# Dietary Hesperidin Suppresses Lipopolysaccharide-Induced Inflammation in Male Mice

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**ABSTRACT:** Depressive disorders are partially attributed to chronic inflammation associated with the tryptophan (Trp)-kynurenine (Kyn) pathway. Recent evidence suggests that anti-inflammatory agents may reduce the risk of depression. The present study aimed to elucidate the potential of the citrus flavonoid hesperidin, which exhibits anti-inflammatory activity, in suppressing the Trp-Kyn pathway in the brain, using a lipopolysaccharide (LPS)-induced inflammation mouse model. Dietary hesperidin was found to suppress activation of the Trp-Kyn pathway in the prefrontal cortex. In addition, it reduced systemic LPS-induced signs of illness, such as low skin temperature and enhanced leukocyte count in the blood. However, dietary supplementation with hesperidin did not improve body weight loss, food intake, water intake, or splenic increases in leukocyte numbers in the LPS model. Collectively, the results suggest that dietary hesperidin can partially regulate central and peripheral events linked to inflammation in LPS mouse models.

**KEYWORDS:** Hesperidin, kynurenine, skin temperature, leukocyte, inflammation, depression, mouse, lipopolysaccharide

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## Introduction

Depression is a common illness that currently affects more than 264 million people worldwide.[1] Recent studies have suggested that inflammation of the central nervous system (CNS) and peripheral tissues plays a critical role in the development of depression.[2] Clinical studies have found that levels of inflammatory cytokines are abnormally elevated in the peripheral blood and cerebrospinal fluid of patients with depression.[3] Meta-analyses have suggested that antidepressant drug treatment may decrease peripheral levels of inflammatory cytokines.[4] Similar results have been noted in animal studies; abnormal increases in inflammatory cytokine levels have been observed in chronic stress models of depression.[5,6] Recently, the relationship between the kynurenine (Kyn) pathway and depression has attracted research attention.[7] In addition, indoleamine 2,3-dioxygenase (IDO), an enzyme involved in Trp metabolism, has been reported as a key factor linking inflammation with depression.[8,9] Therefore, in this study, we focused on the Trp-Kyn pathway in the brain to investigate the associations between inflammation and depression.

We performed screening tests on foods and crops that demonstrated anti-inflammatory activity. In our previous study, we found that dietary hesperidin suppresses Kyn levels in the hippocampus and prefrontal cortex, and inhibits depression-like behavior in subchronic and mild social defeat stress (sCSDS) models of depression.[10] Hesperidin is a flavanone glycoside (a subclass of flavonoids) that is abundant in citrus fruits.[11] It has many health benefits, including anti-inflammatory, antioxidant, anti-obesity, anti-anxiety, and neuroprotective effects.[11] Furthermore, it has been reported to show antidepressant-like effects in an acute depression rodent model.[12-15] However, there is a lack of information regarding the central effects of dietary hesperidin on the Trp-Kyn pathway under conditions of stress-induced inflammation. The inflammatory cytokine inducer, lipopolysaccharide (LPS), is commonly used to produce animal models of inflammation and depression.[16] Therefore, we utilized a LPS-induced inflammation mouse model to investigate novel functions of hesperidin on the central Trp-Kyn pathway and other health deficits.

This study aimed to examine whether dietary intake of hesperidin affected Kyn levels in the hippocampus and prefrontal cortex in an LPS-induced inflammation model. In addition, we analyzed the skin temperature, number of leukocytes in the blood and spleen, and protein levels of IDO and tryptophan 2,3-dioxygenase (TDO) in the hippocampus and prefrontal cortex of the model.

## Materials and Methods

### *Animals*

All experimental procedures involving animals were reviewed and approved by the Animal Care and Use Committee of

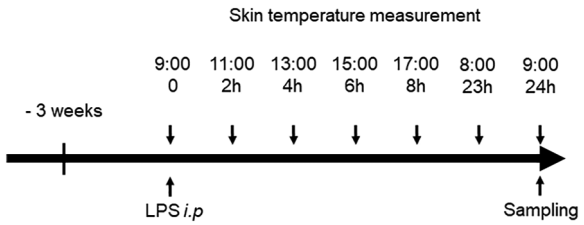


**Figure 1.** Experimental design of lipopolysaccharide (LPS) exposure and sampling.

Ibaraki University (approval no. 19230, Ibaraki, Japan), and conformed to the guidelines of the Ministry of Education, Culture, Sports, Science and Technology, Japan (Notification, No. 71).

Male C57BL/6JJcl (B6) mice (7 weeks old, weighing 21-23 g) were purchased from CLEA Japan (Tokyo, Japan). Feed and water were provided ad libitum. Animals were maintained at constant temperatures (23°C ± 1°C) under a 12-hour light-dark cycle (lights on at 7:00 am). For acclimation, all mice were housed individually under the above conditions for 1 week prior to the experiments.

### *Experimental design*

The experimental design is illustrated in Figure 1. B6 mice (n = 15) were divided into 3 bodyweight-matched groups, namely, the control group, which was fed semi-purified diet powder (AIN-93G diet, Oriental Yeast, Tokyo, Japan) without LPS injection (n = 5), the LPS group, which was fed AIN-93G and subjected to LPS injection (n = 5), and the Hes + LPS group, which was fed AIN-93G containing 0.1% hesperidin (Sigma-Aldrich, St. Louis, MO, USA) and subjected to LPS injection (n = 5). The mice were initially fed their respective diets for 3 weeks before intraperitoneal LPS injection. LPS from *Escherichia coli* O55:B5 (Sigma-Aldrich) was used, as described previously.[17] LPS was fully solubilized in phosphate-buffered saline (PBS), and was administered (0.54 mg/kg) by intraperitoneal injection at a volume of 10 mL/kg bodyweight; the vehicle (VEH) treatment included only PBS.

### *Trp and Kyn measurements*

Brain tissues were homogenized at an approximately 1:5 wet weight-to-volume ratio in 0.1 mol/L HCl. A 100-μL aliquot of the homogenate was acidified with 20 μL of 30% trichloroacetic acid. After centrifugation (15 minutes, 15 000×*g*, 4°C), an aliquot of the supernatant was used for high-performance liquid chromatography (HPLC) of Trp and Kyn. To determine plasma Kyn levels, 100 μL of plasma was acidified with 20 μL of 30% trichloroacetic acid. After centrifugation (15 minutes, 15 000×*g*, 4°C), an aliquot of the supernatant was used to evaluate the Trp and Kyn levels. L-Trp (M5M3023; Nacalai Tesque, Kyoto, Japan) and L-Kyn (BCBH7844V; Sigma-Aldrich) were used as Trp and Kyn standards, respectively. The concentrations of plasma Kyn were determined by HPLC using a diode array detector (SPD-M20A, Shimadzu, Kyoto, Japan) at a wavelength of 365 nm. Free Trp levels in the brain and plasma were evaluated by HPLC using a diode array detector (SPD-M20A, Shimadzu) at a wavelength of 280 nm.

### *Skin temperature measurement*

We measured the skin temperature of mice using thermography (FLIR i5, Flir Systems, Boston, MA, USA) to evaluate the effects of LPS (Figure 1). Skin temperature was measured every 2 hours during the light period (9:00 am-7:00 pm); it was subsequently assessed after a dark period (8:00 am on day 2), approximately 23 hours after starting the treatments.

### *Leukocyte isolation*

Leukocytes were isolated from 200 μL of whole blood using Ficoll-Paque PLUS (GE Healthcare Bioscience, Piscataway, NJ, USA). A sample of cells was stained with trypan blue (Sigma-Aldrich) to assess their viability, and counted with a hemocytometer (Sunlead Glass Corp, Saitama, Japan). Spleen leukocytes were isolated via mechanical dissociation.[18] They were incubated in ammonium-chloride-potassium (ACK) buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, and 100 mM EDTA) and then counted using a hemocytometer.

### *Protein preparation and western blotting*

Mice brains were rapidly sampled after euthanasia and then chilled on ice; thereafter, the hippocampus and prefrontal cortex were removed. Tissues were homogenized in ice-cold buffer using a Polytron homogenizer (IKA Japan, Osaka, Japan). The homogenate was centrifuged at 800×*g* for 15 minutes at 4°C and the supernatant was eventually collected. Proteins were detected using ECL Prime Western blotting Detection Reagents (GE Healthcare Bioscience) and a Fusion SL4 imaging system (Vilber Lourmat, Marne La Vallee, France). The following primary antibodies were used: anti-actin (1:1000; sc-1616; Santa Cruz Biotechnology, Dallas, Texas, USA), anti-IDO1 (1:1000; 12816; Santa Cruz Biotechnology), and anti-TDO2 (1:1000; 15880-1-AP; Proteintech Group, Chicago, IL, USA). Western blots were analyzed quantitatively using ImageJ software.

### *Statistical analysis*

Statistical analyses of all data were performed using one-way analysis of variance (ANOVA), followed by a Tukey–Kramer post hoc test for comparison between groups. Results with $P < .05$ were considered statistically significant. Results with $P < .1$ were considered marginally significant. Data were analyzed using Excel Toukei 2006 for Windows (Social Survey Research Information Co. Ltd., Tokyo, Japan).

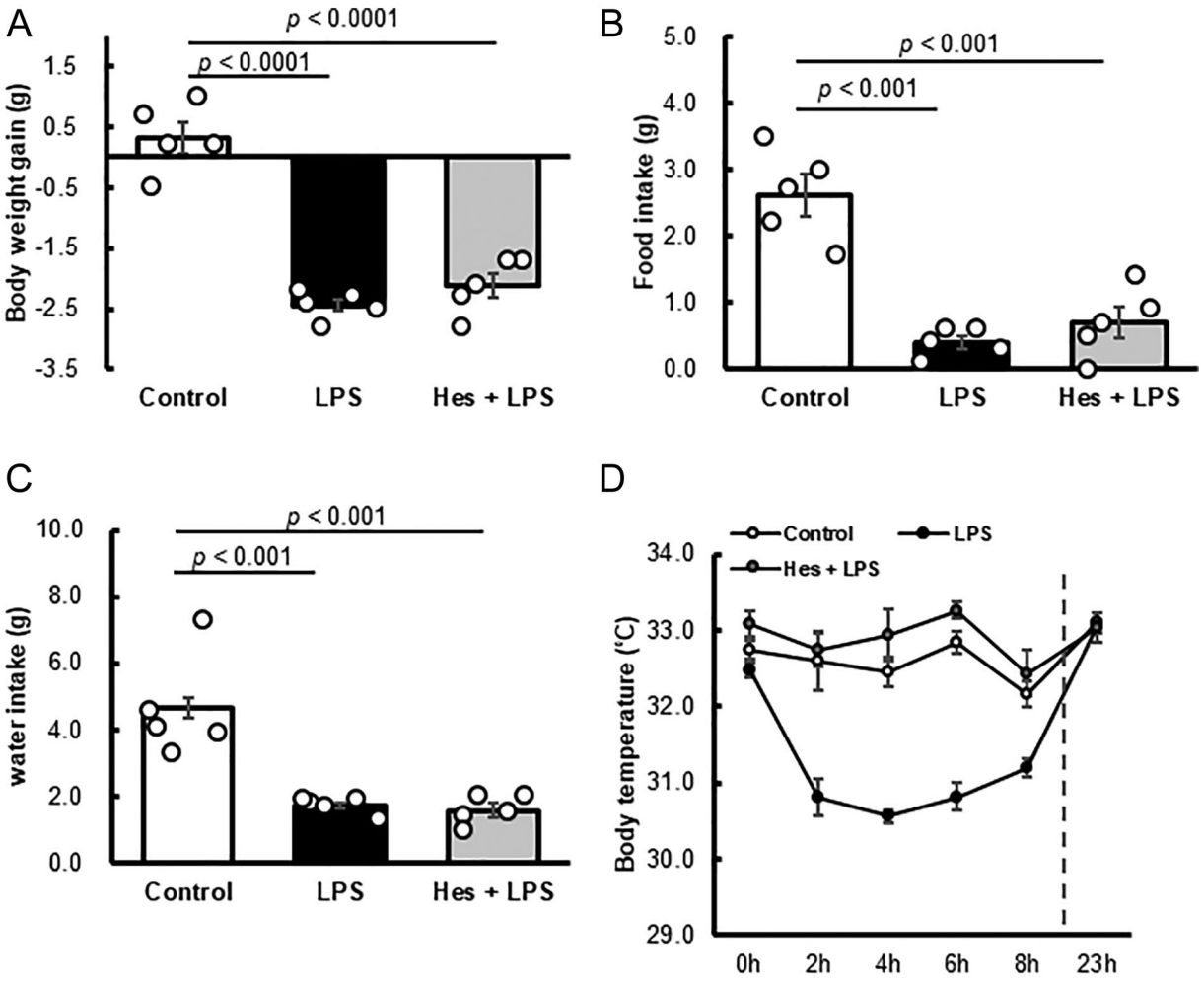


**Figure 2.** Effects of hesperidin on the general physiological features of mice treated with lipopolysaccharides (LPS). Changes in bodyweight gain (A), food intake (B), water intake (C), and skin temperature (D) in mice in the control (Control group; n = 5), LPS (LPS group; n = 5), and hesperidin with LPS (Hes + LPS group; n = 5) groups are shown. Control: AIN-fed mice without LPS injection; LPS: AIN-fed mice with LPS injection; Hes + LPS: AIN containing 0.1% hesperidin-fed mice with LPS injection. Data are presented as mean ± SEM.

## Results

### *Body weight, food intake, and water intake during the 24 hours period following LPS treatment*

Results regarding body weight, food intake, and water intake are shown in Figure 2 and Table S1. There was a significant difference in body weight gain across the groups, with the LPS and Hes + LPS groups showing significantly lower weight gain than the control group (Figure 2A, Table S1). Furthermore, there were differences in food and water intake across the groups, with the LPS and Hes + LPS groups showing lower intake than the control group (Figure 2B and C, Table S1).

### *Trp, Kyn, and Kyn-to-Trp (Kyn/Trp) ratio in the brain*

Trp and Kyn levels and Kyn/Trp ratios in the hippocampus and prefrontal cortex are shown in Figure 3. There was a significant difference in Trp levels in the hippocampus between groups (Figure 3A and Table S2), with levels being significantly lower in the LPS and Hes + LPS groups than in the control group. There was also a significant difference in Kyn levels in the hippocampus (Figure 3B and Table S2), with levels in the LPS and Hes + LPS groups being significantly higher than those in the control group. Accordingly, there was a significant difference in Kyn/Trp ratios in the hippocampus (Figure 3C and Table S2), with ratios of the LPS and Hes + LPS groups being significantly higher than those of the control group.

In contrast, there was no significant difference in Trp levels in the prefrontal cortex across the groups (Figure 3D and Table S2). However, there was a significant difference in Kyn levels in the prefrontal cortex (Figure 3E and Table S2), with levels in the LPS and Hes + LPS groups being significantly higher than those in the control group. Although the Kyn levels in the Hes + LPS group were lower than those in the LPS group, the differences were not statistically significant. There was a significant difference in Kyn/Trp ratios (Figure 3F and Table S2), with ratios in the LPS group being significantly higher than

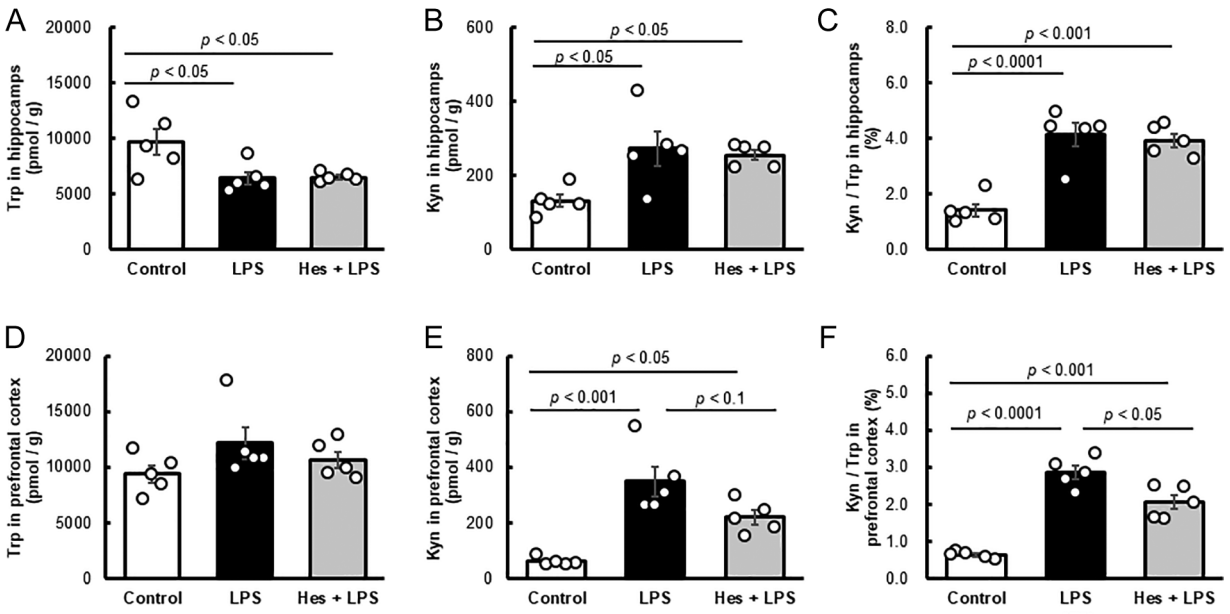


**Figure 3.** Effects of hesperidin on tryptophan (Trp) and kynurenine (Kyn) levels, and Kyn/Trp ratios in the hippocampus and prefrontal cortex of mice treated with lipopolysaccharide (LPS). The hippocampus levels of Kyn (A), Trp (B), and Kyn/Trp (C) and the prefrontal cortex levels of Kyn (D), Trp (E), and Kyn/Trp (F), in mice in the control (n = 5), LPS (n = 5), and hesperidin with LPS (Hes + LPS; n = 5) groups are shown. Control: AIN-fed mice without LPS injection; LPS: AIN-fed mice with LPS injection; Hes + LPS: AIN containing 0.1% hesperidin-fed mice with LPS injection. Data are presented as mean ± SEM.

those in the control and Hes + LPS groups. The Kyn pathway in the prefrontal cortex was significantly suppressed by dietary hesperidin under LPS-induced inflammation.

### *Skin temperature*

Changes in skin temperature over time are shown in Figure 2D and Table 1. There was a significant difference in skin temperature across the groups at 2, 4, and 6 hours after LPS administration. On the other hand, there was no significant difference in skin temperature across the groups after 8 and 23 hours of LPS administration.

### *Blood and spleen leukocytes and spleen weight*

Blood and spleen leukocyte counts and spleen weight are shown in Figure 4 and Table S3. There was a significant difference in the number of leukocytes across the groups in both the blood (Figure 4A, Table S3) and spleen (Figure 4B, Table S3). LPS-induced increases in the number of blood leukocytes could be suppressed by dietary hesperidin.

There was a significant difference in spleen weight (% body weight) across the groups (Table S3). LPS-induced increases in the number of spleen leukocytes and spleen weight could not be suppressed by dietary hesperidin.

**Table 1.** Summary of statistical analysis for skin temperature.

| TIME AFTER LPS ADMINISTRATION (H) | CONTROL (°C) | LPS (°C) | LPS + HES (°C) | ONE-WAY ANOVA | | TUKEY–KRAMER POST HOC TEST | | |
|---|---|---|---|---|---|---|---|---|
| | | | | *F* VALUE | *P* VALUE | CONTROL VS LPS | CONTROL VS HES + LPS | LPS VS HES + LPS |
| 2 | 32.6 ± 0.4 | 30.8 ± 0.2 | 32.7 ± 0.2 | $F_{2,14}$ = 5.549 | *P* = .0197 | *P* = .0247 | *P* = .9148 | *P* = .0505 |
| 4 | 32.5 ± 0.2 | 30.6 ± 0.1 | 32.9 ± 0.3 | $F_{2,14}$ = 9.031 | *P* = .0040 | *P* = .0109 | *P* = .9516 | *P* = .0064 |
| 6 | 32.8 ± 0.1 | 30.8 ± 0.2 | 33.3 ± 0.1 | $F_{2,14}$ = 5.408 | *P* < .0001 | *P* = .0003 | *P* = .9557 | *P* = .0002 |
| 8 | 32.2 ± 0.2 | 31.2 ± 0.1 | 32.4 ± 0.3 | $F_{2,14}$ = 3.263 | *P* = .0739 | *P* = .0936 | *P* = .9788 | *P* = .1301 |
| 23 | 33.1 ± 0.1 | 33.1 ± 0.0 | 33.0 ± 0.2 | $F_{2,14}$ = 2.252 | *P* = .1477 | *P* = .7435 | *P* = .3960 | *P* = .1330 |

One-way ANOVA followed by Tukey–Kramer post hoc test (control vs LPS, control vs Hes + LPS, LPS vs Hes + LPS).

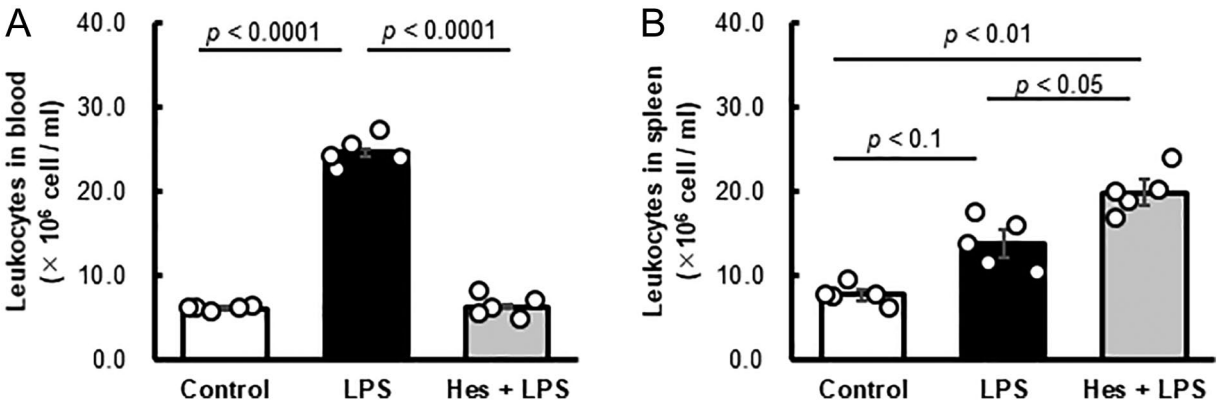


**Figure 4.** Effects of hesperidin on blood and spleen leukocytes in mice treated with lipopolysaccharide (LPS). Changes in blood leukocytes (A) and spleen leukocytes (B) in mice in the control (n=5), LPS (n=5) and hesperidin with LPS (Hes + LPS; n=5) groups are shown. Control: AIN-fed mice without LPS injection; LPS: AIN-fed mice with LPS injection; Hes + LPS: AIN containing 0.1% hesperidin-fed mice with LPS injection. Data are presented as mean ± SEM.

*IDO and TDO protein levels*

IDO and TDO protein levels in the hippocampus and prefrontal cortex in the LPS model are shown in Figure 5 and Table S4. There was no significant difference in IDO or TDO (Figure 5A and Table S4) protein levels in the hippocampus across the groups. Similarly, there was no significant difference in IDO or TDO (Figure 5B and Table S4) protein levels in the prefrontal cortex across the groups.

## Discussion

In this study, we focused on the effect of the citrus flavonoid hesperidin on mouse models of LPS-induced inflammation, focusing on effects on the Kyn pathway in the CNS.

Dietary supplementation with hesperidin suppressed LPS-induced increases in Kyn/Trp ratio in the prefrontal cortex (Figure 3F). However, our results did not show an increase in IDO and TDO protein levels in the prefrontal cortex or hippocampus of the LPS model (Figure 5). According to O'Connor et al,[19] LPS mice models showed increased expression of IDO mRNA in the brain. Numerous mechanisms by which LPS influences brain Kyn activation have been proposed. For example, increased production of cytokines such as TNF-α, IL-6, and interferon-γ (IFN-γ) may activate brain microglia and IDO.[20,21] LPS increases Kyn levels in the blood by activating peripheral inflammatory cytokines and IDO in the liver and lungs.[20,21] In addition, excess Kyn in the blood can cross the blood–brain barrier (BBB) and accumulate in the brain.[22] In the present study, the LPS mouse models were produced using relatively low doses of LPS and thus might not have exhibited upregulated brain IDO levels. Our data suggest that hesperidin may reduce Kyn/Trp ratios in the prefrontal cortex by

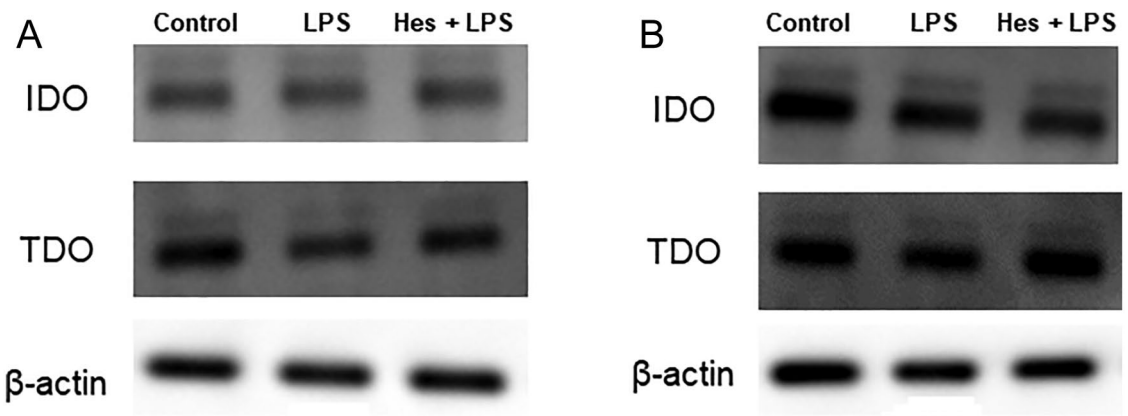


**Figure 5.** Effects of hesperidin on indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) protein levels in the hippocampus and prefrontal cortex. Relative protein expression of IDO and TDO in the hippocampus (A). Relative protein expression of IDO and TDO in the prefrontal cortex (B). Control: AIN-fed mice without LPS injection; LPS: AIN-fed mice with LPS injection; Hes + LPS: AIN containing 0.1% hesperidin-fed mice with LPS injection.

suppressing LPS-induced CNS and peripheral inflammation. The Kyn pathway in the CNS is fully present in macrophages and microglial cells and partially in astrocytes.[23] In addition, Kyn pathway activity in the CNS arises via activation of IDO by inflammatory cytokines, such as IL-2, IL-6, TNF-α, and IFN-γ.[20] Hesperidin prevents microglial activation (Iba-1-positive cells) induced by chronic unpredictable mild stress.[24] Hesperetin, the aglycone portion of hesperidin, also suppresses microglial activity and TNF-α and IL-1β levels in the hippocampus and prefrontal cortex in the LPS model.[25] Intraperitoneal administration of hesperidin increases the expression level of miRNA-132, which suppresses the expression of inflammatory cytokines such as IL-6 and TFN-α in the prefrontal cortex in the LPS model.[26] However, according to André et al[22] lung IDO in the LPS mouse model is activated by leukocyte recruitment in the lungs, increasing Kyn levels in the blood. Hesperidin inhibited recruitment of inflammatory cells such as monocytes and T cell subpopulations as well as lung inflammatory responses in the LPS model.[27] In addition, dietary hesperidin suppressed increases in leukocyte count in the blood of the LPS model (Figure 4A). Hesperidin may lower Kyn/Trp ratios in the prefrontal cortex by suppressing peripheral and CNS inflammation.

Dietary supplementation with hesperidin inhibited decreased skin temperatures seen in the LPS model (Figure 2D). The LPS model showed increased leukocytes that mainly consist of neutrophils, via the activity of secretin and chemokines.[28,29] According to Martino et al[30] excessive increases in leukocytes cause decreased body temperature by transporting leukocytes to the liver, kidneys, and lungs. Hesperidin may prevent decreased body temperatures by affecting the transmigration of leukocytes across blood vessels. Dietary supplementation with hesperidin inhibited the increase in blood leukocytes in the LPS model (Figure 4A). In contrast, dietary hesperidin increased the leukocyte count in the spleen of the LPS model (Figure 4B). Ruiz-Iglesias et al[31] reported similar results after dietary supplementation with hesperidin, and observed suppressed leukocyte numbers in the blood and increased natural killer cells in the spleen in exercise-induced oxidative-stress rat models. Hesperidin has been proposed to suppress leukocyte accumulation in the lungs by suppressing IL-8, which is associated with leukocyte transport.[32]

This study has several limitations. First, we used only male mice in the study. In general, depression is a more prevalent health problem in females; therefore, sex-specific hesperidin activity against brain inflammation should be examined. Second, we did not evaluate the effects of dietary hesperidin on depression-like behaviors in the LPS model mice. Third, we have not investigated the mechanism by which hesperidin decreases brain Kyn levels in the inflammation model. In the future, we aim to elucidate the mechanisms underlying the action of hesperidin on the Kyn pathway in the brain.

Overall, the current study demonstrates that dietary hesperidin suppresses the Kyn pathway in the prefrontal cortex of LPS-induced inflammation model. In addition, hesperidin reduced systemic LPS-induced signs of sickness, such as decreased skin temperature and excessive leukocyte counts in the blood. Dietary hesperidin shows potential in inhibiting LPS-induced central and peripheral inflammation. These results indicate the critical roles of anti-inflammatory substances in modulating inflammatory pathway mechanisms involved in various chronic diseases, including cancer and neurodegenerative and psychological disorders.[33]

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## Author Contributions

A. Toyoda and M. Sato designed the study. M. Sato, A. Okuno, K. Ishisono, and Y. Yajima performed the experiments. A. Toyoda and M. Sato analyzed the data. A. Toyoda and M. Sato wrote the manuscript.

## Supplemental Material

Supplemental material for this article is available online.

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