Preemptive Effects of Administration of Tualang Honey on Inflammatory Responses in Adult Male Rats

Che Aishah Nazariah Ismail[1,*], Che Maraina Che Hussin[2], Mahaneem Mohamed[1] and Che Badariah Abd Aziz[1]

[1]Physiology Department; [2]Immunology Department, School of Medical Sciences, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia

Abstract: *Background*: Honey was reported to reduce pain and inflammation from burn wound. To date, no study has compared between the effects of Tualang honey and prednisolone on inflammatory responses in rats. This study has examined the effects of Tualang honey and prednisolone on inflammatory pain and its associated inflammatory responses secondary to formalin injection.

Methods: Twenty-one Sprague-Dawley male rats were randomised into control, Tualang honey (1.2 g/kg) or prednisolone (10 mg/kg) groups. Formalin test was conducted and the rats were sacrificed at four-hours post-formalin injection. Serum was collected for measurement of leukocyte counts and interleukins level. All data were analysed using one-way ANOVA with post-hoc Scheffe's or Dunnet's C test. Significance level was taken as less than 0.05.

Results: Tualang honey and prednisolone groups had significantly reduced pain behaviour and paw edema compared to control group. Tualang honey group demonstrated a significant increase in blood neutrophil count while prednisolone group had significant reduction in blood lymphocyte and monocyte counts compared to control group. Only interleukin-6 level was significantly reduced in honey group. Both interleukin-6 and -8 levels were significantly reduced in prednisolone group.

Conclusions: Tualang honey is comparable to prednisolone in modulating the inflammatory pain responses in rats; however, with regards to local and systemic inflammatory responses, it has differential effects compared to prednisolone.

Keywords: Tualang honey, prednisolone, pain, inflammation, interleukin-6, interleukin-8.

INTRODUCTION

There are several types of Malaysian honey including Acacia, Gelam, Pineapple, Kelulut and Tualang [1]. Tualang honey is a wild multifloral honey which is produced by *Apis dorsata,* and it can be collected from the hives which are built on branches of tall Tualang tree (*Koompassia excelsa*) [2]. Several reports have shown the anti-nociceptive and anti-inflammatory properties of various types of honey including Pakistani honey [3], Yemeni-Sidr honey [4], Manuka honey [5] and Gelam honey [6]. Tualang honey has been shown to modulate the nociceptive responses secondary to formalin injection in the adult male offspring exposed to prenatal stress [7]. Formalin injection may give rise to inflammatory pain and a report has shown that different doses of Tualang honey have differential effects on the inflammatory pain [8].

A well-known anti-inflammatory drug, such as prednisolone, is widely used in treating psoriasis, allergic conditions, respiratory disorders and ulcerative colitis. Several studies have shown its effectiveness in improving pain in rheumatoid arthritis, chronic bladder syndrome and athralgia [9-14]. However, the administration of a higher dose of prednisolone or administration for a long period may lead to various adverse effects [15]. Immunosuppressive effects, muscle weakness, acne, increased skin pigmentation, vague abdominal distress, growth failure and delayed puberty in children are amongst the common complained side effects of prednisolone [16, 17]. In addition, prednisolone is frequently used pre-emptively to prevent inflammatory-related diseases such as asthma. The onset of action of prednisolone is delayed and may take several days before it shows its anti-inflammatory effects. This property is similar to Tualang honey which also has slow onset of action. To date, there is not a single study which compared the effects of Tualang honey with prednisolone in an inflammatory pain model. Hence, this study aimed to compare the effects of Tualang honey and prednisolone on inflammatory pain and its associated inflammatory responses secondary to formalin injection in Sprague-Dawley rats.

MATERIALS AND METHODS

Study Design

This study was approved by Animal Ethics Committee of Universiti Sains Malaysia (USM/Animal

*Address correspondence to this author at the Physiology Department, School of Medical Sciences, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia; Tel: +609-7676168/+6019-9722559;
E-mail: aishahnazariah@yahoo.com

ISSN: 2223-3806 / E-ISSN: 1927-5951/17

Ethics Approval/2010/(63)(266). This study was an experimental study conducted in Physiology Department Laboratory, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia. The rats were given either Tualang honey (1.2 g/kg) [8] or prednisolone (Dynapharm, Malaysia) (10 mg/kg) [18] or distilled water orally for 10 days before the experiment was conducted. The administration of Tualang honey was given from a single batch of honey. The selected dose of Tualang honey (1.2 g/kg) was based on the results by Abd Aziz *et al.* [8]. Tualang honey was supplied by Federal Agricultural Marketing Authority (FAMA), Ministry of Agriculture and Agro-based Industry, Malaysia. Distilled water was used as a vehicle for the preparation of honey and prednisolone, and also served as a control in the study.

Animals

Twenty-one Sprague-Dawley adult male rats (n=7 per group) with the average weight of 250-300 g (8-10 weeks old) were obtained from Animal Research and Service Centre, Health Campus, Universiti Sains Malaysia. They were maintained in a 12-hours dark-light cycle (light phase 07:00 to 14:00) and fed with standard food pellet and water *ad libitum*. All the rats were kept in the individual cages and acclimatised to the environment [20] in the Physiology Department laboratory for 4 days. The experiments were carried out from 08.00 to 16.00.

Formalin Injection

Formalin test was conducted in an observation transparent chamber measuring 26 cm x 20 cm x 20 cm [20] with a mirror placed at 45° beneath the floor to allow unobstructed view of the paw. The rats were individually placed in a transparent chamber for 30 minutes before the test to allow adaptation to the test environment. Each rat was given subcutaneous injection of 1% formalin (1 mL) (HmbG® Chemicals, Germany) at the plantar region of rat's right hind paw. The rat's behaviour was recorded for one hour immediately following the injection using a video camera. The tape was viewed later and the pain behaviour score was given from 0 to 3 [21], depending on the severity of pain shown by the rats. The pain behaviour score was tabulated at each minute and averaged at 5-minutes interval [20].

Assessment of Paw Edema

Each rat was restrained in a plastic cylinder (20 x 30 cm) and its right hind paw was pulled through a hole at the base of cylinder. A caliper (Duratool, China) was used to measure paw diameter (dorsal-plantar axis). Measurement of paw circumference was obtained by looping a thread round the injected paw and gently tightened. The loop was carefully removed and its length was measured to the nearest millimetre with a metric ruler. Both paw diameter and circumference was measured before and 4-hours after formalin injection. The change of paw diameter or circumference was calculated according to the formula below:

Change of paw diameter/circumference = Paw diameter/circumference at 4-hours after formalin injection – paw diameter/circumference before formalin injection

Measurement of Leukocytes Count and Serum Interleukin-6 and -8 Levels

Four hours after formalin injection, laparotomy was conducted under anaesthesia and blood was collected from inferior vena cava. The rats were then sacrificed with overexposure to diethyl ether (HmbG® Chemicals, Germany) [22]. A volume of fresh blood was collected and half a mL of blood was kept in EDTA tube. The neutrophils, lymphocytes and monocytes in the blood were counted using a lymphocyte subset machine (Sysmex, Malaysia). The remaining blood was left at room temperature for two hours and centrifuged to obtain serum for further analysis. The serum was stored at -80ºC before measurement of interleukin levels. Interleukin-6 and -8 were measured in rat serum using ELISA kits (Cusabio, China).

Statistical Analysis

The data were analysed using Statistical Package for Social Sciences (SPSS) version 19. Repeated measured analysis of variance (ANOVA) was used to analyse pain behaviour score in formalin test while a way ANOVA was used to analyse paw diameter and circumference, blood leukocytes count and serum interleukins level. Post-hoc tests applied in the present study relied on the homogeneity of variances assumption. Post-hoc Scheffe's test was employed when the homogeneity of variances was met while post-hoc Dunnet's C test was applied when the homogeneity of variances was not met [23]. The data were presented as mean ± standard error of mean (S.E.M). The result with *P*-value of less than 0.05 was considered as significant.

RESULTS

Formalin Test

The pain behaviour from minute-0 to 10 represented the phase 1 of formalin test. Meanwhile, the pain behaviour from minute-15 to 35 represented the early phase 2 and the pain behaviour from minute-40 to 60 represented the late phase 2 of formalin test [24]. The analysis on pain behaviour score by one-way ANOVA demonstrated significant differences in phase 1 ($F(2,33)=6.413$, $P<0.05$), early phase 2 ($F(2,33)=7.086$, $P<0.05$) and late phase 2 ($F(2,33)=16.699$, $P<0.05$) between all groups.

Prednisolone administration was associated with significant reduction in the pain behaviour score during phase 1 and phase 2 ($P<0.05$) compared to control group by post-hoc Scheffe's test (Figure **1**). Oral administration of Tualang honey was associated with reduction in pain behaviour score during phase 1 ($P<0.05$), late phase 2 compared to control ($P<0.001$) and prednisolone ($P<0.05$) groups. There was a significant difference in pain behaviour score between Tualang honey and prednisolone groups in both early and late Phase 2 ($P<0.05$).

Paw Edema

The analysis with one-way ANOVA revealed significant differences in the changes of paw diameter ($F(2,33)=20.248$, $P<0.001$) and paw circumference ($F(2,33)=48.964$, $P<0.001$) (Table **1**) between the groups. Post hoc Scheffe's test revealed significant reduction in the paw edema in Tualang honey and prednisolone groups compared to control group ($P<0.001$) in both paw diameter and paw circumference. There was no significant difference between Tualang honey and prednisolone groups in both paw diameter and circumference.

Blood Leukocytes Count

One-way ANOVA revealed a significant difference in blood neutrophil count between the groups ($F(2,21)=6.387$, $P<0.05$) (Table **1**). Post hoc Scheffe's test revealed a higher level of neutrophil count in Tualang honey group compared to control and prednisolone groups ($P<0.05$). There was no significant alteration of blood neutrophil count in prednisolone group when compared to control group ($P>0.05$).

There were significant differences in blood lymphocyte ($F(2,22)=41.381$, $P<0.001$) and monocyte ($F(2,21)=13.368$, $P<0.001$) counts (Table **1**) when compared between the groups. Tualang honey group did not demonstrate significant changes on lymphocyte and monocyte counts, however, prednisolone group showed significant reduction in both lymphocyte and monocyte counts in the blood ($P<0.001$ and $P<0.05$, respectively) compared to Tualang honey and control groups.

Serum Interleukin-6 and -8 Levels

The results showed that there were significant differences in serum interleukin-6 ($F(2,18)=11.059$, $P<0.05$) and interleukin-8 levels ($F(2,18)=7.249$, $P<0.05$) (Table **1**) when compared between the groups. The analysis by post-hoc Scheffe's test revealed that

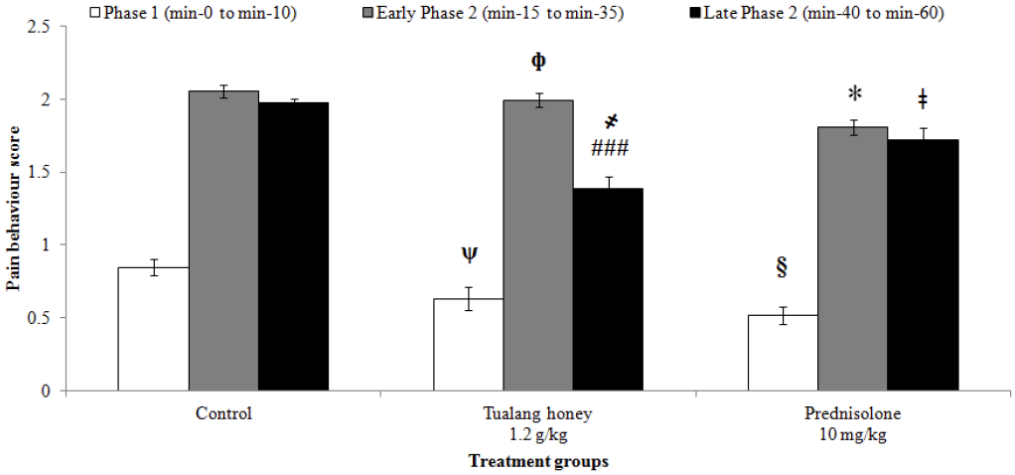

Figure 1: Effects of Tualang honey and prednisolone compared to control on pain behaviour score in each phase of formalin test (mean ± SEM, n=7). Tualang honey group has significantly reduced pain behaviour score during phase 1 ($^{\psi}P<0.05$) and late phase 2 ($^{\#\#\#}P<0.05$) compared to control group. Prednisolone has significantly reduced pain behaviour score during phase 1 ($^{\S}P<0.05$), early phase 2 ($^{*}P<0.05$) and late phase 2 ($^{\ddagger}P<0.05$) compared to control group. There were significant differences between Tualang honey and prednisolone during early phase 2 ($^{\Phi}P<0.05$) and late phase 2 ($^{\neq}P<0.05$).

Table 1 Effects of Tualang Honey and Prednisolone on Changes of Paw Diameter/Circumference, Blood Leukocytes Count and Serum Interleukin Levels (Mean ± SEM, n=7)

Group	Changes of paw measurement (mm)		Blood leukocytes count (10^3 µL)			Serum interleukins level (pg/mL)	
	Diameter	Circumference	Neutrophil	Lymphocyte	Monocyte	IL-6	IL-8
Control	1.54 ± 0.13	3.88 ± 0.36	1.96 ± 0.09	4.61 ± 0.47	0.58 ± 0.05	10.65 ± 0.63	4.33 ± 0.34
Tualang honey 1.2 g/kg	0.82 ± 0.12**	0.94 ± 0.18**	2.98 ± 0.38‡	5.18 ± 0.41	0.62 ± 0.08	6.69 ± 0.55*	3.61 ± 0.49
Prednisolone	0.54 ± 0.09**	0.63 ± 0.19**	1.90 ± 0.15	1.02 ± 0.15§	0.26 ± 0.02‖	7.15 ± 0.76*	2.36 ± 0.23¶

*P<0.05, **P<0.001 Comparison between Tualang honey and prednisolone groups with control group.
‡P<0.05 Comparison between Tualang honey group with control and prednisolone groups.
§P<0.001 Comparison between prednisolone with control and Tualang honey groups.
‖P<0.05 Comparison between prednisolone group with control and Tualang honey groups.
¶P<0.05 Comparison between prednisolone and control group.

the group which received Tualang honey showed significant reduction in serum interleukin-6 level (P<0.05) but showed no significant effect on serum interleukin-8 level compared to control group (Table **1**). Prednisolone administration was associated with significant reduction in both serum interleukins level (P<0.05) compared to control group (Table **1**). There was no significant difference in both interleukins level when compared between between Tualang honey and prednisolone groups.

DISCUSSION

The present study compared between the effects of Tualang honey and prednisolone on pain and inflammatory responses in an acute inflammatory pain model. The injection of formalin on the rat's hindpaw produces inflammation and two phases of pain behavior. Phase 1 pain is partly due to release of histamine, serotonin and kinins that lead to sensitization of peripheral receptors and activation of C-fibres afferents [4, 25, 26].

Meanwhile, phase 2 pain behavior is due to the combined effects of persistent afferent inputs and central sensitisation in the central nervous system [27]. The phase 2 pain is also mediated by the release of kinins and prostaglandins in the spinal cord [26]. The present study showed suppression of phase 1 pain in both Tualang honey and prednisolone groups. It was reported that high total phenolic acid in Tualang honey such as gallic acid [28] was associated with inhibition of histamine release and other pro-inflammatory cytokine production by mast cells in rats [29]. Previous animal and clinical studies have shown that prednisolone suppressed the release of serotonin and histamine during inflammatory conditions [30].

In phase 2 of formalin test, suppression of pain behavior score in prednisolone group was more pronounced in early phase 2 while the effects of Tualang honey were more prominent during late phase 2. Prednisolone might have induced the release of anti-inflammatory product such as annexin-1 which down-regulated spinal prostaglandin E_2 [31] and inhibited nociceptive transmission and led to analgesia in early phase 2. However, prednisolone was not able to suppress the release of kinin in the periphery [32] which was partly responsible for late phase 2 of formalin test. Antioxidants such as catechin [33] and chlorogenic acid [34] found in honey were reported to inhibit kinins and other inflammatory mediators released peripherally during late phase 2 [35] and this mechanism might contribute to the pain suppression in Tualang honey group.

In the present study, both Tualang honey and prednisolone administration was associated with significant suppression of paw edema. Phenolic compounds in honey are reported to reduce paw edema by suppressing polymorphonuclear leukocytes functions, scavenging activities of superoxide anions and suppressing myeloperoxidase release and activity [36]. The phenolic compound in Tualang honey was shown to be higher compared to other Malaysian honey such as Gelam and Borneo honey [33]. Meanwhile, the inhibition of paw edema by prednisolone is due to inhibition of phospholipase A_2 and histamine synthesis in mast cells [37] and via suppression of gene transcription for cyclooxygenase-2, cytokines and iNOS [38].

Oral administration of Tualang honey was associated with significant increase in blood neutrophil count following formalin induced inflammation. The increase of neutrophil in the blood by Tualang honey

was also found by Chepulis [39] and Shaaban *et al.* [40]. Scientists have suggested that sucrose in honey might be responsible for causing the change [41]. The increase in blood neutrophil level in the present study is a beneficial effect of Tualang honey suggesting its role in boosting the immune system in pathological situation in animals [42, 43].

In the present study, administration of Tualang honey was not associated with significant effect on blood lymphocyte and monocyte counts. Previous studies have revealed that honey stimulated the production of monocytes and lymphocytes in human subjects [44, 45]. The difference in their results compared to ours could be contributed by the different duration and method of Tualang honey supplementation, and may be contributed by the difference in the subjects used.

The present study has shown that the blood lymphocyte and monocyte counts were significantly lower in the prednisolone group. Prednisolone inhibited the release of interleukin-1 from macrophage and interleukin-2 from activated T-lymphocyte helper cells [46] and suppressed the release of other lymphokines and granulocyte macrophage colony-stimulating factors [47]. These effects subsequently led to inhibition of activation, production and survival of newly-synthesised lymphocytes [46, 47]. Prednisolone also selectively inhibited $CD14^+$ $CD16^+$ type of monocytes and this effect was probably mediated by intracellular glucocorticoid receptors (GCR) which were found to be highly-expressed in these cells [48]. The higher expression of GCR in $CD14^+CD16^+$ monocytes might have contributed to the increased sensitivity of $CD14^+CD16^+$ monocytes to glucocorticoid-induced cell death [48]. However, in the present study, prednisolone has shown no significant effect on blood neutrophil count. Other studies have shown a suppression in the neutrophil count [49,50]. The difference in the results could be contributed by the difference in the prednisolone doses used and length of prednisolone administration as well as experimental design.

In the inflammatory pain model used in this study, Tualang honey administration was associated with significant reduction in serum interleukin-6 level but there was no significant change in serum interleukin-8 level. Tualang honey was suggested to down-regulate interleukin-6 signaling pathway [51] and suppressed its production [52]. Previous studies have reported that flavonoids in honey such as quercetin [53], chrysin [52], fisetin and rutin [54] inhibited interleukin-8 promoter and gene expression which led to suppression of interleukin-8 [55]. With the dose used in the present study, Tualang honey was associated with the suppression of interleukin-6 but it was not able to suppress interleukin-8 level.

In the present study prednisolone has markedly reduced both interleukin-6 and interleukin-8 levels. Glucocortocoid such as prednisolone suppressed IL-6 and IL-8 productions by inhibiting gene transcription of both cytokines [56]. The direct binding of glucocorticoid or GCR to glucocorticoid responsive elements in the promoter region of the gene may eventually modulate several genes expression involving in inflammatory responses [57-59].

In conclusion, pre-emptive administration of Tualang honey and prednisolone was able to modulate the pain and inflammatory responses induced by formalin injection but the Tualang honey's anti-inflammatory effects are different from those of prednisolone's. The present study provides novel knowledge regarding the potential role of Tualang honey as an anti-inflammatory agent.

ACKNOWLEDGEMENT

The financial assistance for this study was provided by Short Term Grant, Universiti Sains Malaysia (304/PPSP/61311065). We would like to thank all staffs from Physiology, Central Research and Immunology laboratories for their technical support.

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Received on 03-12-2016 Accepted on 09-01-2017 Published on 14-02-2017

DOI: https://doi.org/120.29169/1927-5951.2017.07.01.2