewaxed and hydrated by gradient of alcohol, and then washed in phosphate buffered saline (PBS). The excess PBS was removed from the slide by gently tapping the edge of the slide on a tissue paper. Subsequently, the slides were treated with blocking solution and incubated for 20 minutes in the dark. After 20 minutes incubation, the area around the sample was carefully wiped and washed in PBS and 25 µL buffer was added and further incubated for 10 minutes. Next, 25 µL propidium iodide reagent was added to the slide for 30 minutes and washed in PBS. Slides were then mounted using fluorescent mounting medium (Prolong Gold Antifade Mountant, Molecular Probes, P10144, ThermoFisher Scientific, USA), covered with coverslip (HmbG, G0543, HmbG Inc. Germany) and viewed immediately using fluorescent microscope with image analyzer (Olympus, BX-14-32PO2, Olympus Corporation, Japan) under green filter (20× objective lens power) by 3 blinded investigators.

**Statistical analysis**

Weekly differences in behavioural scores, food intake and body weight were first calculated. Then, one-way ANOVA tests were used to examine the differences in
behavioural scores, food intake and body weight among the experimental groups. Differences were taken to be significant at $P<0.05$.

**RESULTS**

*Changes in Food intake*

There were no differences in food intake in normoxia animals whereas hypoxia treated with sucrose and honey animals showed significant ($P<0.05$) reduction in food intake (Figure 1).

*Changes in Body weight*

Following exposure to hypoxia, the body weight of the animals was significantly ($P<0.05$) reduced in both sucrose and honey treated groups (Figure 1).

*Novel Object Recognition (NOR) Test*

The results suggest that hypoxia adversely affect STM more than LTM. There was significant improvement in discrimination indexes of STM and LTM tests following honey treatment indicating that honey pretreatment was able to prevent the adverse effects of hypoxia on recognition memory functions especially the STM (Figure 2).

*T-maze*

The result suggests that hypoxia also affects the number of alteration in the T maze. Similar to NOR test, significant improvement in mean number of alteration in the T maze was noted following honey treatment suggesting the protective effects of honey pretreatment on spatial memory functions (Figure 2).

*Morphological changes*
Following behavioural studies, hippocampal morphology was analysed using cresyl violet (Figures 3-6) and propidium iodide staining (Figures 7-8). The quantity analysis of CA1, CA2, CA3 and DG of hippocampus showed significant (p<0.05) dead cells in sucrose-treated hypoxic group whereas Tualang honey-treated hypoxic group of animals comparatively showed fewer number of dead cells indicating that Tualang honey prevented neuronal damage (Figure 7). Cytoplasmic shrinkage and pyknotic nucleus are features indicative of a dead cell. For qualitative analysis to further confirm neuronal damage especially in hypoxia treated with sucrose and hypoxia treated with honey groups, PI staining was carried out. The results displayed and reconfirmed that there was considerable neuronal damage in hypoxia treated with sucrose group but not many in hypoxia treated with Tualang honey group (Figure 8).

**DISCUSSION**

There are three important findings in this study. First, continuous normobaric hypoxia for 7 days exerted adverse effects on food intake, body weight gain and memory functions. Second, with regards to the memory function, STM, LTM and spatial memory were significantly affected. Third, Tualang honey pre-treatment was able to protect against hypoxia-induced memory deficits and hippocampal neuronal damage. Regarding food intake, hypoxic animals treated with sucrose and honey, consumed food significantly less than normoxia animals treated with sucrose and honey. Numerous previous studies support the present findings that high altitude exposure is associated with reduction in food intake. Westerterp-Plantenga et al. reported that high altitude causes hypophagia and reporting that as being more specific to carbohydrate and protein. Not only human subjects exposed to high altitude show hypophagia, but also animal’s feeding behaviour changes following exposure to hypobaric hypoxia whereby decreases in food intake is directly related to the degree of simulated altitude.

As a consequent to the reduction in food intake, hypoxia-exposed animals show significant reduction in body weight as well in both sucrose and honey treated groups. Loss of appetite could be one of the possibilities for the reduction in body weight. Hypoxia also affects body weight regulation as noted in human as well as in animals.
Similar findings were noted in the present study whereby the food intake and body weight gain were lower in rats exposed to hypoxia compared to the non-hypoxic rats. The poor appetite and decreased food intake often produce an imbalance in the energy equation that leads to low weight gain and changes in body composition. Among the proposed mechanisms include changes in leptin, glucagon-like peptide 1, protein synthesis, intestinal absorption, and hypoxia-regulated genes. Previous work suggested leptin as the main candidate for the reduction in food intake following hypoxia. However, the role of leptin and food intake during hypoxia is still a matter of debate and the mechanisms responsible for the decrease in energy intake in hypoxia conditions still remain unclear.

Despite the hypoxic groups of animals displaying reduction in food intake and body weight, these groups did not show any kind of locomotor deficits in the open field test. Hence, the recognition objective memory test was carried out following exposure to hypoxia. Interestingly, the hypoxia treated with Tualang honey group of animals showed improvement in recognition objective memory performance whereas hypoxia treated with sucrose failed to retain memory function. There were no significant changes found in normoxia treated with sucrose and Tualang honey. Many previous studies used hypoxic chamber set at 6-8% of oxygen content for a shorter period of time and found significant memory deficit. Lowering the oxygen contents of inspired air to 6% impaired acquisition of the avoidance response, and the difference between the performance (percentage of avoidance responses) of animals kept under normoxic and hypoxic conditions was significant on day 3 (69.2% and 38.0% conditioned avoidance response, respectively). This finding was in line with earlier study findings by Saligaut et al., where the acquisition of a conditioned avoidance response was impaired under 300 torr hypobaric hypoxia (8% oxygen content).

Our results, along with those of previous studies, clearly indicated that both normoxic and hypobaric hypoxia were able to induce memory deficits. Thus, this “equivalent air altitude model” can also be used to study memory function despite earlier reported physiological differences between acute exposures to normobaranic and hypobaric hypoxia. In the present study, two behavioural tests were carried out i.e., spatial working memory and recognition memory and the rats that were exposed to hypoxia displayed deficits in STM, LTM and spatial memory. These findings are consistent with earlier reports that recognition memory and spatial memory were affected following hypoxic exposure. Interestingly, this study revealed that pretreatment with Tualang
honey was able to protect the rats from hypoxia-induced memory deficits as shown by the behavioural performances which were comparable to controls. Numerous studies confirmed that hypoxia exposure causes memory deficits through involvement of different mechanisms. In particular, neuronal apoptosis in cortex, striatum and hippocampal cells, imbalance in oxidative and anti-oxidative enzymes, cholinergic neurotransmission alteration, and changes in glutamate neurotransmission, could be the root cause for spatial working memory disturbance during hypoxia exposure. Nevertheless, possible therapeutic approach has not been evaluated to mitigate these changes during hypoxia. In fact, the present results suggested that Tualang honey treatment could improve memory following exposure to hypoxia. Hence, Tualang honey could be one of the economical ways to mitigate deleterious effects following hypoxia.

A broad range of studies have been carried out in Asian countries to analyse the chemical composition of Tualang honey as well as its functional properties. Tualang honey has been reported to possess high flavonoid content; among them are quercetin, luteolin, kaempferol, apigenin, chrysin, and galangin. Likewise, honey in general contains enzymatic and nonenzymatic antioxidants. Based on the present results, memory performance improved after hypoxia exposure in Tualang honey treated groups through modulation of oxidative stress. Previous studies reported that Tualang honey decreased oxidative stress caused by kainic acid. It also shows antioxidant properties in streptozotocin-induced diabetic rats. In human subjects, Tualang honey treatment in healthy postmenopausal women shows improvement in memory. Al-Rahbi et al. also reported that Tualang honey treatment improved memory in animals subjected to instability stress. Al-Rahbi et al. reported that Tualang honey supplement improve memory performance and protect neuronal damage in hippocampal regions. A recent study reported that in female athletes, Tualang honey showed antioxidant activity and oxidative stress in a dose-dependent manner. Tualang honey was also found to protect the rat midbrain from oxidative stress. Azman et al. demonstrated that Tualang honey prevented memory deficits following noise stress. An in-vitro study also stated that Tualang honey improved cell migration and resistance against oxidative stress. Not only does Tualang honey control oxidative stress, but it also enhances the level of BDNF which could be one of the mechanisms involved in improvement of memory during hypoxia. However, it has to be studied elaborately.
The brain weight and morphological changes were also studied following exposure to hypoxia. The results suggested that weight of the brain following hypoxia significantly increased in hypoxia treated with sucrose group but not in hypoxia treated with Tualang honey. Morocz et al.\textsuperscript{71} reported that brain volume changes occur in patients following exposure to hypoxia due to changes in cerebral blood volume. Another important factor is occurrence of brain oedema due to inflammatory process following acute exposure to hypoxia.\textsuperscript{72} In the present study, we did not evaluate the factors which are involved for oedema but the increased brain weight indicates this. The hippocampal morphological changes were analysed in CA1, CA2, CA3 and DG. Hypoxia treated with sucrose shows significant number of dead cells as compared to hypoxia treated with Tualang honey. It is assumed that Tualang honey prevents neuronal damage through reduction of oxidative stress. It is possible that its antioxidant content contributed to minimizing neuronal damage and improving memory.\textsuperscript{31} It is believed that Tualang honey prevents hippocampal neuronal damage through phenolic acid which has antioxidant property.\textsuperscript{73} Phenolic antioxidants attenuate the hippocampal neuronal cell damage induced by excitotoxicity.\textsuperscript{24} This is suggested that better therapeutic performance seen in the Tualang honey treated rats in hypoxic condition was not due to only the sugar content but also many antioxidant components and other components involved to improve memory through prevention of neuronal damage.

Taking together all findings in the present study, Tualang honey could improve memory and prevent neuronal damage due to hypoxia. It is assumed that Tualang honey antioxidant properties are responsible for these effects. In our knowledge, this is the first time that Tualang honey is evaluated under hypoxic condition using rats. For better understanding regarding the underlying mechanisms whereby Tualang honey improves memory and prevents neuronal damage, further studies are warranted.

**CONCLUSION**

It could be suggested that Tualang honey pre-treatment has protective effects against hypoxia-induced memory deficits possibly through its antioxidant contents.
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FIGURE LEGENDS

Figure 1. Food intake (A), body weight (B) and brain weight (C) following exposure to hypoxia. The data are displayed as mean (SEM). *p < 0.05 vs normoxia with sucrose, #p<0.05 vs normoxia with honey.

Figure 2. Short term memory (A) long term memory (B) and spatial memory (C) following exposure to hypoxia. The data are displayed as mean (SEM). *p < 0.05 vs normoxia with sucrose.

Figure 3. Morphological changes in CA1 of hippocampus in normoxia sucrose (NS), normoxia honey (NH), hypoxia treated with sucrose (HS) and hypoxia treated with honey (HH). Neuronal damage indicated with red arrow. Cytoplasmic shrinkage and pyknotic nucleus indicate dead cells. Bar scale 200µm.

Figure 4. Morphological changes in CA2 of hippocampus in normoxia sucrose (NS), normoxia honey (NH), hypoxia treated with sucrose (HS) and hypoxia treated with honey (HH). Neuronal damage indicated with red arrow. Cytoplasmic shrinkage and pyknotic nucleus indicate dead cells. Bar scale 200µm.

Figure 5. Morphological changes in CA3 of hippocampus in normoxia sucrose (NS), normoxia honey (NH), hypoxia treated with sucrose (HS) and hypoxia treated with honey (HH). Neuronal damage indicated with red arrow. Cytoplasmic shrinkage and pyknotic nucleus indicate dead cells. Bar scale 200µm.

Figure 6. Morphological changes in DG of hippocampus in normoxia sucrose (NS), normoxia honey (NH), hypoxia treated with sucrose (HS) and hypoxia treated with honey (HH). Neuronal damage indicated with red arrow. Cytoplasmic shrinkage and pyknotic nucleus indicate dead cells. Bar scale 200µm.
Figure 7. Quantification of neuronal damage in CA1, CA2, CA3 and DG of hippocampus. The data are displayed as mean (SEM). *p < 0.05 vs normoxia with sucrose.

Figure 8. The apoptotic dead cells using PI staining in PFC and CA1, CA2, CA3 and DG of hippocampus in hypoxia treated with sucrose (HS) and hypoxia treated with honey (HH). Neuronal damage indicated with arrows. Cytoplasmic shrinkage and pyknotic nucleus indicate dead cells. Bar scale 200µm.
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