SHORT COMMUNICATIONS

Tualang and Kelulut Honey Reduced Lipopolysaccharides-stimulated Inflammatory Responses of Microglia

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ABSTRACT

Microglial activity is crucial in maintaining the central nervous system (CNS) homeostasis. However, prolonged microglial activation have been implicated in the pathology of neurodegenerative diseases. Activated microglia will increase the production of inflammatory cytokines, reactive oxygen species (ROS) and alter their surface marker expression levels. This study used Malaysian honey, Tualang honey (TH), and Kelulut honey (KH) to determine lipopolysaccharide (LPS)-stimulated inflammatory responses of microglia. TH and KH at 0.1% were used in the current study as our findings showed no significant difference in the cell viability between BV2 cells treated with 0.1 % of TH and KH and control group. TH and KH reduced the ROS level significantly by 41.62±1.06% and 49.16±0.63%, respectively, and slightly reduced the expression of co-stimulatory molecules, CD40 and CD11b in LPS-activated BV2 cells. Our preliminary findings proposed an in-depth future study on the anti-inflammatory effect of TH and KH on microglial activation.
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Keywords: Tualang honey, Kelulut honey, Microglia, Neurodegenerative disease, Inflammation

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INTRODUCTION

Neurodegenerative diseases are characterized by the loss of neuronal population in the CNS. Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) are examples of neurodegenerative diseases. The trends in the incidence and prevalence of these diseases are increasing worldwide. Standard pathological features shared among these neurodegenerative diseases are neuronal cell death, gliosis, atrophy, and pathological protein inclusions. Gliosis is a non-specific change of glial cells in the CNS involving the activation, proliferation, and hypertrophy of glial cells, such as microglia and astrocytes. Since the initial description of microglia by del Rio-hortega in 1932, the role of microglial activation in immune stimulation remains the subject of debate (1).

Although microglial activation is proven to help in preventing brain infection and maintaining brain homeostasis, researchers also report that over-reactive microglia might be crucial in the pathogenesis of neurodegenerative diseases. Therefore, it might be interesting to manipulate microglial activation status from cytotoxic to neuroprotective by drugs, genetic modification, or even natural raw materials to prevent neurodegenerative diseases. Due to various modern neuroprotective drugs' side effects, researchers are now more interested in raw materials with anti-inflammatory properties. The immune-modulatory effect of honey was first introduced by Tonks and colleagues (2). They reported that 1% (v/v) Manuka honey induces the release of tumor necrosis factor-alpha (TNF-α) but also inhibit the production of reactive oxygen species (ROS) in human monocyte *in vitro*.

Besides Manuka honey, tropical honey such as TH and KH started to gain the attention of researchers. TH is produced by *Apis dorsata* (2), while Trigoma species produce KH (3). Several studies have reported the anti-inflammatory effects of these honey on various cancers, such as colon, breast, and cervical cancer, using mouse models (4–6). Besides, KH has been proven to have chemoprotective properties in azoxymethane-induced rats (7), while TH was shown to inhibit inflammation and DNA damage in murine keratinocyte cells by preventing the overproduction of pro-inflammatory cytokines (8). However, no study has explored the effect of TH and KH on microglial activation, and lack of evidence on the potential immunoregulatory effects of these honey by limiting ROS production in microglia. Since microglial activation is correlated to the upregulation of CD40,

CD86, and CD11b surface markers (9–11), this present study is undertaken to explore the anti-inflammatory effect of TH and KH on LPS-activated microglia (BV2 cells) by evaluating the ROS level and expression levels of CD40, CD86, and CD11b surface markers.

MATERIALS AND METHODS

Cell culture and Honey treatment

BV2 cells were a kind gift from Prof. Dr. Sharmili Vidyadaran, University Putra Malaysia (UPM), were cultured in DMEM supplemented with 5% FBS and 1% Penicillin-Streptomycin-Amphotericin B (PSA) and maintained at 37°C with 5% CO2. Raw TH and KH were also provided by Assoc. Prof. Dr. Yong Yoke Keong (UPM) and were originated from Federal Agricultural Marketing Authority (FAMA) and Malaysian Agricultural Research and Development Institute (MARDI), respectively. 20% (v/v) of TH and KH stocks were prepared freshly and diluted to working concentrations of 5, 1, 0.5, 0.1, 0.05, and 0.01% (v/v) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA). 0.1% (v/v) of TH and KH were added simultaneously with 1 µg/mL of LPS (Sigma-Aldrich, German) to cultures 24 hours before evaluating the surface markers profiles. Before evaluating the ROS production level, 0.1% (v/v) of TH and KH were added to cultures for 21 hours before stimulation with 1 µg/mL LPS for 3 hours.

Determination of lowest observed effect level (LOEL)

LOEL of TH and KH were determined using MTT assay. The monolayer BV2 cells were seeded in 96-well plates and left overnight. Cells were stimulated by LPS and different concentrations of TH and KH treatment in triplicates. Cells were treated for 24 hours at 37°C with 5% CO2 under similar conditions for downstream assays. At the end of the incubation period, 20 µL of MTT solution (Sigma-Aldrich, German) was added to each well. The microplate was kept at 37°C for 4 hours and 20 µL of DMSO (Sigma-Aldrich, German) was added into each well. The optical density of the wells was measured at 570 nm using a microplate reader. The dose-response curve was plotted using GraphPad Prism.

Detection of reactive oxygen species (ROS) level

BV2 cells were treated with 0.1% TH and KH for 21 hours before stimulating with 1 µg/mL LPS for 3 hours. The anti-inflammatory property of TH and KH was evaluated by measuring ROS production. ROS production was assayed using a ROS detection kit from Sigma-Aldrich according to the manufacturer's instructions. The ROS production was detected using fluorescence-based and flow cytometry analysis.

Immunophenotyping of microglia

The monolayer BV2 cells were seeded in 6-well plates and left overnight. Cells were stimulated by LPS together with different concentrations of TH and KH treatment. Expression of microglial activation markers CD40, CD11b, and CD86 were assessed by flow cytometry. Briefly, BV2 cells were stimulated by LPS and treated with 0.1% (v/v) TH and KH simultaneously. Cells were harvested after 24 hours, washed with 0.1% BSA/PBS, and fixed with 4% paraformaldehyde. After that, cells were incubated with anti-mouse-CD40, anti-mouse-CD11b, and anti-mouse-CD86 primary antibodies (BioLegend, USA) at a ratio of 1:1000 separately for 45 mins at 4°C. Subsequently, samples were stained with FITC-conjugated goat anti-rat IgG (H+L) secondary antibodies (Invitrogen, USA) for 30 mins at 4 °C. After incubation, cells were resuspended in 0.1 BSA/PBS and further analyzed with BD Accuri C6 Plus flow cytometer. Ten thousand gated events were recorded, and data were analyzed using FCS Express 6 Flow cytometry Software. The gating was determined based on appropriate isotype-stained control.

Statistical analysis

All data were presented as the mean ± SEM of three independent experiments. Student t test and one-way analysis of variance (ANOVA) were used to analyse significant differences, followed by Tukey's post hoc test using GraphPad Prism 9. $P<0.05$ was considered to indicate a statistically significant result.

RESULTS AND DISCUSSION

Lowest observed effect level of TH and KH on BV2 cells

LOEL of TH and KH on BV2 cells was evaluated using an MTT assay. Our findings show LPS (1 µg/mL) did not affect BV2 cell viability. Based on Fig. 1, there was no significant difference in the cell viability between BV2 cells treated with 0.1 % of TH and KH and untreated control in the LOEL experiment. Thus, 0.1 % of TH and KH were selected to be used in downstream assays. In the recent subacute toxicity study of TH and KH, researchers reported that TH at 200 mg/kg/day and KH at 2,000 mg/kg/day are considered safe in vivo (12,13). Thus, suggesting that these honey can be consumed at high doses and highlighting the importance of the medicinal effects of these honey.

TH and KH reduced the ROS production of LPS-activated BV2 cells

BV2 cells treated with 1 µg/mL of LPS significantly increased ($p<0.01$) the ROS production level by 20.79% and 30.67%, detected by fluorescence microplate reader and flow cytometer respectively (Fig. 2A and Fig. 2B). Microglia detect and response to pro-inflammatory triggers by shifting to an activated phenotype to produce cytotoxic factors such as tumor necrosis factor (TNF-α), nitric oxide (NO), and ROS; this explains the surge in ROS production when BV2 cells encountered the membrane components of bacteria, LPS. Overproduction of ROS, such as hydrogen peroxide, superoxide, and hydroxyl radicals, could lead to oxidative stress and might cause severe diseases. In neurodegenerative disease, microglia in the CNS are highly activated and responsible for

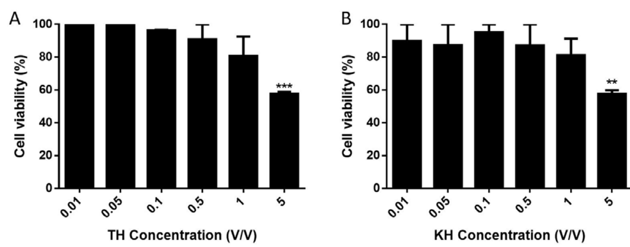


Figure 1: The effects of TH and KH on the cell viability of BV2 cells using MTT assay. BV2 cells were treated for 24 hours with different concentrations of (A) TH and (B) KH [0.01, 0.05, 0.1, 0.5, 1, and 5% (v/v)]. Notes: Data presented as mean ± SEM from three independent experiments. (**p<0.01, ***p<0.001) indicates a significant difference compared to the negative control (BV2 cells without honey treatment).

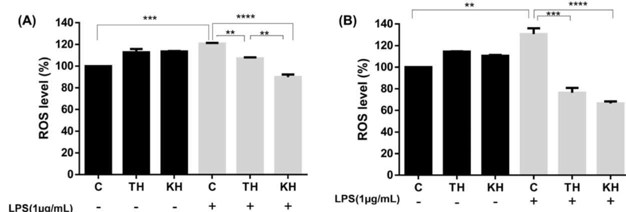


Figure 2: The effect of TH and KH on the reduction of ROS production using (A) fluorescence microplate reader and (B) flow cytometer. BV2 cells were treated with 0.1% TH and KH for 21 hours before stimulating with 1 μg/mL LPS for 3 hours. The control group (C) did not receive any honey treatment (relatively 100% ROS level). Notes: Data presented as mean ± SEM from three independent experiments. C: BV2 cells; TH: BV2 cells treated with TH; KH: BV2 cells treated with KH. **p<0.01, ***p<0.001, ****p<0.01.

the overproduction of ROS in the brain, which is harmful to neurons. Interestingly, as a natural source of various antioxidants, honey was reported to have ROS scavenging properties, and this might reduce the ROS level in the CNS, thus preventing the deterioration of neurodegenerative diseases.

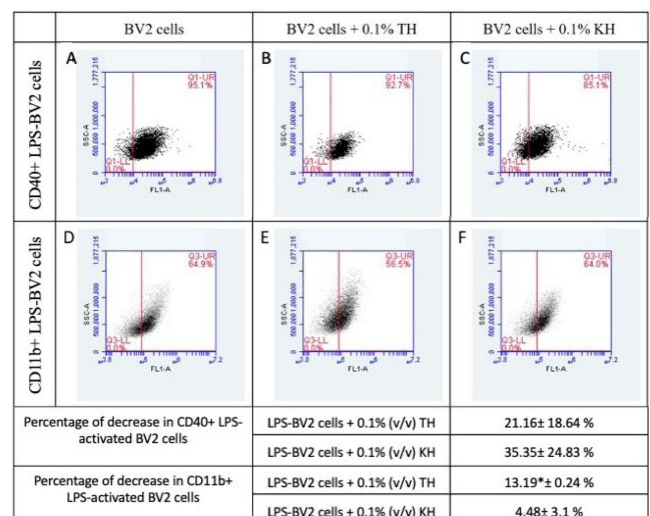
Treatment of 0.1% of TH and KH on unstimulated BV2 cells produced slightly higher amounts of ROS (p>0.05) measured by a fluorescence microplate reader and flow cytometer (Fig. 2A and Fig. 2B) compared to the control group (BV2 cells only). This might be due to stimulants in these honey, which cause minor activation of BV2 cells. This stimulative effect of honey was also previously reported to promote the activation of leukocytes to release cytokines (14).

Treatment of 0.1% TH and KH significantly reduced the ROS production of LPS-activated BV2 cells by 11.13 ± 1.36 % (p<0.01) and 25.59 ± 2.35 (p<0.001), respectively, when the fluorescence intensity was measured by a fluorescence microplate reader (Fig. 2A). To verify this further, ROS production was measured using flow cytometer (Fig. 2B). Data show 0.1 % of TH and KH reduced the ROS production of LPS-activated BV2 cells by 41.62 ± 1.06 % and 49.16 ± 0.63%, respectively (Fig. 2B; p<0.001). Our findings show that 0.1 % TH and KH significantly reduced the ROS accumulated in BV2 cells. This might be due to the high polyphenol content, such as flavonoids in the honey, which were reported to prevent ROS formation by sequencing Fe catalases and peroxidases (15).

Fig. 2 demonstrates that KH treatment leads to a more significant ROS level reduction than TH in LPS-activated BV2 cells in both methods (ROS measured by fluorescence microplate reader and flow cytometer). This can be explained by the high total phenolic content of KH compared to TH (16). Thus, KH might be a better ROS sequencer to reduce ROS production in activated microglia than TH. In this study, we showed that TH and KH can potentially limit microglial ROS production as Manuka and Gelam honey, which have been reported to inhibit other pro-inflammatory mediators such as TNF-α (17).

TH and KH slightly reduce the expression level of inflammatory surface markers

The flow cytometry immunophenotyping assay revealed that TH and KH reduced CD40 expression in LPS-activated BV2 cells by 21.16± 18.64% and 35.35± 24.83 %, respectively (Fig. 3 A-C). However, these results were not statistically significant. Besides, TH and KH also reduced the expression level of CD11b in LPS-activated BV2 cells by 13.19± 0.24% (p<0.05) and 4.48± 3.1%, respectively (Fig. 3 D-F). These results demonstrated that TH and KH could slightly limit the CD40 and CD11b expression levels in LPS-activated BV2 cells. CD40 and CD11b are co-stimulatory molecules highly expressed on antigen-presenting cells (APC), including microglia, especially during their activated state (18). Thus, our



Percentage of decrease in CD40+ LPS-activated BV2 cells	LPS-BV2 cells + 0.1% (v/v) TH	21.16± 18.64 %
	LPS-BV2 cells + 0.1% (v/v) KH	35.35± 24.83 %
Percentage of decrease in CD11b+ LPS-activated BV2 cells	LPS-BV2 cells + 0.1% (v/v) TH	13.19*± 0.24 %
	LPS-BV2 cells + 0.1% (v/v) KH	4.48± 3.1 %

Figure 3: The effect of TH and KH on the reduction of activation makers: CD40 and CD11b expression level in LPS-activated BV2 cells via immunophenotyping assay using flow cytometry. LPS-activated BV2 cells were treated with TH and KH for 24 hours. Numbers within the upper right region of plots indicate the percentage of $CD40^+$ and $CD11b^+$ LPS-BV2 cells. Results are representative of 3 independent experiments.

results indicate that TH and KH might potentially prevent microglial activation. However, the expression level of CD86 in LPS-activated BV2 cells remains similar after treatment with TH and KH (data not shown).

These results revealed that TH and KH could limit the expression level of CD40 and CD11b but not CD86 in LPS-activated BV2 cells. Since honey is rich in flavonoids, these active compounds in TH and KH may contribute to reducing the expression level of inflammatory surface markers in this study. This is supported by Rezai-Zadeh and his colleagues (19) who proved that flavonoids such as apigenin and luteolin could affect STAT1 signaling and the CD40 receptor's gene transcription. Furthermore, catechin found in various honey was proven to inhibit adhesion and migration of B cells by suppressing the expression of CD11b (20). Due to the limited reduction in $CD40^+$ and $CD11b^+$ of LPS-stimulated BV2 after TH and KH treatments, the speculation thereby these honey could regulate the inflammatory state of activated microglia cells is still inconclusive. Further work is underway to assess the expression of other surface molecules, such as MHC receptors, and the production of pro-inflammatory cytokines such as TNF-α, interleukins (IL)-1, and reactive nitrogen species (RNS).

CONCLUSION

Our results provide insight into the role of TH and KH in modulating microglial responses. TH and KH reduced the ROS level significantly and slightly reduced the expression of CD11b in LPS-activated BV2 cells. Future studies need to be done to elucidate the use of Malaysian honey as a complementary treatment option for neurodegenerative diseases.

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