Original article:

Tualang Honey prevents neuronal damage in medial prefrontal cortex (mPFC) through enhancement of cholinergic system in male rats following exposure to normobaric hypoxia

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Abstract:

Background: Medial prefrontal cortex (mPFC) is considered to be involved in human cognition to mPFC in terms of learning and memory. Hypoxia is one of the crucial factors causing secondary damage in cerebral hemorrhage and traumatic brain injury. However, the underlying mechanisms and possible therapeutic approach to prevent neuronal damage has not been attempted yet. Therefore, the present study aimed to investigate the role of Tualang honey on medial prefrontal cortical neuronal morphology and cholinergic markers such as acetylcholine (ACh) and acetylcholinesterase (AChE) following exposure to normobaric hypoxia in rats. ***Material and methods:*** Adult male Sprague-Dawley rats were divided into four groups: (i) sucrose treated non-hypoxia, (ii) sucrose treated hypoxia, (iii) Tualang honey treated non-hypoxia and (iv)Tualang honey treated hypoxia. Rats received sucrose (1 mL of 7.9%) and Tualang honey (0.2 g/kg), respectively, for 2 weeks prior to hypoxia exposure. Morphological study was performed by using Nissl staining and cholinergic markers were estimated by ELISA technique. ***Results and discussion:*** Sucrose treated hypoxia group showed significantly lower mean ACh and higher mean AChE concentrations ($P<0.05$) compared to sucrose and honey treated non-hypoxia groups. Interestingly, mean ACh concentration was significantly increased and mean AChE concentration was significantly decreased in Tualang honey treated hypoxic rats compared to sucrose treated hypoxic rats. Morphological data showed that hypoxia caused neuronal damage in mPFC in sucrose treated hypoxia group whereas Tualang honey treated hypoxia group significantly prevent neuronal damage. ***Conclusion:*** Tualang honey protects hypoxia-induced mPFC neuronal damage through improvement of the brain cholinergic markers in male rats exposed to normobaric hypoxia.

Keywords: medial prefrontal cortex; acetylcholine; acetyl cholinesterase; normobaric hypoxia; Tualang honey

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Introduction:

The prefrontal cortex (PFC) is a crucial nerve center of thinking and behavior regulation in the brain[1] and is divided into two subregions: ventromedial prefrontal cortex (vmPFC) for regulating the affection and dorsolateral sectors (dlPFC) for mediating the cognitive functions[2,3]. In rodents, the medial part of the PFC (mPFC), has been shown to be important for goal-directed action[4], working memory[5] and attention[6-9]. This part of the PFC roughly corresponds to the dlPFC in humans and other primates[10-13].

The interactions within the neuronal networks

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centered on the hippocampus and PFC are closely related to information transfer and storage[14-16]. The hypoxia-ischemic injury causes the disruption of interactions within the prefrontal-hippocampal networks[17], which could affect the thinking and behaviour. Furthermore, hypoxia disrupts glutamate (Glu) reuptake causing the extracellular high concentrations of Glu[18] which apparently facilitates the entry of Ca^{2+} and CI^{-} into neurons via Glu-gated channels resulting in mitochondrial dysfunction, protease activation, lipase activation, osmotic swelling, and eventually neuronal death[19].

In addition, hypoxia also has been linked with various changes in brain neurotransmitter metabolism, both *in vitro* and *in vivo* models[20-26]. One of the neurotranmitters is acetylcholine which is essential for learning and memory[27]. Although the mechanism(s) underlying the irreversible hypoxic neuronal damage is still unclear, efforts have been made to develop agents to prevent or reverse hypoxia-induced neuronal damage.

Tualang honey (TH) commonly consumed as nutritional and medicinal products in Malaysia. It is generated by *Apis Dorsata* bees that inhabits Tualang tree, a tropical tree also known as Koompassia Excelsa. Researchers have found that TH has significant cholinergic, antioxidant, anti-inflammatory, antibacterial and antitumor properties[28-31]. Exogenously administered TH promotes recovery of brain biochemistry, morphology, and function following social stress[32,33] and aging in human[34] as well as in rats[35]. The proposed mechanisms for the protective action of TH against secondary degenerative changes include stimulation of BDNF release[36], reduction of oxidative stress-induced neuronal injury[32] and alteration of the cholinergic system[37]. In this article we demonstrate that TH treatment protects against changes to mPFC morphology and the brain cholinergic system that follow normobaric hypoxia exposure in adult rats.

Materials and methods:

Animals:

A total of 48 adult male Sprague-Dawley rats aged approximately eight weeks and weighed 200 g ± 20 g were obtained from the Animal Research and Service centre, Universiti Sains Malaysia (USM), Malaysia. All rats were housed in polypropylene cages (40 cm × 25 cm × 16 cm), exposed to 12 h light-dark cycles, maintained at a room temperature of 23˚C and provided with free access to food and water. The

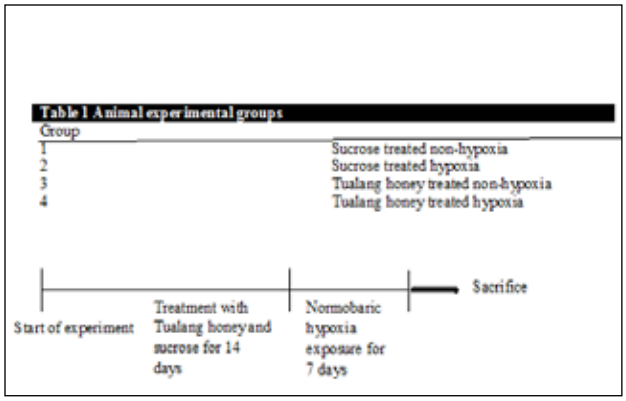

Figure 1 Experimental design

experimental design is illustrated in Figure 1.

TH was used from a single batch honey supplied and processed by Federal Agricultural Marketing Authorities (FAMA), Malaysia. The honey was filtered to remove solid particles, heated in an oven at 40˚C, evaporated to achieve 20% water content and finally irradiated using γ- waves at 25 kGy at Sterile Gamma (M) Sdn. Bhd. (Selangor, Malaysia). The honey was packed in a bottle with a net weight of 230 grams (1.3 g/ml). The 7.9% sucrose was supplied by Sigma-Aldrich, Inc., St. Louis, MO, USA. The sucrose was packed in a bottle with a net weight of 1 kg. The environmental hypoxia at an O_2 content of ~ 11% was generated by the HCA HYPO-002 high-altitude simulation system. The rats were placed in the acrylic air-tight chamber with constant temperature (~ 25˚C) and humidity (~ 76%). The rats had free access to food and water for one week i.e. during week 4.

Estimation of ACh:

The ACh concentration was measured using commercially available ACh assay kit (Elabscience Biotechnology, Wuhan, China). Briefly, 50µL of ddH_2O was added into standard and blank tubes followed by addition of 10 µL of 1 mg/mL ACh standard into standard tube. A volume of 25 µL was added into sample and sample blank tubes. Then, 100 µL of reagent 1 application was added into blank, standard, and sample tubes. Incubation for 15 min at room temperature was done. A volume of 50 µL of reagent 2 was poured into blank, standard, sample and sample blank tubes. Then, 50 µL of reagent 3 was added into blank, standard, sample and sample blank tubes followed by addition of 100 µL of reagent 1 application into sample blank tube. Finally, a volume of reagent 4 equivalent to 25 µL was added into sample and sample blank tube. Incubation for

10 min was done. A volume of 200 µL was pipetted from reaction mixture into 96-well plate followed by measurement of OD value on microplate reader at 550 nm.

Estimation of AChE:

The AChE concentration was measured using commercially available AChE assay kit (Elabscience Biotechnology, Wuhan, China). Briefly, a volume of 50 µL of sample was added into sample tubes. Then, 50 µL of standard application solution was added into standard tube followed by addition of 50 µL double distilled water into blank tube. A volume of 500 µL substrate buffer was added into sample, control, standard and blank tubes. Then, a volume of chromogenic agent application solution equivalent to 500 µL was added into sample, control, standard and blank tubes. Incubation for 6 min at 37˚C was done. Addition of 30 µL of inhibitor reagent into sample, control, standard and blank tubes was done followed by addition of 10 µL of transparent agent into same tubes. Finally, 50 µL of sample was poured into control tubed. Incubation for 10 min was conducted and then, 200 µL of each reaction mixture was pipetted into 96-well plate. Measurement of OD was done at 412 nm wave length.

Morphological study:

The right brain hemispheres were fixed in formalin 10% for one week, processed, embedded in paraffin, cut coronally into 5 µm thickness by a rotary microtome (HM505E; Microm International GmbH, Walldorf, Germany), mounted on slides and stained using standard procedure of Nissl staining. Two continuous fields of each mPFC were selected and the images were captured (Olympus biological microscope; CX41, Japan). The arrangement and number of pyramidal neurons were recorded using different magnifications with a high-definition medical image analysis program (Analysis docu 5.0, Germany). The mean of two fields was taken as the neuron number of each section, and the mean of the four sections was taken as the neuronal number of each group. Cells with a shrunken or unclear body with surrounding empty spaces were excluded.

Statistical Analysis:

One-way ANOVA was used to evaluate the effects of hypoxia and treatment on concentrations of ACh and AChE and the number of pyramidal cell in mPFC among groups. *P* value less than 0.05 ($P<0.05$) was considered significant.

Ethical clearance: The experimental protocol was approved by the Animal Ethics Approval: USM/ Animal Ethics Approval/2015/ (95) (609).

Results:

Effects of hypoxia and treatment on number and arrangement of pyramidal cell in mPFC:

One-way ANOVA showed significant difference between experimental groups in the number of pyramidal cells in mPFC [$F (3, 47) = 102.36$; $P<0.05$]. Post-hoc analysis revealed that sucrose treated hypoxia group had significantly lower number of pyramidal neurons ($P<0.05$) compared to sucrose and honey treated non-hypoxia groups. Also, the arrangement of pyramidal neurons in mPFC in sucrose treated hypoxia group was sparse and the Nissl substances were decreasing or disappearing. Interestingly, TH treated hypoxia group had significantly higher number of pyramidal cells ($P<0.05$) compared with sucrose treated non-hypoxia group. Similarly, the arrangement of pyramidal neurons in mPFC was preserved and the Nissl substances in the cytoplasm were clearly visible (Figure 2 & 4).

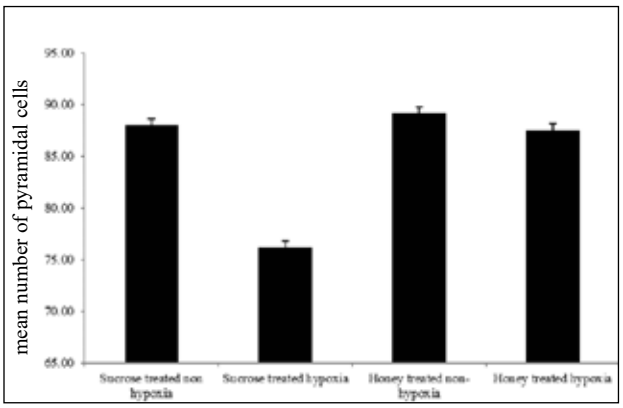

Figure 2 Effects of sucrose and Tualang honey treatment on the mea number of pyramidal cells in mPFC. The data are expressed as mean ± SEM. *$P<0.05$; significant difference between sucrose treated hypoxia and non-hypoxia group. #$P<0.05$; significant difference between honey treated and sucrose treated hypoxia group

Effects of hypoxia and treatment on ACh and AChE concentrarions:

In addition, one-way ANOVA revealed that the mean ACh and AChE concentrations in left hemisphere homogenates were significantly different between different experimental groups [$F (3, 47) = 32.29$;

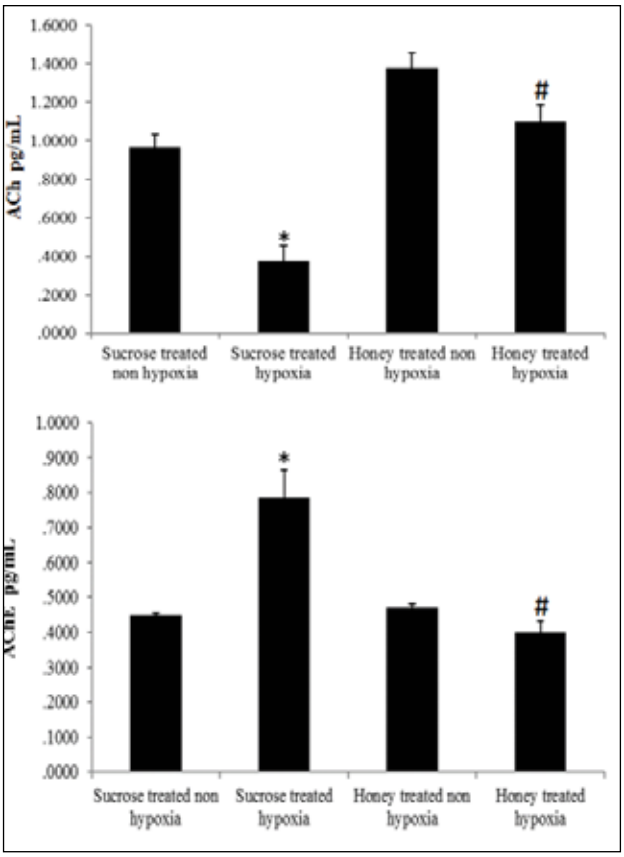

Figure 3 Effects of hypoxia and Tualang honey treatment on mean concentrations of ACh (top) and AChE (bottom) among different experimental groups. The data are expressed as mean ± SEM. **P*<0.05; significant difference between sucrose treated hypoxia and control group

# *P*<0.05; significant difference between honey treated and sucrose treated hypoxia groups

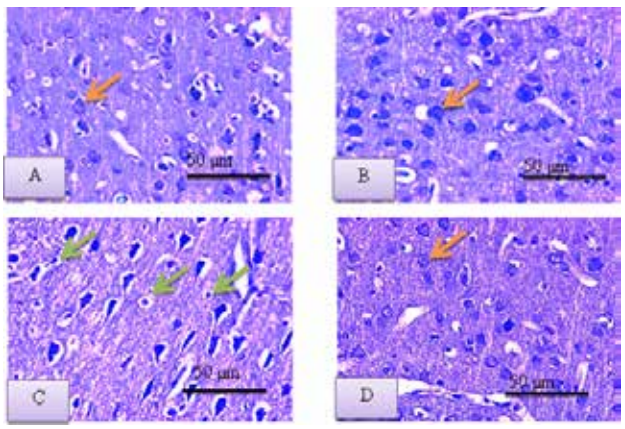

Figure 4 Arrangement of mPFC pyramidal neurons among groups; A) sucrose treated non-hypoxia, B) honey treated non-hypoxia, C) sucrose treated hypoxia, D) honey treated hypoxia. The yellow arrows indicate the normal cells and the green arrows indicate the cells of interest (Nissl staining × 40, scale bar: 50µm)

P<0.05] & [F (3, 47) = 17.29; *P*<0.05]. Sucrose treated hypoxia group revealed significantly lower mean ACh and higher mean AChE concentrations (*P*<0.05) compared to sucrose and honey treated non-hypoxia groups. Interestingly, TH treated hypoxia group showed more significant increase in the mean ACh and decrease in the mean AChE concentrations (*P*<0.05) compared with sucrose treated non-hypoxia group (Figure 3).

Discussion:

Medial prefrontal cortex (mPFC) is particularly involved in the retrieval of long term memory[38]. Other previous studies reported that mPFC plays important role in fear memory and consolidation of memories[39,40]. The present results suggested that TH treatment prevents neuronal damage in mPFC following exposure to normobaric hypoxia in rats. In our previous studies demonstrated that TH improved hippocampal neuronal damage in aged rats[35] and stressed ovariectomized rats[33]. Recent studies from other laboratory also reported that TH protects neuron in midbrain and minimizes neurodegeneration in the cortex[41,42]. Based on the previous literature, TH mitigates neuronal damage through increased level of antioxidant enzymes and reduced oxidative stress[32,42] and increased level of BDNF and acetylcholine[36-37]. In the present result also suggests that TH treatment increased level of ACh following exposure to hypoxia in mPFC of rats.

Hypoxia is well known to induce impairment of brain function and cholinergic system which is an indispensable element for learning and memory[27]. Gibson and Blass[43] reported that the cholinergic system is particularly susceptible to the brain hypoxia. Similar finding was reported cholinergic neurons appeared to be the most vulnerable compared with other neurons and also found that loss of ACh level and their transport in the frontal cortex, hippocampus and striatum.

The previous literature documented that decreased oxygen availability leads to reduced glucose oxidation into pyruvate resulting in decreased synthesis of acetyl CoA which is precursor of ACh synthesis[43]. This could be one of the reasons to reduce ACh synthesis and release as observed in in-vitro and in-vivo studies[44,45]. Another possible enzyme to reduce the ACh synthesis is that choline acetyltransferase (ChAT) which helps to synthesis ACh in presynaptic neurons. Interestingly, studies in rodents found that intermittent hypoxia could decrease the expression of ChAT in the basal forebrain[46,47]. These are all possible mechanisms lead to reduce ACh synthsis and release during hypoxia.

Apart from reduced ACh synthesis, increased breakdown of ACh neurotransmitter i.e. increased AChE level, an enzyme responsible for acetylcholine catabolism, may also lead to reduced ACh level[48]. In the present study, a sucrose-treated hypoxia group was used to evaluate the effects of hypoxia on cholinergic system. It was observed that continuous hypoxia exposure (equivalent to 11% of O_2) for 7 days significantly decreased ACh and increased AChE levels in brain homogenates compared to sucrose- and honey-treated non-hypoxia groups. Based on the previous findings, it is possible that the reduced ACh observed in our study is due to both reduced in synthesis and increased in breakdown of ACh.

AChE is also available in tissues that are not innervated by cholinergic nerves such as erythrocytes and megakaryocytes where overexpression of this enzyme leads to abnormal megakaryocytopoiesis[49]. Moreover, Zhang et al.[50] has proposed that AChE overexpression lead to apoptosis in different cell types. We, therefore, hypothesized that the increased AChE levels in brain homogenates may exert neuronal damage. We proceeded with morphological study of the mPFC which is important for goal-directed action[4], working memory[5] and attention[6-9]. Our findings are in agreement with previous studies that used either hypoxic rats' model or other types of stress such as ovariectomised rats[51-53]. Muthuraju et al.[52] showed pyknosis and tangle-like appearance along with large dot-like structures in cortex and hippocampus in conjunction with decreased ACh and increased AChE levels in rats exposed to hypobaric hypoxia equivalent to 6100 m for 7 days.

Our research group has earlier proposed that the improvement in learning and memory following TH supplementation is due to the significant improvement in brain morphology and enhancement of brain cholinergic system secondary to reduction in brain oxidative damage and/or upregulation of BDNF concentration in various rat models[54]. It is possible that TH exerts the same effects in rats exposed to hypoxia. In the present study, TH treatment significantly increased the pyramidal cells count and preserved their morphology and arrangement compared to sucrose-treated hypoxia group. In addition, TH-treated group showed significantly higher ACh and lower AChE levels compared to sucrose-treated hypoxia group. These findings are in line with previous study by Al Rahbi et al.[37] Findings from the study showed reduced concentrations of ACh and increased AChE in the brain homogenates of stressed OVX rats compared with non-stressed sham-operated controls and the effects were reversed after treatment with TH. The observed findings could be attributed to the choline and ACh content in honey[55].

Conclusion:

TH pretreatment at a dose of 0.2 g/kg exerts its protecting effects on the mPFC morphology by decreasing AChE level and increasing ACh level in the brain of male rats exposed to normobaric hypoxia. Future studies should be conducted to elucidate the underlying molecular mechanism(s) of TH on neurogenesis and cholinergic system.

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Conflict of interest: Nil

Authors' Contribution:

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Study design: Qaid EYA, Zakaria R, Yusof NAM, Sulaiman SF, Shafin N, Ahmad AH

Writing and submitting manuscript: Qaid EYA, Zakaria R, Yusof NAM, Sulaiman SF, Shafin N

Editing and approval of final draft: Qaid EYA, Zakaria R, Yusof NAM, Sulaiman SF, Shafin N, Ahmad AH, Othman Z, Al-Rahbi B, Muthuraju S

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