Investigating anti-neuroinflammatory mechanism of orientin in lipopolysaccharide-induced BV2 microglia cells

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Abstract. Chronic neuroinflammation in central nervous system (CNS) can lead to neurodegenerative diseases (ND). This was due to the over-activated microglia, which releases excessive pro-inflammatory mediators. The molecular mechanisms of orientin as anti-neuroinflammatory are yet to be fully elucidated. In order to investigate the effect of orientin on LPS-stimulated BV2 microglial cells, the cells were pre-treated with orientin at maximum non-toxic dose (MNTD) (15 μ M) or half MNTD ($1/2$ MNTD) (7.5 μ M) for 3 hours, followed by incubation with 0.1 μ g/mL of LPS for 24 hours. The LPS-stimulated cells were then subjected to three series of studies, including the determination of ROS level using 2',7'-dichlorofluoresceindiacetate (DCFH-DA) methods and the determination of mRNA of nuclear factor (NF)- κ B, Signal transducer and activator of transcription 1 (STAT1), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and heme oxygenase-1 (HO-1) via real-time PCR (qPCR). The findings from this study demonstrated the probable mechanism of orientin in treating neuroinflammation via the downregulation of ROS level, STAT1, NF- κ B, iNOS and COX-2 whilst upregulating HO-1. Validation of molecular mechanism of orientin suggested that it could be a potential therapeutic agent in treating ND.

Keywords: anti-neuroinflammatory, microglia, neurodegenerative diseases, neuroinflammation, orientin

INTRODUCTION

Neurodegenerative diseases (ND), such as Alzheimer's disease (AD), Parkinson's diseases (PD), Huntington disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, etc. are diseases characterized by progressive damage in the nerve cells. The deterioration will gradually result in the loss of motor and also cognitive functions such as memory and decision making (Chen *et al.*, 2016). According to National Institute of Health (NIH), ND affect millions of people worldwide, with AD and PD as the most

common types (Katzman, 2008). It was reported that there are more than 5 million American living with AD, while roughly 500,000 living with PD (Katzman, 2008). The cases were expected to increase from 13.5 million in 2000 to 36.7 million in 2050 (Katzman, 2008). Even though the etiologies of ND remain unclear, recent studies indicated that neuroinflammatory processes are closely related to several neurodegenerative pathways leading to ND (Chen *et al.*, 2016; Frank-Cannon *et al.*, 2009; Streit *et al.*, 2004).

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Microglia cells are said to be the key cellular mediators of neuroinflammatory processes, which result in ND (Frank-Cannon *et al.*, 2009, Streit *et al.*, 2004; Rojo *et al.*, 2008; Rogers *et al.*, 2007). Studies suggested that when there are stimulus such as lipopolysaccharide (LPS), hypoxia, trauma or neurotoxins acting on microglia cells, it will initiate the neuroinflammatory response in central nervous system (CNS) by releasing pro-inflammatory cytokines, such as tumour necrosis factor- α (TNF- α), interleukin-1 α (IL-1 α), and IL-6 (Chen *et al.*, 2016, Frank-Cannon *et al.*, 2009; Nakajima & Kohsaka, 2001). The release of these pro-inflammatory cytokines will eventually lead to the activation of transcription factors, such as nuclear factor kappa-B (NF- κ B) and signal transducer and activator of transcription 1 (STAT1) (Lawrence, 2009), with subsequent upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in order to get rid of the intruders and tissue damage, hence promoting the neuronal survival (Chen *et al.*, 2016; Frank-Cannon *et al.*, 2009; Nakajima & Kohsaka, 2001; Hsieh & Yang, 2013). In short, the transmembrane receptor in CNS will firstly recognize the inflammatory stimulus followed by the transmission of the signal to the nucleus for pro-inflammatory gene activations (Ahmed *et al.*, 2015). This activation of pro-inflammatory genes is then controlled by the selective binding of transcription factors to the promoters of these genes (Ahmed *et al.*, 2015).

However, when the cells are left untreated, it will eventually result in sustained activation of the microglia cells, causing uncontrolled release of pro-inflammatory factors that lead to continuous activation of NF- κ B and STAT1, which then result in high expression of iNOS and COX-2 and eventually contribute to chronic neuroinflammation. In addition, over-activated microglia cells may also produce excessive amounts of reactive oxygen species (ROS) leading to oxidative stress (Hsieh & Yang, 2013; Yune *et al.*, 2004; Massa *et al.*, 2006). As a normal feedback mechanism, this oxidative stress will trigger the upregulation of heme oxygenase-1 (HO-1), regulating the imbalance ROS levels, hence, exerting an anti-oxidative and anti-neuroinflammatory effects (Hsieh *et al.*, 2013; Chau, 2015). In short, the uncontrolled release of

pro-inflammatory factors and the oxidative stress will lead to chronic neuroinflammation, causing cumulative neuronal dysfunction, neuronal death and eventually neurodegenerative disorders (Luu & Block, 2010). Therefore, targeting the microglia cells could be one of the potential therapeutic approaches in treating ND.

The current anti-inflammatory agents are nonsteroidal agents (NSAIDs), such as indomethacin and rofecoxib, which are shown to have anti-neuroinflammatory effects (Kulkarni *et al.*, 2005; Ajmone-Cat *et al.*, 2010). However, these drugs were known to cause adverse effects in long term treatment (Kulkarni *et al.*, 2005). For instance, Kanatani *et al.* suggested that indomethacin is associated with the damage of gastric mucosal (Kanatani *et al.*, 2004). Besides, rofecoxib was shown to possess cardiotoxicity that will lead to heart failure (Mamdani *et al.*, 2004). Due to all these adverse effects of NSAIDs, researchers started to divert their focus on some active constituents derived from medicinal plants, particularly the flavonoids. For example, studies have shown that flavonoid quercitrin extracted from *Juglans mandshurica* could be a potent anti-neuroinflammatory agent (Kulkarni *et al.*, 2005). Besides, flavonoid such as cudraflavanone D and paeonol have shown their anti-neuroinflammatory responses in activated microglia cells through the inhibition of nitric oxide (NO), ROS production and the expression of NF- κ B, iNOS and COX-2 (Lin *et al.*, 2015; Himaya *et al.*, 2012; Kim *et al.*, 2016), whereas curcumin was found to induce the HO-1 expression in microglia cells, providing an anti-oxidative effects (Parada *et al.*, 2015). In addition, both oroxylin A and luteolin have also shown to suppress the expression of NF- κ B and STAT1 in LPS-induced BV2 microglia cells, which resulted in its anti-neuroinflammatory effects (Liu *et al.*, 2012; Kao *et al.*, 2011).

In this study, orientin was chosen among all the flavonoids. Orientin is a water-soluble *c*-glycoside flavonoids that can be found or isolated from various medicinal plants such as *Ocimum sanctum* (holy basil), *Phyllostachys* species (bamboo leaves), *Passiflora* species (passion flowers), *Trollius* species (Golden Queen), and *Jatropha gossypifolia* (Bellyache Bush) (Lam *et al.*, 2016). Orientin are widely reported to have beneficial properties such as anti-oxidative (An *et al.*, 2012), anti-thrombotic

and anti-platelet (Lee & Bae, 2015), anti-viral and anti-bacterial (Li *et al.*, 2004), anti-inflammation (Bae, 2015), vasodilation (Fu *et al.*, 2005), neuroprotective (Law *et al.*, 2014), radioprotective (Nayak *et al.*, 2006), anti-adipogenesis (Kim *et al.*, 2010) and antinociceptive (Da Silva *et al.*, 2010), anti-inflammatory and anti-oxidative properties (An *et al.*, 2012; Bae, 2015; Kim *et al.*, 2010; Yu *et al.*, 2015; Seo *et al.*, 2012) that has stimulated the interest to further study this compound in the present study. At the molecular level, Yu *et al.* showed that orientin could alleviate oxidative stress in β -amyloid ($A\beta$)-mediated mouse model with Alzheimer's disease through the activation of Nrf2/HO-1 signaling (Seo *et al.*, 2012). The activation of this signaling pathway induced the translocation of Nrf2, which then upregulated the expression of HO-1, activating the redox signaling pathway by exerting an anti-oxidative property (Seo *et al.*, 2012). In addition, Seo *et al.* suggested that orientin isolated from *Spirodela polyrrhiza* ethanolic extract possesses anti-inflammatory effect through the inhibition of iNOS and COX-2 expression by attenuating the expression of NF- κ B p65 in LPS-induced RAW264.7 cells (Kim *et al.*, 2010; Matsuo *et al.*, 2005).

Even though orientin has demonstrated various bioactivities, its anti-neuroinflammatory activity is not well studied or elucidated. Thus, this is a worthwhile study as elucidation of molecular mechanism could contribute largely in the development of new therapeutics that are capable of preventing neuroinflammation. As a potential therapeutic agent in treating ND, the bioavailability of orientin in crossing the blood-brain barrier (BBB) is a concern. There has been study suggesting that orientin have difficulty in crossing the BBB (Li *et al.*, 2008). However, studies showed that the function of BBB is altered in ND (Ohtsuki *et al.*, 2010; Jaeger *et al.*, 2009). Therefore, although orientin have difficulty in crossing BBB under normal physiological condition, the BBB permeability of compound might be altered and enhanced in neurodegenerative conditions. Hence, with the objectives to determine the effects of intracellular reactive oxygen species (ROS) and the mRNA and protein expression of iNOS, COX-2, STAT1, NF- κ B and HO-1 upon treatment with orientin, these findings could reveal the

possibility of orientin to be used as potential therapeutic agent in neuronal diseases.

MATERIALS AND METHOD

Preparation of orientin stock solution. The pure orientin compound was purchased from Sigma Aldrich, USA. It was then dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) to prepare a 22.3 mM of stock solution. Then, the stock solution was further diluted to the desirable concentrations using complete Dulbecco's Modified Eagle Medium (DMEM) medium (GIBCO, USA) before treating the cells.

Cell culture and maintenance. The BV2 microglia cells used in this study was provided by Dr. Sharmili of University Putra Malaysia (UPM). BV2 cells are a primary form of active immune defense in the central nervous system and had been reported to be a valid model and substitute for primary microglial in many experimental settings (Henn *et al.*, 2009; Ackerman *et al.*, 2015). The cells were cultured in complete DMEM medium supplemented with 10% fetal bovine serum (FBS) (GIBCO, South America), 1% Penicillin and Streptomycin (GIBCO, South America), 0.1% Fungizone (GIBCO, South America) and 0.1% gentamycin (GIBCO, China), and were maintained in a 5% CO₂ incubator (RS Biotech, UK) at 37°C.

For continuous maintenance, the cells were subcultured into new tissue culture flasks (Corning, USA) when the confluency reached 70%. The procedures were initiated with the removal of culture medium, followed by washing with 2 mL of sterile phosphate buffer solution (PBS) (AMRESCO, USA). Next, 1 mL of 0.25% trypsin-EDTA (GIBCO, Canada) was added and incubated for few minutes in the 5% CO₂ incubator at 37°C. As soon as the cells detached, 1 mL of fresh culture medium was added into the flask in order to inactivate the trypsin. Then, the cell suspension was centrifuged at 0.4 g for 5 min. After centrifugation, the supernatant was then discarded and the pellet was resuspended with 2 mL culture medium. Lastly, 1 mL of the cell

suspension was added into each new T-flask containing 4 mL fresh culture medium.

As for storage purpose, the pellet was resuspended with 1 mL of freezing medium consisting of 10% DMSO and 90% FBS after the centrifugation step. It was then transferred to cryovial and stored at -80°C or liquid nitrogen.

Determination of maximum non-toxic dose (MNTD). About 100 µL of BV2 microglial cells at the density of 8×10^4 cells per mL were seeded in 96-well plates (Corning, USA). Once the confluency of the cells reached about 70%, the medium was removed from each well and the cells were treated with a range of orientin concentrations (0, 0.032, 0.16, 0.80, 4, 20 and 100 µM) to determine its cytotoxic effect on BV2 microglial cells. After 24 hours of incubation, the viability of the cells was assessed using MTT assay by adding 20 µL of 5 mg/L MTT (Bio Basic, Canada) into the wells containing BV2 microglial cells. After 4 hours of incubation at 37°C, the solution was removed carefully. Then, 100 µL of solvent grade DMSO (Friendemann Schmidt, Germany) was added and mixed well to dissolve the formazan. The absorbance was then measured at 570 nm using microplate reader (Dynex, USA). Based on the absorbance measured, the percentage of cells viability as well as percentage of cytotoxicity were calculated using the following formulae:

$$\text{Percentage of viability (\%)} = \frac{\text{Absorbance reading of treated cells} - \text{Absorbance reading of medium}}{\text{Absorbance reading of control cells} - \text{Absorbance reading of medium}} \times 100\%$$

$$\text{Percentage of cytotoxicity (\%)} = 100\% - \text{percentage of cell viability (\%)}$$

Determination of optimal lipopolysaccharide (LPS) concentration. A total of 1×10^5 BV2 microglial cells were seeded in each well of 24-well plate (Nuclon, Denmark). At 70% confluent, the cells were treated with 0.0, 0.1, 1.0 or 10.0 µg/mL of LPS (Sigma, USA) and incubated for 24 hours. After 24 hours, the level of nitric oxide (NO) being released was measured. Griess test was used to measure the nitric oxide level, whereby 50 µL of the supernatant was mixed with 50 µL Griess reagent (Sigma, Germany). After 15 minutes, the absorbance reading at 540 nm was measured using microplate reader (Molecular Devices, USA). In this study, fresh culture medium was used as the

control. The effects of LPS on the morphology of the cells were observed under inverted microscope (Nikon Eclipse Ti-80, Japan) at 100x magnification and compared with the untreated cells.

Cell treatment and stimulation. BV2 microglia cells at density of 5×10^5 cells per well was seeded in 60 mm petri dishes (Thermo Scientific, USA) and incubated for 24 hours. Once the confluency of the cells reach about 70 to 80%, the cells were treated with orientin at MNTD (15 µM as determined above) or half MNTD (7.5 µM) and were incubated for 3 hours. Next, 0.1 µg/mL of lipopolysaccharide (LPS) (Sigma Aldrich, USA) was added to the treated cells to stimulate inflammation and was further incubated for 24 hours. After 24 hours, the cells were subjected to determination of intracellular ROS level, the mRNA expression of iNOS, COX-2, STAT1, NF-κB and HO-1 through real time PCR. An independent set was conducted for the determination of ROS as well as mRNA expression, respectively. In this study, indomethacin (Sigma Aldrich, USA) at 25 µM was used as the positive control. All the studies were conducted with 7 treatment groups: (1) untreated cells, (2) cells treated with ½ MNTD (7.5 µM), (3) cells treated with MNTD (15 µM), (4) cells treated with 0.1 µg/mL LPS, (5) cells treated with ½ MNTD (7.5 µM) and 0.1 µg/mL LPS, (6) cells treated with ½ MNTD (7.5 µM) and 0.1 µg/mL LPS, (7) cells treated with 25 µM Indomethacin and 0.1 µg/mL LPS, (8) cells only.

Determination of reactive oxygen species (ROS) level. After 24 hours of incubation with LPS, the medium and cells were collected and centrifuged at 0.4 g for 5 min. After centrifugation, the pellet was resuspended in PBS. Next, 100 µL of each cell suspension was added into 96-well plates (Corning, USA), which then followed by the addition of 100 µL of 40 µM 2',7'-dichlorofluoresceindiacetate (DCFH-DA) (Sigma Aldrich, USA). The contents were mixed well and were measured at excitation wavelength of 485 nm and emission wavelength of 538 nm using Tecan Infinite F200 Multifunctional Microplate Reader (Tecan, USA) after 10 min of incubation.

Then, the cell concentration was determined using trypan blue exclusion methods, in which 10

μL of the sample was mixed with 10 μL of trypan blue (Thermo Scientific, USA). A total of 10 μL of the suspension was then placed on the haemocytometer (Abcam, USA) for cell counting and the cell concentration of each treatment was calculated. The ROS level in each treatment was expressed as Relative Fluorescence Unit (RFU) per unit of cells.

Determination of mRNA expression. After 24 hours of incubation with LPS, RNAs were extracted and purified from the treated and untreated BV2 microglia cell lines by PureLink® RNA Mini Kit (Ambion, USA). Then, cDNA was synthesized from the RNA in Peltier Thermal Cycler-100 (MJ Research, USA) through qPCRBIO cDNA Synthesis Kit (PCR Biosystems, UK) following the manufacturer's instructions. Measurement of cDNA was then carried out

using Tecan Infinite F200 Multifunctional Microplate Reader at Nanoquant filter wavelength of 260 nm. Then, equal amount of cDNA samples (50 μg) with 2X qPCRBIO SyGreen and specific iNOS, COX-2, STAT1, NF- κB (subunit p65), HO-1 and β -actin forward and reverse primers (Table 1) were added and mixed well in semi-skirted 96-well PCR plates (Molecular Bioproducts, USA). Quantitative real-time PCR was performed using iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, US). Lastly, the mRNA expression level of iNOS, COX-2, STAT1, NF- κB , HO-1 and β -actin were analysed and CT values were obtained. In this study, β -actin was used as the housekeeping gene in normalization of data. Relative mRNA expression were determined using Double Delta CT analysis (Kenneth & Thomas, 2002).

Table 1. Forward and reverse primers sequences of genes analyzed in real time PCR.

Gene	Forward	Reverse
iNOS	5'-CCTCCTCCACCCTACCAAGT-3'	5'-CACCCAAAGTGCTTCAGTCA-3'
COX-2	5'-TGGGTGTGAAGGGAAATAAGGA-3'	5'-GAAGTGCTGGGCAAAGAATG-3'
STAT1	5'-CTGAATATTTCCCTCCTGGG-3'	5'-TCCCGTACAGATGTCCATGAT-3'
NF- κB	5'-CTGGTGGACACATACAGGAAGAC-3'	5'-ATAGGCACTGTCTTCTTTCACCTC-3'
HO-1	5'-AGCCCCACCAAGTTCAAACA-3'	5'-CATCACCTGCAGCTCCTCAA-3'
β -actin	5'-TCCTCCTGAGCGCAAGTACTCT-3'	5'-GCTCAGTAACAGTCCGCCTA-3'

The abbreviations of genes; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; STAT1: signal transducer and activator of transcription 1; NF- κB : nuclear factor kappa-light-chain-enhancer of activated B cells; HO-1: heme oxygenase-1; β -actin: Beta-actin.

Statistical analysis. The data obtained from three independent experiments with triplicates each were analysed and represented as mean $\pm$ SD (standard deviation). Significant differences at $p < 0.05$ were examined using One way analysis of variance followed by Tukey's multiple comparison test using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Determination of MNTD. MNTD is the highest concentration of orientin that did not cause any cytotoxic effect on BV2 microglia cells. It was determined by plotting a graph of percentage of cytotoxicity against concentrations

of orientin (Figure 1). From Figure 1, there was a very clear relationship between the orientin concentrations and its cytotoxic effect on the cells. The studies demonstrated that at $\leq 15 \mu\text{M}$ of orientin, the concentrations were not cytotoxic to BV2 microglia cells. Thus, the MNTD and $\frac{1}{2}$ MNTD of orientin that were determined in this study were 15 μM and 7.5 μM , respectively. At these concentrations, BV2 cells showed a normal morphology of small soma with distal arborization, characteristic of "ramified" microglia (Dai *et al.*, 2015). This concentration is lower than the MNTD of orientin (20 μM) towards SH-SY5Y cells (Law *et al.*, 2014). By comparing MNTD of orientin in these two cells, it seems to indicate that orientin is more toxic towards BV2 microglia cells compared to SH-SY5Y cells. Based on this comparison, it proved

that the cytotoxic effect of orientin might depended on the treated cell types, most probably due to structure-cytotoxicity relationship between orientin and the cells (Matsuo *et al.*, 2005). It was proposed that cytotoxic effect of flavonoids will be higher if the permeability of the cells towards them is higher (Matsuo *et al.*, 2005).

Figure 1. Percentage of cytotoxicity of orientin on BV2 microglial cells. After 24 hours of incubation with orientin at the range of 0 to 100 μM , the viability of the cells was measured by MTT assay. At $\leq 15 \mu\text{M}$ of orientin, the concentrations were not cytotoxic to BV2 microglia cells. MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

Meanwhile, at lower concentration, particularly at $0.16 \mu\text{M}$, negative cytotoxicity values were recorded that indicated that orientin exhibited promoting growth effects on BV2 microglia cells. At this concentration, the growth of BV2 cells was promoted by 4.19% as compared to the untreated cells. This observation might be due to the antioxidant activity of orientin that helped to scavenge the excessive free radicals produced in normal cells during ATP production in mitochondria, which eventually promote the growth of the cells (Praveena *et al.*, 2014; Middleton *et al.*, 2000; Khan, 2012; Spencer *et al.*, 2012; Droge, 2002). The high antioxidant activity of orientin are known to be due to the presence of ring B, C-glucoside, maximum numbers of hydroxyl group and the ability to form intramolecular hydrogen bonding in its molecular structure (Praveena *et al.*, 2014; Middleton *et al.*, 2000).

However, as the concentration of orientin increased above $15 \mu\text{M}$, there was a marked increase in the percentage of cytotoxicity of BV2 microglial cells. For instance, approximately 37%

of an increase was recorded in treatment using $100 \mu\text{M}$ relative to $15 \mu\text{M}$. The cytotoxic effect of orientin above MNTD might be related to the generation of intracellular ROS, which was demonstrated by some other flavonoids (Matsuo *et al.*, 2005). The findings by Matsuo and her colleagues proved that at higher concentrations, flavonoids would have an increased ability to be incorporated into the cells thus, increased the intracellular ROS generating ability as well as the levels of few oxidative metabolites, which subsequently contributed to cell death (Matsuo *et al.*, 2005).

Determination of optimal LPS concentration.

To determine the concentration of LPS that could induce maximum inflammation on BV2 microglia cells, two parameters were analysed: the release of NO by the cells and cell cytotoxicity. The optimal concentration of LPS that was selected in this study is the concentration that would cause the cells to release a significant amount of NO but insignificantly cytotoxic to the cells.

Under physiological conditions, NO acted as neuromodulator and neurotransmitter in the brain that helped to maintain the function of neuronal and vascular cells (Lowenstein *et al.*, 1994). There are two types of NO synthase (NOS) enzymes involved in the production of NO: neuronal NOS (nNOS) and endothelial NOS (eNOS) (Bredt & Snyder, 1990). They are calcium (Ca^{2+})-dependent enzymes, which are constitutive forms of NOS (Bredt & Snyder, 1990). Nevertheless, NO has been selected as a parameter of neuroinflammation in many studies, including the present study due to its well-known role under pathological conditions in CNS (Ghasemi & Fatemi, 2014). The inflammatory stimuli will induce the expression of iNOS in activated microglial cells and lead to NO production (Dello Russo *et al.*, 2004). Overproduction of NO has been correlated with many neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis (Yuste *et al.*, 2015).

Findings from this study showed a significant dose-dependent increase in the release of NO by BV2 microglia cells (Figure 2A). As LPS concentration increased to 0.1, 1.0, and 10.0

$\mu\text{g/mL}$, the produced NO were 2.33, 3.98, and $4.10 \mu\text{M}$, respectively, which were higher than NO produced by the cells in the control group. It was most probably due to the upregulation of iNOS in LPS-induced BV2 microglia cells through various pathways, such as NF- κB , c-Jun NH2-terminal kinase (JNK) MAPK, and p38MAPK (Lee *et al.*, 2012; Svensson *et al.*, 2010; Oh *et al.*, 2010). On the other hand, the results also demonstrated that even in the absence of LPS, NO was still being produced. Under normal conditions, the neuron cells will produce a low concentration of NO, in which it will act as neurotransmitter that involves in many biological effects (Snyder, 1995).

As for the effect of LPS on the viability of BV2 microglial cells, the present study demonstrated an insignificant decrease in the percentage of cell viability in a dose dependent manner (Figure 2B). Figure 2B illustrates that as the concentration of LPS increased from 0 to 0.1, 1.0, and $10.0 \mu\text{g/mL}$, the percentage of cell viability decreased to 10.76, 11.52, and 14.18 %, respectively. In terms of its morphology, the observation was in accordance with the findings by Dai *et al.*, whereby the untreated cells showed small soma with distal arborization, characteristic of “ramified” microglia (Figure 2C) while the $10.0 \mu\text{g/mL}$ LPS-treated BV2 cells showed fewer branches that were shorter (Figure 2D) and/or appeared to be resorbed into the cell body (Dai *et al.*, 2015). It is postulated that the cytotoxic effect of LPS on BV2 microglial cells could be via co-expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Mander & Brown, 2005). The NADPH oxidase is an enzyme responsible for the production of intracellular ROS, such as superoxide (O_2^-), which is important to kill the pathogens that infected the body (Qin & Crews, 2012; Gandhi & Abramov, 2012). However, a prolonged inflammation process will lead to the excessive production of several other potent free radicals, such as peroxynitrite, which will kill all cells nearby the inflammation site through protein oxidation, lipid peroxidation and DNA damage, eventually leading to neuronal death (Mander & Brown, 2005; Wilkinson & Landreth, 2006; Possel *et al.*, 2000).

Figure 2. Effect of various concentrations of LPS on (A) concentration of NO (B) cell viability (C) morphology of the untreated BV2 microglial cells under 100x magnification (D) morphology of the cells treated with $10.0 \mu\text{g/mL}$ LPS under 100x magnification. The concentration of NO was measured by Griess reagent while the cell viability was measured by MTT assay. “*” denotes significantly different from control at $p < 0.05$ using one way analysis of variance followed by Tukey's multiple comparison test. LPS, lipopolysaccharide; NO, nitric oxide.

As illustrated in Figure 2A, a significant inflammation as exhibited through 112% of increase in NO level was observed in cells treated

with 0.1 $\mu\text{g}/\text{mL}$ LPS. This same concentration of LPS did not cause any significant cytotoxicity towards BV2 cells (Figure 2B). Thus, 0.1 $\mu\text{g}/\text{mL}$ of LPS is considered as the optimal concentration to be used in the following studies.

Determination of ROS level. ROS is a redox signal that can be produced by various enzymatic reactions and chemical processes (Hsieh *et al.*, 2013). Low concentration of ROS are known to be essential in physiological functions and acted as second messengers (Hsieh *et al.*, 2013). However, excessive production of ROS have implicated the pathogenesis of human diseases, such as ND. This is probably due to the imbalance redox states resulting in oxidative stress. Studies had shown that cumulative oxidative stress can lead to cellular damage, impairment of DNA repair system and mitochondrial dysfunction, which eventually accelerated the aging process and the development of ND (Gandhi & Abramov, 2012; Federico *et al.*, 2012; Patten *et al.*, 2010). Hence, it is important to suppress the release of ROS levels in order to prevent ND.

The release of LPS-induced ROS levels in different treatments were determined in this study. As exhibited in Figure 3, the LPS-stimulated microglia cells showed significant upregulation of ROS levels compared to the untreated cells. This suggested that LPS plays an important role in the upregulation of ROS level. Studies suggested that this could be due to the upregulation of NADPH oxidase (Patten *et al.*, 2010). Han *et al.* (2012) showed the upregulation of the transcriptional levels of two NADPH oxidase components, p47^{phox} and $\text{gp91}^{\text{phox}}$ by LPS, suggesting the upregulation of NADPH oxidase upon LPS treatment, which resulted in an increase in the production of ROS levels (Qin *et al.*, 2004).

On the other hand, present studies also suggested that orientin at both MNTD and $\frac{1}{2}$ MNTD showed significant downregulation of LPS-induced ROS levels by 35.75% and 47.04% respectively (Figure 3). Of which, orientin at $\frac{1}{2}$ MNTD was found to be more effective than its MNTD, possibly due to the fact that an increase in the concentration of orientin could also lead to an increase in the intracellular stress of the cells, thus increases the ROS levels. The probable mechanism of orientin in reducing the LPS-induced ROS levels might be through the

suppression of the two NADPH oxidase components, p47^{phox} and $\text{gp91}^{\text{phox}}$. The study by Ganet *al* showed that glaucocalyxin B (GLB) markedly inhibited the expression of p47^{phox} and $\text{gp91}^{\text{phox}}$, which resulted in the inhibition of NADPH oxidase with subsequent downregulation of ROS production (Gan *et al.*, 2015). In addition, quercetin and isorhamnetin were shown to inhibit the expression of p47^{phox} subunit, with subsequent decreased in O_2^- production, suggesting the correlation between NADPH oxidase and the production of ROS levels (Sanchez *et al.*, 2007). Furthermore, there were increasing evidences suggesting the upregulation of HO-1 in response to the oxidative stress as a natural anti-oxidant (Loboda *et al.*, 2016). For instance, orientin was shown to alleviate oxidative stress through the induction of Nrf2 translocation to nucleus, which then upregulated the expression of HO-1 and activated the redox signaling pathway (Shih *et al.*, 2015). Consequently, downregulating the ROS level in $\text{A}\beta 1\text{-42}$ -induced mouse model of AD (Shih *et al.*, 2015). In short, the probable mechanisms of orientin in downregulating the ROS level might be through the inhibition of NADPH oxidase, and the upregulation of HO-1 warrants further study.

Determination of NF- κ B expression. NF- κ B is a transcription factor that are responsible for the transcription of genes, such as chemokines, pro-inflammatory cytokines, pro-inflammatory enzymes, and other factors in order to regulate the inflammatory responses upon stimulation (Shih *et al.*, 2015). NF- κ B resides in the cytoplasm as a heterodimer formed by p50 and p65, physically linked to an inhibitory molecule, $\text{I}\kappa\text{B-}\alpha$. Upon stimulation, NF- κ B signaling pathway will be activated firstly through the phosphorylation of $\text{I}\kappa\text{B-}\alpha$ subunit, with subsequent proteolytic degradation after detachment from the main NF- κ B unit (Shih *et al.*, 2015). Then, the NF- κ B p65 unit will translocate into nucleus and bind to promoter of pro-inflammatory cytokines and enzymes, which eventually resulted in the transcription into respective cytokines or proteins, such as $\text{TNF-}\alpha$, IL-1B, IL-6 iNOS and COX-2, and hence their upregulation expressions (Shih *et al.*, 2015).

Figure 3. Relative reactive oxygen species (ROS) level in LPS-stimulated BV2 cells upon treatment with $\frac{1}{2}$ MNTD and MNTD of orientin. ROS level after 24 hours was measured by 2',7'-dichlorofluoresceindiacetate (DCFH-DA) method. Bars indicate the means $\pm$ standard deviation. '*' indicates that the treatment was significantly different from the untreated cells using one way analysis of variance followed by Tukey's multiple comparison test at $p < 0.05$. '#' denotes the treatment was significantly different from the LPS-stimulated cells using one way analysis of variance followed by Tukey's multiple comparison test at $p < 0.05$. LPS, lipopolysaccharide; MNTD, Maximum Non-toxic Dose of orientin (15 μ M); $\frac{1}{2}$ MNTD, half value of Maximum Non-toxic Dose of orientin (7.5 μ M); Indo, Indomethacin.

In this study, the expression of LPS-induced NF- κ B were downregulated significantly after the pre-treatment of orientin at both MNTD and $\frac{1}{2}$ MNTD by 85.29% and 85.87% respectively (Figure 4A). Similar finding was shown in Glaucocalyxin B (GLB) whereby the expression of NF- κ B was suppressed. The suppression mechanisms shown by GLB was through the inhibition of IKK phosphorylation, I κ B- α phosphorylation and degradation, as well as the inhibition of NF- κ B promoter (Gan *et al.*, 2015). Besides, both anthocyanins and dihydromyricetin isolated from *Ampelopsis grossedentata* also shown the inhibition of phosphorylation and degradation of I κ B- α in suppressing the inflammatory responses (Jeong *et al.*, 2013; Hou *et al.*, 2015). Olajide *et al.* (2013) also demonstrated that the suppression of NF- κ B expression by cryptolepine was through the inhibition of nuclear translocation of NF- κ B. Hence, the probable mechanism of orientin in suppressing the

expression of NF- κ B might be through the similar pathways. Nevertheless, the activation of these transcription factors was not investigated in the present study and thus, should be validated in the future.

Determination of STAT1 expression. Besides NF- κ B, STAT1 is also a transcription factor. Stimulation by stimulus such as LPS will eventually lead to the activation of STAT1, which then mediated the transcription of STAT1-responsive genes (Reza-Zadeh *et al.*, 2008). Binding of LPS-induced interferons (IFNs) had shown to increase STAT DNA binding activity and eventually resulted in the phosphorylation of STAT1 (Reza-Zadeh *et al.*, 2008). This was then followed by dimerization and nuclear translocation of STAT1, resulting in the transcription of STAT1-responsive genes, such as iNOS, IL-1 β , and IL-6 (Reza-Zadeh *et al.*, 2008).

In this study, STAT1 expression was shown to be upregulated by 4 folds upon LPS stimulation (Figure 4B). Incubation with LPS for 24 hours had resulted in the over-stimulation of BV2 microglia cells and this eventually contributing to the active STAT1, which was responsible for producing pro-inflammatory cytokines or proteins. However, pre-treatment of the BV2 microglia cells with orientin had significantly downregulated the STAT1 expression. The results showed the reduction of STAT1 expression by 71.15% at MNTD of orientin and 60.85% at $\frac{1}{2}$ MNTD of orientin. Up to now, not many studies have been conducted on STAT1. However, based on a study carried out on luteolin, Kao *et al.* demonstrated the inactivation of STAT1 through the attenuation of upstream stimulatory kinases such as IFN- γ , which then inhibited the downstream pathways and eventually downregulated the production of pro-inflammatory cytokines (Kao *et al.*, 2011). Hence, it was postulated that orientin probably also acted through the same way. Nevertheless, the activation of these transcription factors warrants further study.

Determination of iNOS expression. iNOS, an enzyme encoded by Nitric oxide synthase 2 (NOS2) gene, generates nitric oxide (NO) from amino acid L-arginine (Lirk *et al.*, 2002). The production of NO plays an important role in the

pathogenesis of neuroinflammation (Sharma *et al.*, 2007). In normal physiological conditions, NO production gives an anti-inflammatory effects (Sharma *et al.*, 2007). However, excessive NO production will be considered as pro-inflammatory mediators that induces the neuroinflammation and eventually leads to neuronal cell death (Yuste *et al.*, 2015; Lirk *et al.*, 2002; Sharma *et al.*, 2007). Studies suggested the activation of glia cells expressing the enzyme iNOS produced NO, which then triggered calcium mobilization and activated the release of vesicular glutamate from astroglia cells (Yuste *et al.*, 2015). This eventually resulted in progressive neuronal cell death, contributing to ND (Yuste *et al.*, 2015). Besides, Kroncke *et al.* showed that high levels of iNOS protein were found in the brains of post-mortem Alzheimer's and Parkinson's diseases patients. Similarly, elevated iNOS level was found in the degenerating infarct region in transgenic mouse model of Huntington's disease (Chen *et al.*, 2000). In addition, strong iNOS immune-reactivity was also detected in activated astrocytes in ALS (Barbeito *et al.*, 2004). All these studies suggested the lethal effects of high iNOS expression. Hence, it is necessary to suppress the expression of iNOS.

In this study, the LPS-simulated BV2 microglia cells showed approximately 4 folds increase in the iNOS expression (Figure 4C). This was known to be highly related to the activation of NF- κ B and STAT1 pathway as discussed above, which resulted in the transcription and upregulation of iNOS. However, pre-treatment of the cells with orientin at MNTD showed significant downregulation of the LPS-induced iNOS expression by 50.09%, whereas it showed 95.92% at $\frac{1}{2}$ MNTD of orientin (Figure 4C). Effectiveness of orientin at $\frac{1}{2}$ MNTD over its MNTD was in accordance with the present findings on ROS level as iNOS expression could be ROS-dependent. Similar findings were obtained in torilin and geniposide, whereby iNOS expression was suppressed in LPS-induced N9 and BV2 microglia cells, respectively (Choi *et al.*, 2009; Zhang *et al.*, 2012). This downregulation of iNOS expression in both torilin and geniposide was known to be due to the inhibition of NF- κ B pathway (Choi *et al.*, 2009; Zhang *et al.*, 2012). Study by Zhang *et al.* showed that geniposide inhibited the I κ B- α subunit degradation and

subsequent translocation of NF- κ B into nucleus (Zhang *et al.*, 2012). This eventually inhibited the production of iNOS and led to the downregulation of iNOS expression. Therefore, the inhibition of NF- κ B signaling pathway might be a possible mechanism of orientin in downregulating iNOS expression.

Figure 4. Relative mRNA expression (Fold Change) of (A) NF- κ B (B) STAT1 (C) iNOS (D) COX-2 (E) HO-1 in LPS-stimulated BV2 cells upon treatment with $\frac{1}{2}$ MNTD and MNTD of orientin. Cells were treated for 24 hours and gene expression was calculated using Double Delta CT analysis. Bars indicate the means $\pm$ standard deviation. “*” indicates that the treatment was significantly different from the untreated cells using one way analysis of variance followed by Tukey's multiple comparison test at $p < 0.05$. “#” denotes the treatment was significantly different from the LPS-stimulated cells using one way analysis of variance followed by Tukey's multiple comparison test at $p < 0.05$. LPS, lipopolysaccharide; MNTD, Maximum Non-toxic Dose of orientin (15 μ M); $\frac{1}{2}$ MNTD, half value of Maximum Non-toxic Dose of orientin (7.5 μ M); Indo, Indomethacin; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; STAT1, signal transducer and activator of transcription 1; NF- κ B, nuclear factor- κ B; HO-1, hemeoxygenase 1.

Determination of COX-2 expression. COX-2 is an enzyme encoded by prostaglandin-endoperoxide synthase 2 (PTGS 2) gene. It involved in the synthesis of prostaglandin H₂ from arachidonic acid where overproduction will result in neurotoxicity (Ricciotti & Fitzgerald, 2011; Minghetti, 2004). Studies suggested that COX-2 was expressed in the central nervous system (CNS) under normal condition and it contributed to synaptic activity, long term memory and also helped in functional hyperemia (Minghetti, 2004). When there were pro-inflammatory activities, expression level of COX-2 was known to be elevated in the brain and played a crucial role in inflammatory reactions involving in the pathogenesis of ND (Ricciotti & Fitzgerald, 2011). For instance, there were elevated levels of COX-2 mRNA and protein found in post-mortem spinal cords of ALS patients (Yasojima *et al.*, 2001). Besides that, studies also showed increased expression level of COX-2 in microglia cells from idiopathic PD patients (Knott *et al.*, 2000). These suggested COX-2 as one of the potential targets used in treating ND.

As shown in Figure 4D, LPS significantly upregulated the COX-2 expression by 4-fold as compared to the untreated cells. This drastic upregulation of COX-2 expression was also known to be due to the activation of NF- κ B pathway (Rezai-Zadeh *et al.*, 2008). Upon treatment, both MNTD and 1/2 MNTD of orientin significantly downregulated COX-2 expression in LPS-induced BV2 cells by 85.3% and 78.3%, respectively. These findings were in accordance with various studies that suggested the attenuation of LPS-induced COX-2 expression upon treatment with natural compounds. For instance, astaxanthin showed the inhibition of COX-2 expression in LPS-stimulated BV2 microglia cells (Choi *et al.*, 2008). Besides, Kang *et al.* (2004) suggested the suppression of LPS-induced COX-2 expression in BV2 microglia cells by curcumin through the inhibition of NF- κ B and AP-1 DNA bindings. Another study by Yoon *et al.* (2016) showed that Cudraticusxanthone A suppressed the expression of both iNOS and COX-2 in LPS-stimulated BV2 microglia cells and this inhibitory effects were known to be due to the inhibition of NF- κ B and p38 MAPK pathways. Therefore, the probable mechanism of

suppression of COX-2 might be via the inhibition of NF- κ B pathway, which was also the probable mechanism for iNOS as discussed in the previous section.

However, it was noted that the COX-2 expression (Figure 4D) in LPS-stimulated BV2 microglia cells at both MNTD and 1/2 MNTD orientin were independent of iNOS (Figure 4C). There are studies suggesting that the decrease in iNOS would increase the COX-2 activity through the inhibition of NO production (Patel *et al.*, 1999; Clancy *et al.*, 2000; Habib *et al.*, 1997). For instance, study by Patel *et al.* suggested that inhibition of nitrite accumulation with high concentration of iNOS inhibitor (L-NMMA) in activated murine macrophage (RAW 264.7) showed the upregulation of PGE₂ production and the accumulation of COX-2 protein (Patel *et al.*, 1999). Hence, this could be the possible reason that iNOS and COX-2 acted independently in this study.

Determination of HO-1 expression. HO-1 as an anti-inflammatory enzyme, is enzyme encoded by hemoxygenase (decycling) 1 (HMOX1) gene located on chromosome 22q12 (Alzaraz *et al.*, 2003; Schipper *et al.*, 2009). HO-1 are responsible for degrading heme to generate carbon monoxide, biliverdin and free iron. It is a normal mechanism in response to oxidative stress or nitrosative stress caused by the high ROS/RNS levels (Kwon *et al.*, 2017). It will also be induced by cytokines or mediators formed during inflammatory processes in order to exert a negative feedback mechanism for cell activation and mediators production, which eventually regulates the inflammatory response (Kwon *et al.*, 2017).

In this study, orientin at MNTD showed a significant upregulation of LPS-induced HO-1 by approximately 4-fold as opposed to untreated cells (Figure 4E). Similar findings were also demonstrated with flavonolquercetin whereby it showed significant upregulation of Nuclear factor-like 2 (Nrf2) and HO-1 expression after LPS exposure (Kwon *et al.*, 2017). This suggested the induction of HO-1 by orientin most probably occurred via Nrf2 pathway. Besides, Kwon *et al.* (2017) also suggested the reduction of pro-inflammatory cytokines production via Nrf2/HO-1 signaling pathway by trythantrin. Another study by Zhou *et al.* (2014) on Orientin-

2"-O-Galactopyranoside (OGA) in BV2 microglia cells also demonstrated the significant induction in Nrf2 expression, suggesting the induction of HO-1 expression was mediated by Nrf2 activation. Furthermore, xanthohumol was reported to exert anti-inflammatory properties in

BV2 microglia cells through Nrf2-ARE signaling and the upregulation of downstream HO-1 (Lee *et al.*, 2011). Thus, it is also hypothesized that the probable mechanism of orientin was via the Nrf2 pathway.

Figure 5. Probable mechanism of orientin. Orientin inhibit the production of pro-inflammatory components such as STAT1, NF- κ B, iNOS and COX-2, whereas promote the production of anti-inflammatory component, HO-1, which eventually leads to anti-inflammation.

CONCLUSION

In conclusion, this study showed that orientin at both MNTD and $\frac{1}{2}$ MNTD was able to downregulate the ROS level. Orientin was also shown to be able to significantly downregulate the mRNA expression of transcriptional factor, such as NF- κ B and STAT 1, as well as the downregulation of pro-inflammatory mediators, such as iNOS and COX-2 significantly. In addition, orientin was also capable of upregulating HO-1. Based on all these findings, the probable mechanism of orientin was summarized and illustrated in Figure 5, which further validates the potential of orientin as an alternative therapeutic agent in treating ND. Nevertheless, additional studies, for instance, detecting the effects of orientin on other key inflammatory cytokines, such as IL-1 β and TNF- α , *in vivo* study in

evaluating the capacity of orientin to achieve effective concentration in the brain of animal models with neurodegenerative conditions as well as investigating the bioavailability. The neuroprotective effect of orientin in animal models of neuroinflammatory diseases should be further carried out prior to developing orientin as a potential drug in treating ND.

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