



Experimental Article

Tualang honey and DHA-rich fish oil reduce the production of pro-inflammatory cytokines in the rat brain following exposure to chronic stress

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المخلص

أهداف البحث: تهدف هذه الدراسة للتحقق في تأثير عسل توالانج، وزيت السمك الغني-بحمض الدوكوساهيكسانويك ومزيجهم على تركيز السيتوكينات المسببة للالتهابات في دماغ الفئران بعد التعرض لإجهاد مزمن.

طرق البحث: تم تقسيم خمسين فأراً نكراً من نوع سبراغ داوولي إلى ٥ مجموعات؛ (١) التحكم، و(٢) المتعرضين للإجهاد، و(٣) المتعرضين للإجهاد وعولجوا بعسل توالانج (١جم/كجم من وزن الجسم مرتين يومياً عبر التزجيج عن طريق الفم)، و(٤) المتعرضين للإجهاد وعولجوا بزيت السمك الغني بحمض الدوكوساهيكسانويك (٤٥٠ مجم/كجم من وزن الجسم مرتين يومياً عبر التزجيج عن طريق الفم) و(٥) المتعرضين للإجهاد وعولجوا بمزيج من عسل توالانج وزيت السمك الغني بحمض الدوكوساهيكسانويك. يتكون نظام الإجهاد المزمن من مزيج إجهاد ضبط النفس واختيار إجهاد السباحة لمدة ٢٨ يوماً. وتم قياس السيتوكينات المسببة للالتهابات المحددة في تجانس الدماغ (عامل نخر الورم ألفا، وانترلوكين ٦، وانترفيرون جاما) باستخدام تقنية اليزا.

النتائج: تركيز عامل نخر الورم ألفا، وانترلوكين ٦، وانترفيرون جاما في تجانس الدماغ في مجموعة حمض الدوكوساهيكسانويك، ومجموعة عسل توالانج ومزيج المجموعتين حمض الدوكوساهيكسانويك + عسل توالانج كانت أقل بكثير عند مقارنتها لمجموعتي التحكم والإجهاد ولكن ليس بين مجموعات العلاج.

الاستنتاجات: استهلاك زيت السمك الغني-بحمض الدوكوساهيكسانويك وعسل توالانج يمكن أن يكون فعال في خفض السيتوكينات المسببة للالتهابات في دماغ

الفئران تحت حالة الإجهاد المزمن. ولكن، استهلاك هذه العوامل معا لا يعطي فائدة إضافية عن أخذها بشكل منفصل.

الكلمات المفتاحية: عسل توالانج؛ حمض الدوكوساهيكسانويك؛ زيت السمك؛ السيتوكينات؛ الإجهاد

Abstract

Objectives: The aim of the present study was to investigate the effects of Tualang honey (TH), DHA-rich fish oil, and their combination on the concentrations of selected pro-inflammatory cytokines in rat brains following exposure to chronic stress.

Methods: Fifty male Sprague–Dawley rats were divided into (i) control, (ii) stress-exposed, (iii) stress-exposed and treated with TH (1 g/kg body weight twice daily via oral gavage), (iv) stress-exposed and treated with DHA-rich fish oil (450 mg/kg body weight twice daily via oral gavage), and (v) stress-exposed and treated with a combination of TH and DHA-rich fish oil. The chronic stress regimen consisted of a combination of restraint stress and a swim stress test for 28 days. The concentrations of selected pro-inflammatory cytokines in brain homogenates (TNF- α , IL6, and IFN- γ) were measured by ELISA.

Results: The concentrations of TNF- α , IL6, and IFN- γ in brain homogenates from the DHA, TH, and TH + DHA-treated groups were significantly lower compared to the control and stress-only-exposed groups

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($p < 0.05$), but no difference was observed between treatment groups.

Conclusion: Consumption of DHA-rich fish oil and TH can be effective in lowering pro-inflammatory cytokine levels in the brains of rats under chronic stress conditions. However, consuming these agents together does not provide additional benefits compared to taking them separately.

Keywords: Cytokines; DHA; Fish oil; Stress; Tualang honey

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Introduction

Tualang honey is a type of wild multiflora honey found in tropical rainforests in Southeast Asia. Its name is derived from the Tualang tree (*Koompassia excelsa*) on which Asian rock bees (*Apis dorsata*) build their hives. Tualang honey exhibits numerous medicinal properties, including antimicrobial, anti-inflammatory, antitumour, antimutagenic, antioxidant, antidiabetic, and neuroprotective properties.^{1,2} In addition, Tualang honey can potentially provide protection against the harmful effects of stress. A recent study demonstrated that Tualang honey improves pregnancy outcome in rats exposed to chronic stress, as well as serum corticosterone levels and adrenal cortex parameters.³

Fish oil is a concentrated source of omega-3 polyunsaturated fatty acids (PUFAs). Although several different types of omega-3 PUFAs exist, the three types that are important for human physiology and that are the frequent focus of scientific research are docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and alpha-linolenic acid (ALA). Numerous studies have shown that omega-3 is effective in curing or preventing diseases such as cardiovascular disease, cancer, and inflammatory diseases, among others.⁴ Previous studies have also suggested that omega-3 can protect against the negative effects of stress and has been shown to improve peak plasma concentrations of corticosterone, reduced spatial and working memory, learning, and other stress-related symptoms.^{5,6}

Traditionally, DHA and EPA have been considered as equal constituents of omega-3 PUFAs in terms of their mechanisms of action and functions. However, increasing evidence has shown that individual omega-3 PUFAs have unique and specific effects, which, in turn, may elicit differential effects on health outcomes.^{7,8} Numerous studies investigating the effects of omega-3 PUFAs in humans and animals used anchovy oil, which contains a mixture of DHA and EPA at a typical ratio of 12:18 (120 mg of DHA and 180 mg of EPA per g of oil).⁸ Because of the higher levels of EPA in anchovy oil, these studies cannot indicate which of these omega-3 PUFAs is responsible for the evaluated biological effects; consequently, it is important that pure or almost pure DHA or EPA is used. In the present study, we used purified fish oil containing a very high DHA content at

a DHA to EPA ratio of 23:1 (1000 mg of DHA and 44 mg of EPA) as the source of omega-3 PUFAs.

Cytokines are potent endogenous mediators released by a variety of cell types and are involved in numerous biological processes, including cell growth, survival, and development.⁹ Some pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α); interleukin IL1, IL6, IL8, IL12, and IL18; and interferon- γ (IFN- γ) are produced in response to inflammatory stimuli. TNF- α is the central mediator of the inflammatory response and has a critical role in the initiation of a cytokine cascade during inflammation,¹⁰ while IL6 is an extremely important pleiotropic cytokine with wide-ranging activity, including stimulation of acute-phase protein production during inflammatory reactions, and is also involved in specific cellular and humoral immune responses.¹¹ IFN- γ is another pro-inflammatory cytokine involved in innate and adaptive immune responses against pathogens and tumours, as well as in other biological activities.¹² There is a considerable amount of evidence showing that acute and chronic stress can alter the levels of peripheral and brain cytokines. For instance, plasma TNF- α and IL6 levels were found to increase following exposure to acute and chronic stress, whereas those of IFN- γ were decreased.¹³ However, various other factors such as the type, severity, or duration of the stress, gender and age of subjects, and coping strategies can affect the levels of cytokines. Any alteration in the levels of cytokine production following stress may increase the risk for certain conditions such as cardiovascular, neurodegenerative, and autoimmune diseases.¹⁴ Consequently, the objective of the present study was to assess the protective effects of Tualang honey, DHA-rich fish oil, and their combination on the activity of selected pro-inflammatory cytokines in the brains of rats following exposure to chronic stress.

Materials and Methods

Animals

Six-week-old male Sprague–Dawley rats were obtained from the Animal Research and Service Center (ARASC) at the Universiti Sains Malaysia (USM) Health Campus. The animals were housed in pairs in polypropylene cages in well-ventilated rooms of the ARASC housing facility. The animals were maintained under a 12-h light/dark photoperiod and with *ad libitum* access to drinking water and food pellets, except during the experimental period. The rats were acclimatised to the researchers for at least one week prior to the experiment and were observed closely during this period to ensure that they were in good health before starting the experiment.

Experimental design

Fifty male rats were used in the present study, divided into five groups of 10 rats each, as follows:

- Group 1: Non-stressed control group (C) – rats were not exposed to stress but were briefly handled daily.
- Group 2: Stress-treated group (S) – rats were exposed to chronic stress for 28 days.
- Group 3: Tualang honey-treated group (TH) – rats received Tualang honey (1 g/kg body weight

twice a day) and were exposed to chronic stress for 28 days.

Group 4: DHA-rich fish oil-treated group (DHA) – rats received fish oil (DHA at 450 mg/kg body weight twice a day) and were exposed to chronic stress for 28 days.

Group 5: Tualang honey + DHA-rich fish oil-treated group (TH + DHA) – rats received both Tualang honey (1 g/kg body weight twice a day) and fish oil (DHA at 450 mg/kg body weight twice a day) and were exposed to chronic stress for 28 days.

The rats in the control group were placed in a different room throughout the experiment to minimise any possible indirect exposure to stress from the other groups. All procedures were conducted between 8:00 AM and 4:00 PM. For groups 3, 4, and 5, Tualang honey and/or fish oil was administered via oral gavage twice daily before and after the stress procedures. An equal volume of normal saline was administered via oral gavage to the rats in groups 1 and 2 to ensure that all the rats were placed under a similar amount of stress during treatment. Tualang honey (AgroMas®) was purchased from the Federal Agricultural Marketing Authority (FAMA), Kelantan, Malaysia. The honey was previously filtered, evaporated to 20% (w/v) water content at 40 °C, and then sterilised by gamma irradiation (25 kGy). The DHA-rich fish oil was purchased from General Nutrition Corporation (GNC), Pittsburgh, PA, USA (GNC Triple Strength DHA 1000).

Stress procedure

Except for those in the control group, all the animals were subjected to a combination of restraint stress¹⁵ and a swim stress test¹⁶ in a randomly determined order. For restraint stress, rats were immobilised by wrapping them with flexible plastic mesh with both ends fastened with metal clips. The mesh was appropriately adjusted to fit the animals tightly but without restricting their breathing or causing any pain. The animals were restrained for 5 h a day for 28 days.¹⁷ The swim stress test was employed to prevent or minimise habituation associated with the restraint stress procedure.¹⁸ During the swim stress test, rats were placed in a plastic cylindrical container filled with water (23–25 °C) to a depth of approximately 30 cm for 15 min. During the test, the animals could not touch the bottom of the container either with their feet or their tails, but could swim freely. Immediately after swimming, the animals were dried with a paper towel and then returned to their cages.¹⁹

Tissue sample preparation

The rats were sacrificed by decapitation on the day following the end of treatment (on day 29). Trunk blood samples were collected in 20 ml centrifuge tubes without anticoagulant and then left to clot at room temperature before being centrifuged at 1000×g for 20 min. The supernatants were aliquoted into 1.5 ml centrifuge tubes and stored at –80 °C until analysis.

The brain was quickly removed from the skull and washed with an ice-cold saline solution. The cerebrum was separated from the rest of the brain by removing the brainstem and cerebellum, including the olfactory bulbs, weighed, and then

stored at –80 °C. Cerebra were homogenised in sodium phosphate buffer (0.1 M, pH 7.4) using a motor-driven tissue homogeniser fitted with a Teflon pestle (Glas-Col, Terre Haute, Indiana, USA) in ice-chilled glass homogenising vessels at 900 rpm for 90 s, yielding 10% (w/v) homogenates. The homogenates were centrifuged in a refrigerated centrifuge at 3000×g for 15 min at 4 °C, following which the supernatants were aliquoted into 1.5 ml centrifuge tubes and stored at –80 °C until use.

Measurement of cytokine levels

Cytokine concentrations in the cerebral homogenates were measured using sandwich ELISA kits (Fine test, Wuhan Fine Biotech Co. Ltd, Wuhan, China) following the manufacturer's protocols. Measurements were performed in 96-microwell plates coated with antibodies to rat TNF- α , IL6, or IFN- γ . First, the plates were washed twice with wash buffer. Standards of respective antigens (TNF- α , IL6, or IFN- γ) and homogenate samples were then added to wells as determined prior to the start of the procedure. The standards consisted of several serially diluted concentrations of the respective antigens as per the manufacturer's instructions. After a 90-min incubation, the plates were first washed with wash buffer, and a biotin-labelled antibody was then added to all the wells prior to incubation for 60 min at 37 °C. After washing the plates three times, an HRP-streptavidin conjugate (SABC) solution was added to the wells and the plates were incubated for 30 min at 37 °C. The plates were washed five times, following which a TMB substrate solution was added to the wells and the plates were incubated in the dark for 15–30 min at 37 °C. Stop solution was added to the wells to terminate the reaction and the absorbance at 450 nm was read immediately using a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The final cytokine concentrations were expressed as ng/g of wet tissue.

Measurement of serum corticosterone levels

Serum corticosterone levels were measured using an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, Michigan, USA) following the manufacturer's protocol. Corticosterone concentrations were expressed as ng/ml.

Statistical analysis

All data were expressed as means \pm standard error of the means (SEM). Normality was tested using the Kolmogorov–Smirnov test. Statistical analysis was carried out using one-way ANOVA. If ANOVA analysis achieved significance, multiple Turkey's *post hoc* tests were performed; *p*-values < 0.05 were considered significant.

Results

Effects of DHA-rich fish oil, Tualang honey, and their combination on serum corticosterone levels

Figure 1 shows the effects of stress and DHA-rich fish oil, Tualang honey, and their combination on serum

corticosterone concentrations. The results showed that serum corticosterone levels in the stress-only group (35.2 ± 1.9 ng/ml) were significantly higher than those in the control group (12.8 ± 1.1 ng/ml), indicating that a sufficient amount of stress had been administered to the animals throughout the study period. Serum corticosterone levels in the DHA (28.3 ± 1.6 ng/ml), TH (24.5 ± 1.7 ng/ml), and TH + DHA groups (25.1 ± 1.4 ng/ml) were all significantly higher than those in the control group, but lower than in the stress-only group. Other comparisons were not significant. These findings indicated that the DHA-rich fish oil and Tualang honey were effective in reducing the stress-induced increase in corticosterone concentrations; however, consuming a combination of these substances was not superior to consuming them separately.

Effects of DHA-rich fish oil, Tualang honey, and their combination on TNF- α levels

The effects of stress and DHA-rich fish oil, Tualang honey, and their combination on TNF- α levels in the brain are presented in Figure 2. The results indicated that the levels of TNF- α in the stress-only group (33.9 ± 2.4 ng/g) were significantly higher than that in the control group (27.7 ± 1.8 ng/g). Moreover, the results also showed that TNF- α levels in the DHA (18.8 ± 1.7 ng/g), TH (20.2 ± 1.7 ng/g), and TH + DHA groups (22.3 ± 1.5 ng/g) were significantly lower compared to the control and stress-only groups. Other comparisons were not significant. These results indicated the efficacy of DHA-rich fish oil and Tualang honey in lowering TNF- α levels in the brain following exposure to stress. However, compared to each treatment alone, combining the DHA-rich fish oil and Tualang honey did not provide any additional benefit in reducing the brain levels of TNF- α .

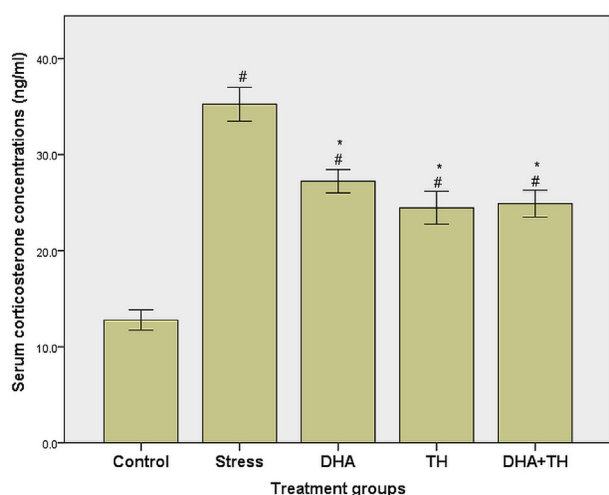


Figure 1: Serum corticosterone levels (ng/ml) in various treatment groups (n = 10 rats per group). Compared to the control group, rats in the stress-only, DHA-treated, TH-treated, and TH + DHA-treated groups presented significantly higher serum corticosterone levels. Data are presented as means \pm standard error of the means. # $p < 0.05$ compared to the control group, * $p < 0.05$ compared to the stress-only group..

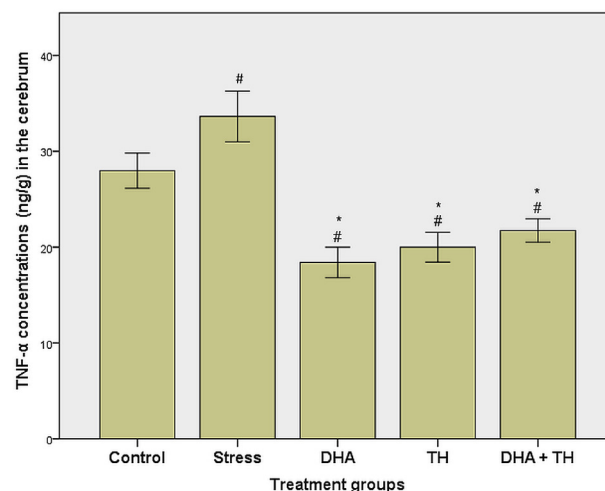


Figure 2: TNF- α levels (ng/g) in rat brain homogenates from various treatment groups (n = 10 rats per group). Compared to the control and stress-only groups, treatment with DHA, TH, and their combination significantly reduced the concentrations of TNF- α in the brain homogenates. # $p < 0.05$ compared to the control group, * $p < 0.05$ compared to the stress-only group. Data represent means \pm standard error of the means..

Effects of DHA-rich fish oil, Tualang honey, and their combination on IL6 levels

Figure 3 shows the effects of stress and DHA-rich fish oil, Tualang honey, and their combination on IL6 levels in the brain. The results demonstrated that IL6 levels in the control group (124.1 ± 9.4 ng/g) were significantly lower than those in the stress-only group (140.1 ± 17.2 ng/g). Our results also indicated that IL6 levels in the DHA (105.4 ± 11.3 ng/g), TH (101.2 ± 10.0 ng/g), and TH + DHA groups (93.3 ± 10.2 ng/g) were significantly lower compared to the control and stress-only groups. Other comparisons were not significant. These findings showed that DHA-rich fish oil and Tualang honey were effective in lowering IL6 concentrations in the brain following exposure to stress. However, consuming the DHA-rich fish oil and Tualang honey in combination was not superior to consuming them separately.

Effects of DHA-rich fish oil, Tualang honey, and their combination on IFN- γ levels

The effects of stress and DHA-rich fish oil, Tualang honey, and their combination on brain IFN- γ levels are illustrated in Figure 4. The levels of IFN- γ in the stress-only group (9.6 ± 0.9 ng/g) were significantly higher than those in the control group (7.8 ± 0.9 ng/g). Moreover, the results also indicated that IFN- γ levels were significantly lower in the DHA (6.4 ± 0.8 ng/g), TH (6.6 ± 0.7 ng/g), and TH + DHA groups (6.7 ± 0.7 ng/g) than in the control and stress-only groups. Other comparisons were not significant. Together, these observations suggested that DHA-rich fish oil and Tualang honey were both effective in reducing the concentration of IFN- γ in the brain following stress exposure. However, the combination treatment appeared not to provide any additional benefit in reducing brain IFN- γ levels compared to the individual treatments.

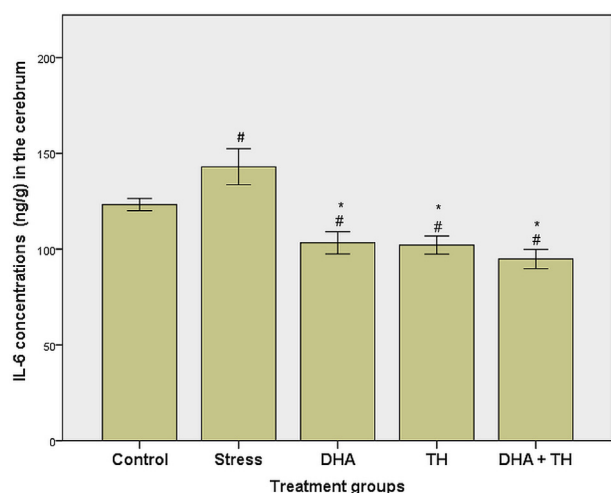


Figure 3: IL6 (ng/g) levels in rat brain homogenates from various treatment groups (n = 10 rats per group). Treatment with DHA, TH, and their combination significantly reduced the concentrations of IL6 in the brain homogenates compared to the control and stress-only groups. [#]*p* < 0.05 compared to the non-stress control group, ^{*}*p* < 0.05 compared to the stress-only group. Data represent means ± standard error of the means.

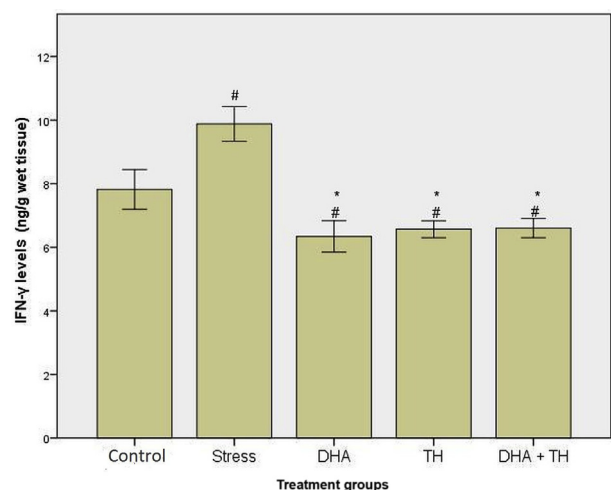


Figure 4: IFN-γ levels (ng/g) in rat brain homogenates from various treatment groups (n = 10 rats per group). Compared to the control and stress-only groups, treatment with DHA, TH, and their combination resulted in significantly reduced IFN-γ concentrations in the brain homogenates. [#]*p* < 0.05 compared to the non-stress control group, ^{*}*p* < 0.05 compared to the stress-only group. Data represent means ± standard error of the means.

Discussion

In the present study, we showed that Tualang honey can reduce the production of TNF- α , IL6, and IFN- γ in the rat brain following exposure to chronic stress. To the best of our knowledge, this is the first report on the effect of Tualang honey on the production of pro-inflammatory cytokines under this condition. Hussein et al. (2012)²⁰ reported the inhibitory effect of a different type of local honey (Gelam honey) on various inflammatory mediators in an

inflammation-induced rat model. The authors showed that Gelam honey inhibited many pro-inflammatory mediators, including TNF- α , IL6, NO, PGE2, iNOS, and COX2. Our study also indicated that Tualang honey can attenuate stress-induced increases in corticosterone levels, suggesting that Tualang honey possesses significant anti-stress properties. A study by Haron et al. (2014)³ similarly showed that Tualang honey can improve stress-induced increases in corticosterone levels, as well as adrenal cortex parameters, in rats exposed to chronic restraint stress.

The results of our study also indicated that DHA-rich fish oil is effective in reducing the production of TNF- α , IL6, and IFN- γ in the rat brain following exposure to chronic stress. This finding is in agreement with several studies which showed that omega-3 PUFAs in fish oil help modulate the negative effects of chronic stress on the body.^{6,21,22} For instance, Hennebelle et al. (2012)⁶ showed that rats exposed to restraint stress for 21 days and fed with an omega-3 enriched diet presented lower stress-induced weight loss, a lower plasma corticosterone peak, and improved behavioural responses compared to controls. Interestingly, rats that lacked omega-3 PUFAs in their diet exhibited the opposite effects, suggesting the importance of omega-3 PUFAs in adaptation to stress. The modulatory action of omega-3 PUFAs during chronic stress is believed to be mostly due to the action of DHA and not EPA. Bradbury et al. (2004)²¹ showed that EPA-rich fish oil could not reduce chronic psychological stress levels among the human participants. The present study also indicated that a combination of Tualang honey and DHA-rich fish oil are effective in reducing pro-inflammatory cytokine levels in the rat brain; however, the effects were similar to those observed when Tualang honey or DHA-rich fish oil were administered alone.

Taken together, our results demonstrated that Tualang honey and DHA-rich fish oil can lower the levels of pro-inflammatory cytokines in the brain and can potentially also lower other types of inflammatory mediators in the body. The balance between inflammatory and anti-inflammatory cytokines is essential for normal health and any imbalance between these two cytokine types can lead to dysregulation of the cytokine network, resulting in the development of certain diseases.²³ The concentrations of TNF α , IL6, and IL1 are elevated in most inflammatory states and are recognised targets for therapeutic intervention.¹¹ In addition to being a potent activator of the immune system, TNF- α is also involved in several normal cellular processes such as cell viability, gene expression, and synaptic integrity.²⁴ Although this cytokine is produced by numerous cell types, activated microglia are the major source of TNF α in the brain.²⁵ However, TNF- α produced by activated microglia can activate the production of TNF- α in resting microglia or astrocytes located in the adjacent microenvironment, resulting in dysregulation of the inflammatory response in the central nervous system.²⁴ As with other pro-inflammatory cytokines, IL6 is also involved in stimulating immune responses; moreover, IL6 is also a potent inducer of the acute phase response and regulates the transition from acute to chronic inflammation.⁹ In the central nervous system, IL6 is produced by different cell types, including neurons, microglia, astrocytes, and endothelial cells. All these cell types can produce IL6 to some extent under normal condition, but the levels of IL6 increase

significantly following inflammation or injury.^{11,26} IFN- γ is a critical pro-inflammatory cytokine for both innate and adaptive immunity against viral infection. This cytokine is secreted by activated immune cells such as T cells and natural killer cells, as well as by activated microglia in the central nervous system.²⁷ A number of studies have shown that all these cytokines play significant roles in the pathogenesis of several neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases.^{24,26–28}

Conclusion

In conclusion, our results indicated that both Tualang honey (1 g/kg) and DHA-rich fish oil (450 mg/kg) can reduce the production of pro-inflammatory cytokines in the brain of rats following exposure to chronic stress. However, combined administration of these two substances is not superior to administration of either substance alone.

Source of funding

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The experimental protocols in the present study were reviewed and approved by the Universiti Sains Malaysia Animal Ethics Committee (ethical approval no. USM/IACUC/2017/105/846).

Authors' contributions

MAA, KNSS, NAMY, and NSMY conceived and designed the study. MAA, MHZ, and NAMY conducted the experiment and analysed and interpreted the data. MAA and MHZ wrote the initial and final drafts of the manuscript. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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