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The effects of Tualang Honey Supplement on Medial Prefrontal Cortex Morphology and Cholinergic System in Stressed Ovariectomised Rats

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Summary. This study evaluated the effects of Tualang honey on the medial prefrontal cortex (mPFC) morphology and the brain cholinergic system in stressed ovariectomised (OVX) rats. Sixty female Sprague-Dawley rats were divided into six groups: (i) unstressed sham-operated control rats, (ii) stressed sham-operated control rats, (iii) nonnstressed OVX rats, (iv) stressed OVX rats, (v) stressed OVX rats treated with 17 β-oestradiol (20 µg daily, sc) and (vi) stressed OVX rats treated with Tualang honey (0.2 g/kg body weight, orally). After social instability stress, the rats were sacrificed, and the right and left brain hemispheres were isolated for histological studies and estimation of acetylcoline (ACh) and acetylcholinesterase (AChE) concentrations. Stressed OVX rats showed reduced concentrations of ACh and increased AChE in the brain homogenates compared with nonstressed sham-operated controls and the effects were reversed after treatment with either 17 β-oestradiol or Tualang honey. The arrangement and number of Nissl positive cells in the mPFC neurons were significantly improved in stressed OVX rats treated with either 17 β-oestradiol or Tualang honey compared to untreated stressed OVX rats. In conclusion, treatment with either 17 β-oestradiol or Tualang honey significantly improved the morphology of mPFC, increased ACh and reduced AChE concentrations in stressed ovariectomised rats.

Industrial relevance. Although the currently available hormone replacement therapy is valuable in the management of postmenopausal symptoms, it needs close monitoring of the side effects. Because of this, there is a need for a safe alternative treatment to alleviate postmenopausal symptoms. In view of the fact that most natural products have little or no known side effects, coupled with previous studies reporting neuroprotective benefits of Tualang honey, it is deemed a suitable alternative therapy to alleviate some of the neurological symptoms in postmenopausal women.

Keywords. medial prefrontal cortex; acetylcoline; acetylcholinesterase; ovariectomy; Tualang honey

INTRODUCTION

The prefrontal cortex (PFC) plays an important role in integrating cortical and subcortical inputs for cognitive processes. Depletion of acetylcholine (ACh) in the PFC produces profound impairments in cognitive functions in primates (Croxson *et al*. 2011) and in rodents (Chudasama *et al*. 2004) and the degeneration of cholinergic neurons in PFC have been implicated in the neurodegenerative disorders such as Alzheimer's disease.

Many effects of chronic stress are mediated by increased in stress-induced levels of glucocorticoids; the major stress hormones (Uno *et al*. 1989). One of the primary targets of glucocorticoids is the prefrontal cortex (McEwen, 2007), a region controlling high-level ''executive'' functions, including working memory, inhibition of distraction, novelty seeking, and decision making (Miller, 1999; Stuss and Knight, 2002). Chronic stress or glucocorticoid treatment has been found to cause structural remodelling and behavioural alterations in the PFC of adult animals such as dendritic shortening, spine loss, and neuronal atrophy (Cook and Wellman, 2004; Radley *et al.* 2004, 2006), as well as impairments in cognitive flexibility and perceptual attention (Cerqueira *et al*. 2005, 2007; Liston *et al.* 2006). In addition, stress significantly reduced ACh concentrations in old animals (24 months) when compared with young animals (6 months) (Segovia *et al.* 2008). Alteration in the morphology of PFC and reduction in ACh concentrations following chronic stress may impair cognitive function and trigger neuropsychiatric disorders (Sarter and Bruno 1999; de Kloet *et al.* 2005).

Oestrogens can affect the serotonergic, cholinergic, noradrenergic and dopaminergic systems. Administration of oestrogens to ovariectomised rats increases the activity of choline acetyl transferase (ChAT), which is involved in the synthesis of ACh in the basal forebrain, and in two of its projection areas - the CA1 region of the hippocampus and the frontal cortex. A de novo synthesis of the enzyme in the basal forebrain has been postulated to elevate the ChAT activity with subsequent axonal transport

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to the CA1 region of hippocampus and the frontal cortex (Luine, 1985). A single photon emission tomography (SPET) study reported an increased index of cortical cholinergic terminal concentrations with increasing years of oestrogen replacement therapy (ERT) use in healthy post-menopausal women (Smith *et al.* 2001) and this may preserve the memory in older women.

Tualang honey, a phytoestrogen, has been used as a natural source of oestrogen in the present study. Tualang honey is one of Malaysia's most well-known honey among others such as Gelam honey, Belimbing honey and Coconut honey. Tualang honey is produced by wild bees of the species *Apis Dorsata* that dwell in tropical forests in the state of Kedah, Malaysia. Tualang honey derives its name from Tualang tree, a tropical tree also known as Koompassia Excelsa. Wild honey produces nectar of high quality and high nutritional content. Traditionally used for general health, recent research has shown that Tualang honey may have a number of systemic benefits on bone structure (Zaid *et al.* 2010), climacteric symptoms (Othman *et al.* 2011), and breast cancer prevention (Ghashm *et al.* 2010; Fauzi *et al.* 2011). In addition, honey has significant antioxidant activity (Al-Mamary *et al.* 2002; Meda *et al.* 2005; Perez *et al.* 2007; Khalil *et al.* 2011; Kishore, 2011) and contains both choline and ACh (Heitkamp and Hauswirtschaft, 1984) which function as neurotransmitters and are essential for brain function.

The present study aims to evaluate the possible beneficial effects of Tualang honey on morphology of the PFC and brain cholinergic system in OVX rats exposed to chronic social stress and these findings were compared with the 17β-oestradiol-treated group.

MATERIALS AND METHODS

Animals. A total of 60 adult female Sprague-Dawley rats aged approximately eight weeks and weighing 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 experimental protocol was approved by the Animal Ethics Committee of the USM; No. of Animal Ethics Approval: USM/Animal Ethics Approval/2011/(64)(272). The rats were randomly divided into six groups as summarised in Table 1. The experimental design is shown in Figure 1.

Table 1 Animal experimental groups

Group	
1	nonstressed sham-operated control rats1
2	sham-operated control rats exposed to stress
3	nonstressed and OVX rats
4	stressed OVX rats
5	stressed OVX rats treated with 17 β-oestradiol (20 μg daily, sc)
6	stressed OVX rats treated for 18 days with Tualang honey (0.2 g/kg body weight, orally)

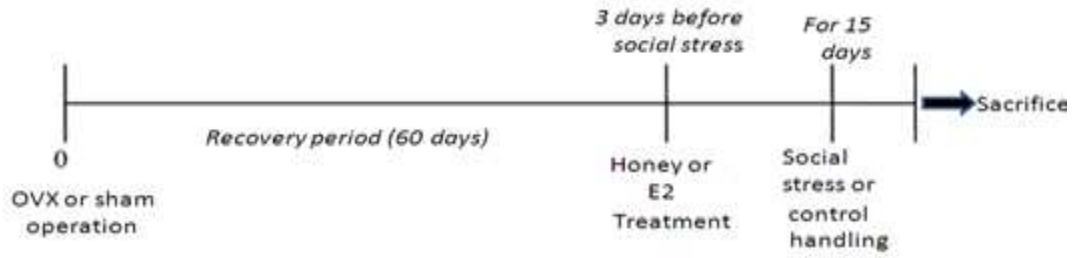

Figure 1 Experimental design

Surgical procedure. Rats were randomly stratified into two groups; one group underwent bilateral OVX and the other group underwent standard sham operation. The surgical procedures were performed under general anaesthesia induced by the intraperitoneal injection of ketamine (Sigma, USA, 60 mg/kg) and xylazine (Sigma, USA, 5 mg/kg). After shaving the area, a small midline incision (about 2 cm) was made on the dorsal area of the 3rd to 5th lumbar vertebrae and cleaned with a chlorhexidine scrub and 70% ethanol. Following surgical procedure, the peritoneal cavity and skin were closed with absorbable sutures (Miralene, B. Braun Melsungen AG, Germany). The entire surgical procedure was performed by aseptic technique and immediately after the procedure the rats were placed under a heat lamp for 1 hour to avoid hypothermia. Following surgery, the rats were housed separately in a clean cage for 10 days to avoid any interactions that might yield bleeding or poor healing.

Social instability stress procedure. The social instability stress procedure was conducted two months after surgery. The procedure consisted of alternating isolation and crowding phases for 15 days as previously described (Haller *et al.* 1999). The experiment started and ended with an isolation phase, and each phase lasted for 24 hours. Eight rats comprise of three males and five females were held in a cage for the crowding phase. The male rats were used to mimic normal social situation in human. The rats were videotaped for the initial 30 minutes of each crowding phase. Records of behavioural changes were made including biting attacks (bite marks), dominant postures and fighting for food behaviour (De Goeij *et al.* 1992).

Treatments. The rats were treated with either subcutaneous injection of 17 β-oestradiol 20 µg/day (Cayman Chemical, Ann Arbor, MI, USA) in 2.5 µl of corn oil (Takuma *et al.* 2007) or oral gavage of 0.2 g/kg body weight/day (Zaid *et al.* 2010) Tualang honey (Agro Mas, Mergong, Kedah, Malaysia) 3 days prior to the procedure and continued throughout the 15 days of treatment.

Preparation of brain tissue. After completion of the social stress procedure, the rats were sacrificed by rapid decapitation. The brains from each group were quickly removed followed by careful dissection of the right and left brain hemispheres in ice-cold saline. The isolated hemispheres were then weighted and brain homogenate (10% w⁄v) was prepared from the left hemispheres in ice-cold 0.1M of phosphate-buffered saline (pH 7.4). The homogenate was then rapidly centrifuged (10,000 × g) for 10 minutes and kept at −80 °C until assay. The right brain hemispheres were preserved in 10% formalin for histology analysis.

Estimation of acetylcholine concentrations. ACh concentrations were measured using a commercially available ACh assay kit (BioAssay, USA) according to the manufacturer's instructions. Briefly, 20 µl of each brain homogenate sample was transferred into separate wells of the plate. Then, 80 µl of working reagent was added to each well. The plate was slightly mixed and incubated for 20 minutes at room temperature. The reactions were terminated by adding 50 µl of stop solution to each well. Finally the amount of ACh was determined using spectrophotometer at 570 nm absorbance (Thermo Fisher Scientific Inc., Waltham, MA, USA). The standard curve demonstrates a direct relationship between optical density and ACh concentration. ACh concentration was expressed as pg of ACh per ml obtained from the brain homogenate. ACh concentrations in pg per ml obtained from the brain homogenate were normalised to total protein in the sample to account for possible differences in protein concentrations between subjects and expressed in pg/µg protein.

Estimation of acetylcholinesterase concentrations. Acetylcholinesterase (AChE) concentrations were measured using a commercially available AChE kit (Cusabio, USA) according to the manufacturer's instructions. Briefly, 100 µl of the samples and standard were added to each well and incubated for 2 hours at 37.5 °C. The liquids were then removed but not washed. Subsequently, 100 µl of anti-AChE antibody was added to each well and incubated for another 60 minutes at 37°C. After serial washing with a wash buffer, 100µl of an HRP-avidin working solution was added to each well and incubated for 60 min at 37°C. The reaction was started by adding 50 µl of tetramethylbenzidine, which made the reaction blue, and was then terminated by adding 50 µl of stop solution to each well. Finally, the amount of AChE was determined using spectrophotometer at 450 nm absorbance (Thermo Fisher Scientific Inc., Waltham, MA, USA). The standard curve was plotted to demonstrate a direct relationship between optical density and AChE concentration. All AChE concentrations were normalised to total protein in the sample to account for possible differences in protein concentrations between subjects and expressed in pg/µg protein.

Histological assessment of mPFC. The right brain hemispheres were embedded in paraffin, cut into 5µm thick coronal sections by a rotary microtome (HM505E; Microm International GmbH, Walldorf, Germany), mounted on slides and Nissl-stained. Nissl staining was performed according to the standard procedure. Two continuous fields of each mPFC were selected and captured (Olympus biological microscope; CX41, Japan). The arrangement of pyramidal neurons was recorded and the neurons were counted at different magnifications with a high-definition medical image analysis program (analySIS docu 5.0, Germany). The mean of the two fields was taken as the neuron number of each section, and the mean of the four sections was taken as the neuron number of each group. Cells with a shrunken or unclear body with surrounding empty spaces were excluded.

Statistical analyses. Two-way ANOVA was utilised to examine the effects of stress (stressed vs. nonstressed) and surgery (sham-operated vs. OVX) on ACh and AChE concentrations, and the number of pyramidal neurons of PFC, followed by one-way ANOVA to examine the effects of 17 β-oestradiol and Tualang honey on ACh and AChE concentrations, and number of pyramidal neurons in mPFC among the groups. Probability values of less than 5% ($P < 0.05$) were considered statistically significant.

RESULTS AND DISCUSSION

Effects of surgery and social stress on number of Nissl-positive cells and concentrations of ACh and AChE . A significant main effect of surgery was found on the number of Nissl-positive cells in mPFC ($F(1, 36) = 53.75$; $P < 0.001$), ACh ($F(1, 36) = 51.12$, $P < 0.001$) and AChE ($F(1, 36) = 58.71$, $P < 0.001$) concentrations, indicating that oestrogen deficiency in OVX rats reduced the number of neuronal cell bodies in mPCF and the cholinergic activity compared with those in sham-operated rats. However, for the main effect of stress only the number of Nissl-positive cells in mPFC ($F(1,36) = 12.66$; $P < 0.05$) was found to be significant. ACh ($F(1, 36) = 0.98$, $P > 0.05$) and AChE ($F(1, 36) = 4.08$, $P > 0.05$) concentrations in the brain homogenate were not significant.. Our data also indicated a significant surgery and stress interaction on the number of Nissl-positive cells in mPFC ($F(1, 36) = 12.90$, $P < 0.05$) and AChE ($F(1, 36) = 0.59$; $P < 0.05$) but not on ACh ($F(1, 36) = 2.21$, $P > 0.05$) concentrations as shown in Table 1.

Effects of 17β-oestradiol and Tualang honey on number of Nissl-positive cells in mPFC and ACh and AChE concentrations

One-way ANOVA revealed a significant difference among the groups in the number of Nissl-positive cells in mPFC ($F(1,36) = 17.91$; $P < 0.001$), and post-hoc analysis revealed that the stressed OVX rats treated with either 17β-oestradiol or Tualang honey had significantly higher number of Nissl-positive cells in mPFC compared to untreated stressed OVX rats (Figure 2).

In addition, one-way ANOVA data showed that the mean ACh and AChE concentrations in the left brain homogenate were significantly different among the groups ($F(5, 54) = 17.79$, $P < 0.001$ and $F(5, 54) = 12.68$, $P < 0.001$, respectively). Both 17β-oestradiol and Tualang honey increased the mean ACh ($P < 0.001$) and reduced AChE ($P < 0.05$) concentrations in the left brain homogenate of the stressed OVX rats compared with those of the sham-operated control rats, as shown in Figure 3.

Table 2 Effects of surgery and social stress on number of Nissl-positive cells in mPFC, ACh and AChE concentrations

Groups	mPFC/0.1mm^2	ACh Concentration (pg/ml).	AChE Concentration (pg/ml).
Sham operated control			
No stress	92.50 ± 0.81	1.16 ± 0.06	1.45 ± 0.03
Stress	89.70 ± 0.36	1.03 ± 0.57	1.49 ± 0.03
OVX			
No Stress	86.50 ± 1.31	0.71 ± 0.16	1.53 ± 0.01
ovx +Stress	81.40 ± 1.04	0.74 ± 0.33	1.71 ± 0.01
Stress effects	$P < 0.05$	$P > 0.05$	$P > 0.05$
Surgery effects	$P < 0.001$	$P < 0.001$	$P < 0.001$
Stress x surgery interaction	$P < 0.05$	$P > 0.05$	$P < 0.05$

Values are expressed as mean ± SEM.

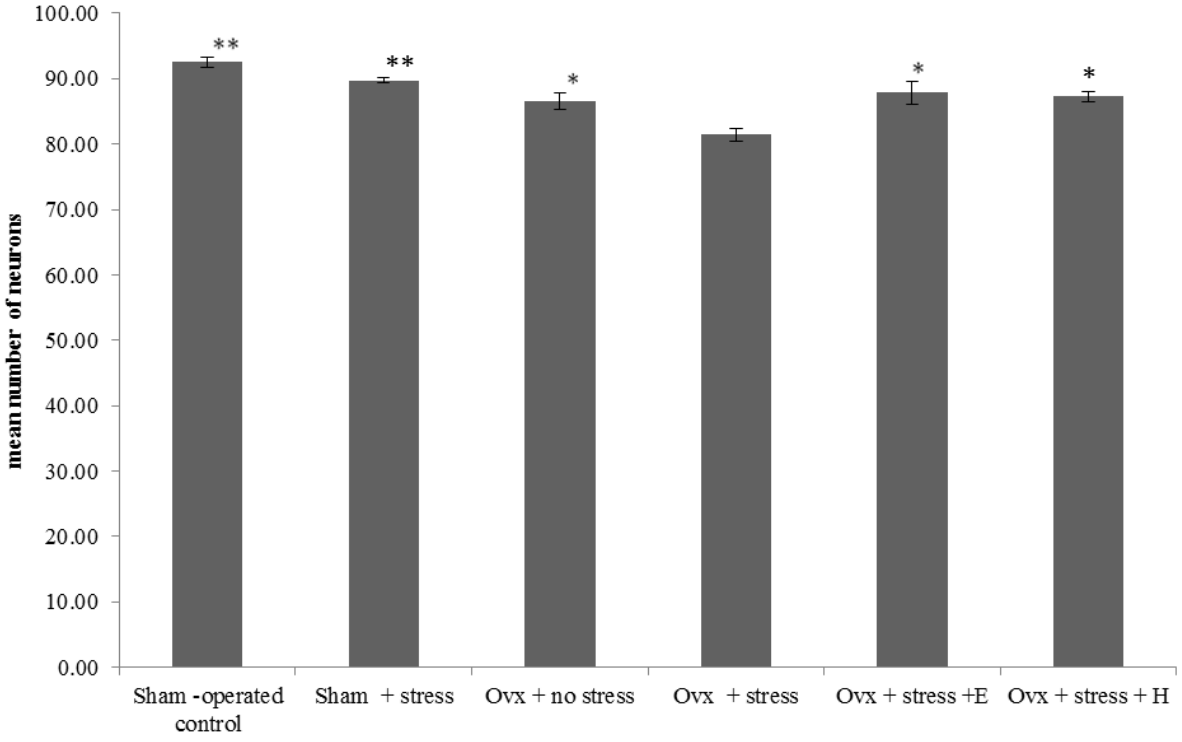

Figure 2 Effects of 17β-oestradiol and Tualang honey on the number of Nissl-positive cells in mPFC. The data are shown as mean ± SEM. * $P < 0.05$, ** $P < 0.001$; significantly different from stressed OVX group

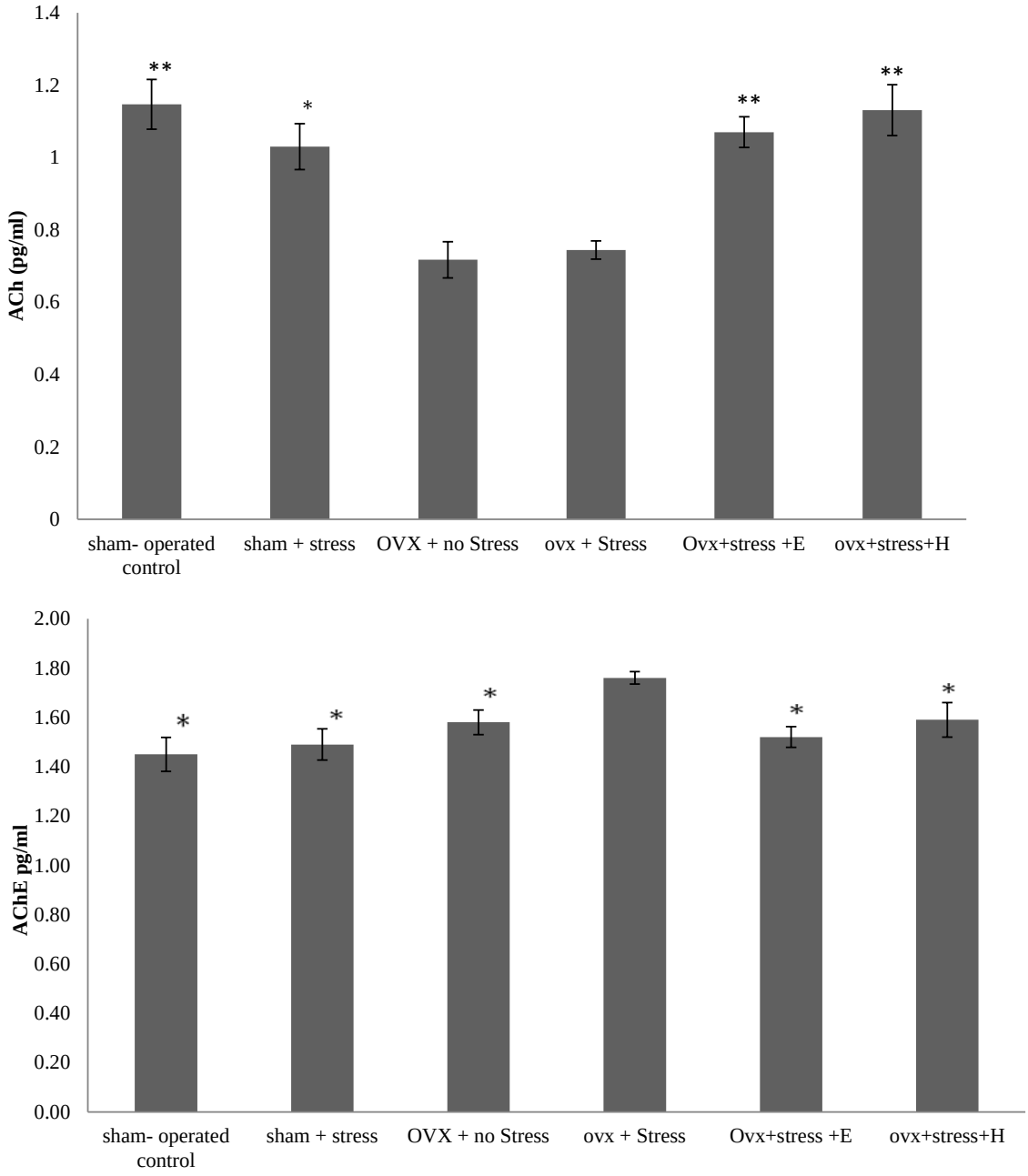

Figure 3 Effects of Tualang honey and E2 treatment on concentrations of ACh (top) and AChE (bottom). The data are shown as mean ± SEM. * $P < 0.05$, ** $P < 0.001$; significantly different from stressed OVX group

Effects of surgery and social stress on arrangement of Nissl-positive cells in mPFC. The arrangement of mPFC pyramidal neurons was unaffected by chronic stress alone as seen in the stressed or nonstressed sham-operated controls. There were abundant pyramidal neuron, the architecture of these neurons was preserved and Nissl substances in the cytoplasm were clearly visible (Figures 4 & 5 A and B). In contrast, the arrangement of mPFC pyramidal neurons of OVX group was sparse and the Nissl substance was decreasing or dissolving. The effects were more obvious in the stressed OVX group as shown in Figures 4 & 5 C and D.

Effects of 17β-oestradiol and Tualang honey on arrangement of Nissl-positive cells in mPFC . Similarly, normal architecture of mPFC pyramidal neurons of the treated stressed OVX (Figures 4 & 5 E and F) was observed compared with that of the untreated stressed OVX group.

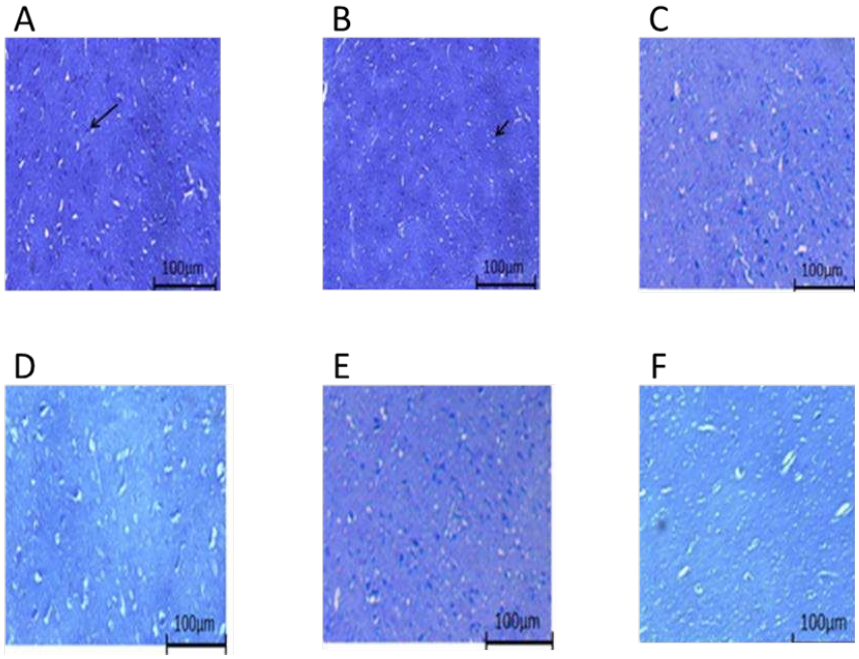

Figure 4 Arrangement of mPFC pyramidal neurons among groups; A) sham-operated control rats, B) stressed sham-operated control rats, C) nonstressed OVX rats, D) stressed OVX rats, E) stressed OVX rats treated with 17 β-oestradiol and F) stressed OVX rats treated with Tualang honey. The arrows indicate the cells of interest (Nissl staining × 100, scale bar: 50µm).

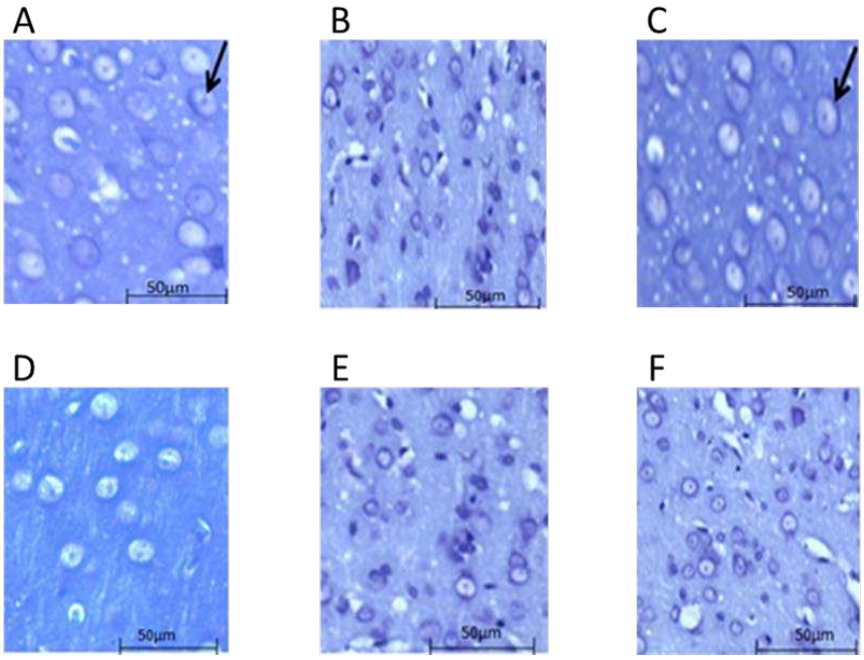

Figure 5 Arrangement of mPFC pyramidal neurons among groups; A) sham-operated control rats, B) stressed sham-operated control rats, C) nonstressed OVX rats, D) stressed OVX rats, E) stressed OVX rats treated with 17 β-oestradiol and F) stressed OVX rats treated with Tualang honey. The arrows indicate the cells of interest (Nissl staining × 400, scale bar: 50µm).

DISCUSSION

In the present study, a stressed OVX rat model was used to examine the effects of chronic stress and oestrogen deficiency on the cholinergic system and mPFC morphology. We found that oestrogen deficiency following OVX significantly affects both the mPFC morphology and the cholinergic system. These findings are consistent with those of earlier reports (Wallace *et al*. 2006; Mitsushima *et al*. 2009). Wallace *et al.* (2006) found significantly lower spine densities (17% to 53%) in the pyramidal neurons of the mPFC and the CA1 region of the hippocampus compared with those of intact rats seven weeks after OVX. In addition, Mitsushima *et al*. (2009) revealed severe reduction in hippocampal ACh release in OVX rats.

On the contrary, chronic stress only significantly affects the mPFC morphology but not the cholinergic system. These findings are in agreement with many previous studies involving different types of stressors, including predator odour (Tanapat *et al.* 2001), social stress (Gould *et al*. 1997; Czed *et al*. 2002), acute and chronic restraint stress (Pham *et al*. 2003; Veena *et al*. 2009 a & b; Rosenbrock *et al*. 2005; Vollmayr *et al*. 2003), footshock stress (Vollmayr *et al*. 2003; Malberg and Duman, 2003) and chronic mild stress (Alonso *et al*. 2004). Radley *et al*. (2006) noted 21 days of repeated restrain stress induced dendritic spine loss in the rat mPFC. Previous functional neuroimaging studies revealed diminished mPFC responses during the symptomatic state (Shin *et al*. 2005) in PTSD patients and reduction in mPFC volume (Rauchs *et al.* 2003). Our findings are also consistent with those of Mizoguchi *et al*. (2001), who revealed that rats exposed to stress for four weeks did not show any change in basal ACh release.

Oestrogen treatment improved the cognitive in object recognition task (Luine *et al*. 2003; Li *et al*. 2004; Walf *et al*. 2006; Scharfman *et al*. 2007) and morphological impairments in the hippocampus (Takuma *et al*. 2007; Al-Rahbi *et al*. 2014a) of rats and mice. Its neuroprotective effects is believed to work via antioxidant mechanisms (Komuro *et al*. 1990; Vedder *et al*. 1999), up-regulation of brain-derived neurotrophic factor (BDNF) expression (Takuma *et al*.2007; Al-Rahbi *et al*. 2014b) and/or augmentation of choline acetyltransferase and acetylcholinesterase activities in specific brain areas (Luine *et al*. 1986; Gibbs and Aggarwal, 1998; Gibbs *et al*. 2004).

The PFC is one of the targets for oestrogen action (Juraska and Markham, 2004; Tang *et al*. 2004). Chronic oestrogen treatment enhanced PFC function in aged female monkeys (Hao *et al*. 2006) and increased the apical dendritic length in PFC of OVX rats (Garrett and Wellman, 2009). Our recent histologic data (Al-Rahbi *et al*. 2014a) confirmed that Tualang honey supplementation to stressed OVX rats enhances neuronal proliferation in the hippocampal CA2, CA3 and DG regions. However, the effects on the PFC have yet to be reported.

In the present study, we found that both oestradiol and Tualang honey treatments significantly increase the number of Nissl-positive cells in mPFC compared to untreated stressed OVX rats. Our findings are in line with the beneficial effects of several dietary phytochemicals or flavonoids on the nervous system by protecting neurons against injury or diseases (Spencer, 2010; Park and Lee, 2011; Yoo *et al*. 2011). Phenolic dietary antioxidant supplements such as curcumin (Park and Lee, 2011) and grape seeds (Yoo *et al*. 2011) have been shown to enhance hippocampal neurogenesis.

In addition, both 17β-oestradiol and Tualang honey treatments significantly increase ACh and reduce AChE concentrations in stressed OVX rats. Our findings are consistent with those of Pan *et al*. (1999) who revealed that oestrogen and soy phytoestrogen significantly increased choline acetyltransferase in the frontal cortex and hippocampus of OVX young rats. In addition, 17β-oestradiol treatment also increases both affinity of choline uptake and ACh synthesis in basal forebrain neurons (Pongrac *et al*. 2004). In another study, Mitsushima *et al.* (2009) found gonadectomy severely reduced the ACh release and activity levels in both male and female rats but testosterone replacement in gonadectomised males or 17β-estradiol replacement in gonadectomised females successfully restores it. The ability of the gonadal steroids in maintaining stress-induced ACh release in the dorsal hippocampus in immobilised rats has also been observed (Mitsushima *et al*. 2008) and these findings were supported by earlier studies that 17β-oestradiol induced choline acetyltransferase in the basal forebrain of OVX rats (Luine *et al.* 1986; McEwen and Alves, 1999).

CONCLUSION

Chronic Tualang honey supplementation exerts beneficial effects on the mPFC morphology and the cholinergic system comparable to those received 17β-oestradiol treatment. Since Tualang honey is rich in flavonoids and contains the highest total phenolic content compared to Gelam, Indian forest and Pineapple honey (Kishore, 2011), it is possible that Tualang honey exerts its positive effects through its antioxidant properties as well as its choline and ACh contents. However, future studies should be conducted to elucidate the exact mechanisms of Tualang honey on the neurogenesis and the cholinergic system, and its choline and ACh concentrations.

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