Original Article
LED enhances anti-inflammatory effect of *luteolin (3',4',5,7-tetrahydroxyflavone) in vitro*

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Abstract: Neuroinflammation is a complex pathological process usually results from abnormal microglial activation, thus, intervention in a microglial stimulation pathway could be a promising approach for the treatment of neurodegenerative diseases. Luteolin is an important bioflavonoid possesses anti-inflammatory properties, which is widely studied over these years. Light emitting diode (LED) therapy is reported to be a potential therapeutic strategy for many diseases including neurodegenerative diseases. However, little is known about the anti-inflammatory effect of LED therapy on activated microglial cells, even less is known whether there is a synergistic anti-inflammatory effect exist in LED and luteolin therapy. In this study, we aimed to confirm the anti-inflammatory effect of luteolin and LED combination therapy in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. We showed that luteolin inhibited LPS-induced cytotoxicity, tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) production through modulation of p38 and extracellular signal-regulated kinase (ERK) signaling in BV2 cells. In addition, LED therapy enhanced the anti-inflammatory effect of luteolin. These results suggest that a synergistic effect between luteolin and LED could be a new effective therapy in relieving neuroinflammation.

Keywords: Neuroinflammation, luteolin, LED, TNFα, IL-6, p38, ERK

Introduction

Increasing evidence suggests that inflammation, specifically neuroinflammation, is a central player of stroke [1], traumatic brain injuries [2], autoimmune diseases [3], psychiatric diseases [4], neurodegenerative diseases [5] including, but not limited to, Alzheimer's disease (AD). It is unknown whether inflammation is a primary cause leading to a particular disease or if it is a secondary response to the disease [6]. A growing body of literature shows that microglia, the main type of inflammatory cells in the brain, could be activated when exposed to certain neurotoxic agents, such as lipopolysaccharide (LPS) [7], interferon-γ (IFNγ) [8], and β-amyloid [9]. Microglial activation induces neuroinflammation in the human brain by recruiting more inflammatory cells such as IL-1, IL-6 and TNFα in the affected tissues as a source of immune protection. But uncontrolled or chronic neuroinflammation [10] induces progression of neurodegeneration and/or secondary injuries to the brain. Clinical evidence suggests that excessive secretion of inflammatory TNFα and insufficient production of anti-inflammatory TGF-β in the CSF significantly increased the conversion risk from mild cognitive impairment (MCI) to dementia. Furthermore, cytokines such as IL-1β, TNFα and others have been linked with functional and structural changes of brain tissue [11]. Therefore, anti-inflammatory drugs that attenuate microglial activation and neuroinflammation might be beneficial for intervention of AD.

We have previously shown that flavonoid compounds such as epigallocatechin-3-gallate [12-17] ameliorate pathology and cognitive impairment in AD mouse models through down-modu-

lation of inflammation. Of these different bioflavonoid compounds, luteolin, a potent anti-oxidant enriched in several plants, (*e.g.,* pepper, broccoli, carrot, celery, thyme, etc.) have disease modifying anti-inflammatory pharmacologic properties. Accumulating experimental evidence indicates the anti-inflammatory, anti-oxidant, anti-tumor and anti-diabetic benefits of luteolin in cell culture and in animal model. Many researchers have found that luteolin exerts its pharmacologic effect through modulation of cell signaling pathways, including mitogen activated protein kinase (MAPK), Akt, nuclear factor-kappa B (NF-kB) [18, 19]. Subsequently, NF-κB modulates the expression of some genes that mediate expression of inflammatory cytokines, such as TNFα, interleukin-1β (IL-1β), and IL-6 [20]. Luteolin has been shown to inhibit LPS-induced TNFα and Nitric Oxide (NO) production in an activated macrophage-like cell line [21]. In addition, it has been shown to regulate the expression of membrane metalloproteinases negatively by suppressing the production of several cytokines. Recently, luteolin, in combination with several anti-cancer drugs has been shown to improve the therapeutic efficacy of those treatments synergistically. This led many groups to investigate the effect of luteolin to be used as a combination therapeutic with other chemical or physical agent more effectively. Yet the exact mechanism of action is largely unknown. The mechanisms of luteolin mediated anti-inflammatory pathways need to be clearly understood to utilize this flavone to treat disease independently or in combination with other therapy.

Recent research showed the potential anti-inflammatory effect of light emitting diode (LED) for managing pain and inflammatory diseases. It has been comfirmed as an effective therapeutic for many diseases, such as allergic lung inflammation in experimental model of asthma [22], burn wound healing [23], skin modality [24] and colitis-induced inflammatory process in mice [25]. Further research showed that LED exerts its anti-inflammatory effect by decreasing the influx of inflammatory cells into the affected area. Interestingly, the same group and others have found decreased mRNA expression of cytokines such as of IL-1β, IL-6, TNF-α in Achilles tendinitis in rat and in osteoarthritis rabbit model [26, 27]. In addition, LED has also been shown to reduce inflammasome-mediated brain damage in experimental ischemic stroke model by suppressing MAPK and NF-kB signaling [28]. Interestingly, LED treatment with a frequency of 40 Hz (Gamma frequency) has been shown to reduce β-amyloid load in an AD model [29]. In parallel, blue light has been shown to modulate the microglial inflammatory gene expression in a cell line of murine model. In agreement with the previous findings, we hypothesized that LED could mediate its anti-inflammatory activity through the modulation of microglial activation.

Here, using a model of microglia cell line, we analyzed the anti-inflammatory effect of luteolin in LPS-stimulated BV2 microglia. In addition, we also investigated whether the combination of LED treatment and luteolin could modulate the anti-inflammatory activity in BV2 microglial cells using TNFα and IL-6 production. To better understand the mechanism in these activities, MAPK P38/ERK signaling was also explored in the study.

Material and methods

Reagents and LED device

Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone. Penicillin-streptomycin and fetal bovine serum (FBS) were purchased from GIBCO-BRL (USA). Luteolin (>95% purity by HPLC), LPS (L2880), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). MAPK P38 inhibitor SB202190, ERK inhibitor PD98059, antibodies against p38 (Thr180/Tyr182), phospho-p38 (p-p38), ERK (Thr202/Tyr204), phospho-ERK (p-ERK) were obtained from Cell Signaling Technology (Danvers, MA, USA). ELISA kits for tumor necrosis factor-α (TNFα) and Interleukin-6 (IL-6) were obtained from R&D Systems (Minneapolis, MN, USA). BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). White LED emitting device at a wavelength of 411 to 777 nm and at a frequency of 40 to 50 HZ was purchased from Intertek (London, United Kingdom).

Cell culture and MTT assay

BV2 microglia cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented
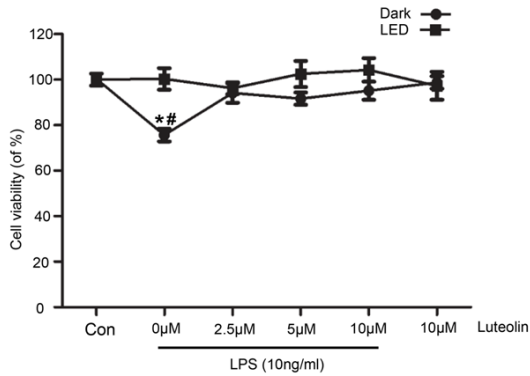
Figure 1. Effects of Luteolin and/or LED on cell viability. The MTT results showed that both luteolin (2.5-10 µM) and luteolin/LED could reverse the inhibitory effects induced by LPS. BV2 cells were treated with various concentrations of luteolin (2.5-10 µM) and/or LPS (10 ng/ml) for 6 h, with or without LED; cell viability was determined by MTT assay. The data represent the means ± standard deviation (SD) (*P<0.05 compared with unstimulated cells; #P<0.05 compared with LED treated cells). The results did not show significant difference between luteolin (2.5-10 µM) groups and luteolin/LED groups.

with 10% FBS, 100 units/ml penicillin-streptomycin at 37°C in a humidified atmosphere under 5% CO_2. The cells were seeded in 96-well culture plates at 1×10^4 cells/well for 24 h, followed by treatment with various concentrations of luteolin (0-10 µM) and LPS (10 ng/ml), with or without LED irradiation. Following incubation of these conditions for 6 h, 100 µl of MTT (0.5 mg/ml final concentration) were added and incubation was continued for a further 4 h. The formazan crystals in each well were dissolved in dimethyl sulfoxide (DMSO) for 10 minutes, and the absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA).

ELISA assay

BV2 cells were seeded in 24-well plate at a density of 1×10^5 cells/well and treated with luteolin (0-10 µM) and LPS (10 ng/ml) for 6 h, with or without LED light. The levels of TNFα and IL-6 in the culture medium were detected using ELISA kits according to the manufacturer's instruction.

Western blot

BV2 cells were seeded in six-well plate incubated with luteolin (0-10 µM) and LPS (10 ng/ml)

for 1 h, with or without LED light. After treatment, cells were harvested and lysed with 20 mM Tris-HCl buffer. The protein concentration was determined by BCA protein assay kit. Equal amount of protein (30 µg) from each group was resolved using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA), washed and blocked for 2 h at room temperature in tris-buffered saline containing 5% (w/v) nonfat dry milk. After blocking, membranes were incubated for overnight at 4°C with primary antibodies of p-p38, p-ERK, p38, ERK, washed and incubated for 1 h with the appropriate HRP-conjugated secondary antibody. Blots were developed using the luminol reagent (Thermo Fisher Scientific, Waltham, MA). Density analysis was performed using Quantity One software (Bio-Rad, Hercules, CA).

Statistical analysis

The statistical analysis results are expressed as the means ± standard deviation (SD) at least from three independent experiments. Comparisons between groups were determined by one-way analysis of variance (ANOVA) followed by the post-hoc least significant difference test. *P* value <0.05 was considered statistically significant.

Results

LED light prevents LPS-induced cell death in BV2 microglial cells

Initially, we examined whether the dark and LED light had any cytotoxic effect on cell viability on LPS-treated BV2 microglial cells. BV2 microglial cells were treated with LPS (10 ng/ml) either in the presence of dark or LED light for 6 h and cell viability were evaluated using lactate dehydrogenase (LDH) MTT assay. As expected, LPS-treatment significantly decreased cell viability in dark condition, whereas LED light inhibited LPS-induced cell death (**Figure 1**).

Then, we wanted to see whether luteolin could restore or prevent the LPS-induced cell death in the absence of LED light. To examine further, BV2 microglial cells co-treated with LPS (10 ng/ml) and luteolin (2.5-10 µM) in the presence of dark and LED light for 6 h. Similarly, cell viability
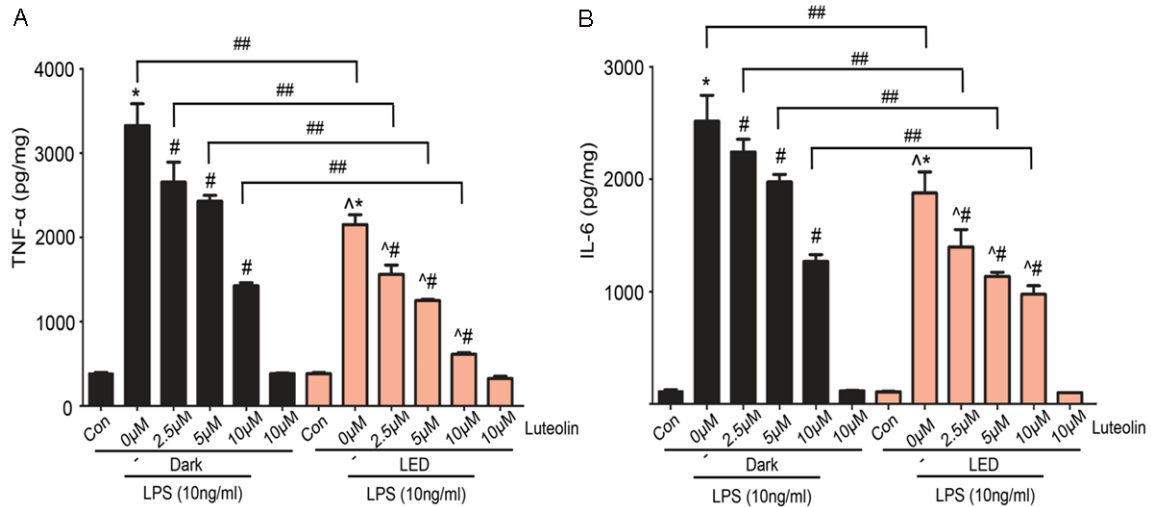
Figure 2. LED light enhances the effect of luteolin in inhibiting LPS-induced TNFα and IL-6 production. BV2 cells were incubated with luteolin (2.5-10 μM) and/or LPS (10 ng/ml) for 6 h, with or without LED light. ELISA was performed after collecting the supernatant in these groups. The results showed that luteolin (2.5 μM, 5 μM, 10 μM) could suppress the TNFα and IL-6 production levels induced by LPS, while LED could enhance the suppressive effects of luteolin. A. TNFα ELISA. *P<0.05, ^*P<0.05 compared with unstimulated cells; #P<0.05, ^#P<0.05 compared with LPS treated cells; ##P<0.05 compared luteolin/LED groups with luteolin groups in counterparts. B. IL-6 ELISA. *P<0.05, ^*P<0.05 compared with unstimulated cells; #P<0.05, ^#P<0.05 compared with LPS treated cells; ##P<0.05 compared luteolin/LED groups with luteolin groups in counterparts. Data are shown as means ± SEM.
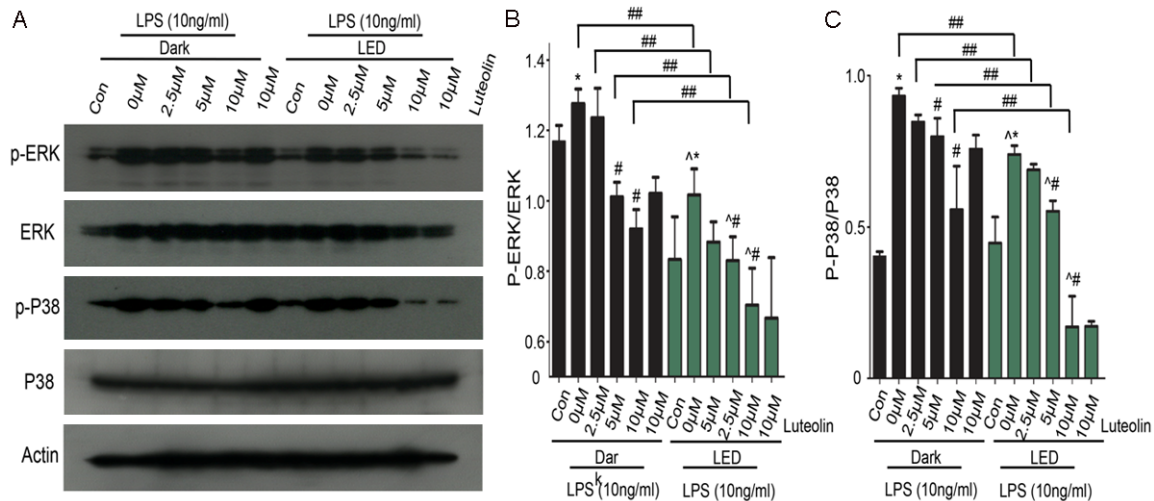

Figure 3. ERK and p38 MAPK pathways are involved in the synergistic effects of LED and luteolin. BV2 cells were incubated with Luteolin (0-10 μM) and/or LPS (10 ng/ml) for 1 h, with or without LED light. The results showed that luteolin (5 μM, 10 μM) could inhibit ERK and p38 phosphorylation levels induced by LPS, while LED could enhance the inhibitory effects of luteolin. A. Western Blotting was performed after collecting the cell lysis in each groups, phospho-ERK (pERK), total ERK (tERK), phospho-p38 (pp38) and total p38 (tp38) were then tested. B. pERK/tERK ratio analysis. *P<0.05, ^*P<0.05 compared with unstimulated cells; #P<0.05, ^#P<0.05 compared with LPS treated cells; ##P<0.05 compared luteolin/LED groups with luteolin groups in counterparts. C. pp38/tp38 ratio analysis. *P<0.05, ^*P<0.05 compared with unstimulated cells; #P<0.05, ^#P<0.05 compared with LPS treated cells; ##P<0.05 compared luteolin/LED groups with luteolin groups in counterparts. Data are shown as means ± SEM.

was measured using MTT activity assay. By MTT analysis, luteolin treatment significantly

inhibited the cell death induced by LPS in dark. No significant difference was found between
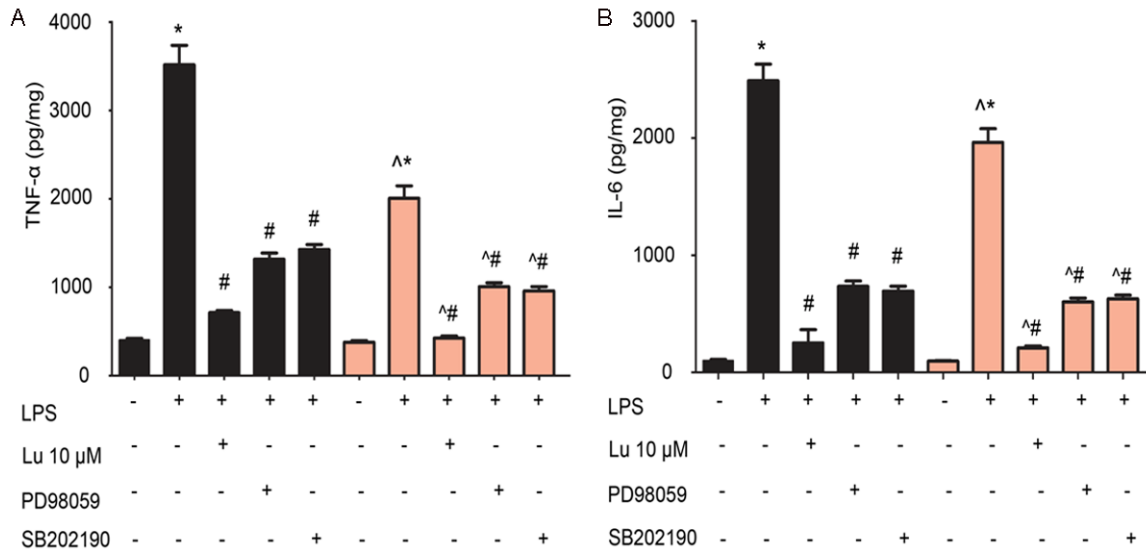
Figure 4. ERK and p38 MAPK pathways are involved in the synergistic effects of LED and luteolin. BV-2 cells were pretreated with MAPK P38/ERK inhibitors for 1h (SB202190/PD98059, 20 μM), then incubated with LPS (10 ng/ml) and/or Luteolin (10 μM) for 6 h, with or without LED light. ELISA was performed after collecting the supernatant in these groups. The results showed that luteolin, SB202190 and PD98059 could suppress the TNF-α and IL-6 production levels induced by LPS. A. TNF-α ELISA. *P<0.05, ^*P<0.05 compared with unstimulated cells; #P<0.05, ^#P<0.05 compared with LPS treated cells. B. IL-6 ELISA. *P<0.05, ^*P<0.05 compared with unstimulated cells; #P<0.05, ^#P<0.05 compared with LPS treated cells. Data are shown as means ± SEM.

luteolin (0, 2.5, 5, and 10 μM) groups and luteolin/LED groups (**Figure 1**).

LED light enhances anti-inflammatory effect of luteolin on cytokines TNF-α and IL-6 production

We hypothesized that pro-inflammatory cytokines production such as TNFα and IL-6 could be down-regulated by LED as well as luteolin treatment in LPS-stimulated BV2 microglial cells. Thus, BV2 microglial cells were co-treated with LPS (10 ng/ml) and luteolin (0, 2.5, 5, and 10 μM) for 6 h in the presence of dark and LED light. LPS treatment of BV2 cells results in significant increases in TNF-α and IL-6 production as compared with the control (**Figure 2**). As expected, luteolin inhibits LPS-induced TNFα and IL-6 production in a concentration-dependent manner. Furthermore, LED significantly enhances the anti-inflammatory effect of luteolin as shown by decreased LPS-induced TNFα and IL-6 production, which indicates there is a synergistic effect of LED and luteolin combination therapy.

Effect of LED light and luteolin on ERK and p38 pathways

ERK and P38 signaling pathways are known to be main regulators of pro-inflammatory cyto-

kines production in LPS-stimulated microglial cells [30]. We wanted to investigate whether LED light and/or luteolin treatment had any effect on p38 and/or ERK signaling pathways in LPS-stimulated BV2 cells. In this study, BV-2 microglial cells were incubated with luteolin (0, 2.5, 5, and 10 μM) and LPS (10 ng/ml) for 1 h in the presence or absence of LED light. LPS treatment significantly increased the phosphorylation of ERK and p38, while luteolin at 5 and 10 μM concentrations inhibited ERK and p38 phosphorylation induced by LPS. In addition, LED light also enhanced the inhibitory effects of luteolin (**Figure 3**). The results indicate that ERK and p38 MAPK pathways could be responsible for the synergistic anti-inflammatory effects of LED light and luteolin combination therapy. In order to better understand the exact role of ERK and P38 signaling, we pretreated BV2 cells with MAPK P38/ERK inhibitors for 1 h (SB202190/PD98059, 20 μM), then incubated the cells with LPS (10 ng/ml) and/or Luteolin for 1 h/6 h, with or without LED light. After that, we performed ELISA and Western blot for these groups. The results showed that luteolin, SB202190 and PD98059 could suppress the TNF-α and IL-6 production levels induced by LPS (**Figure 4**). Furthermore, luteolin (10 μM) and luteolin/LED combination could inhibit ERK
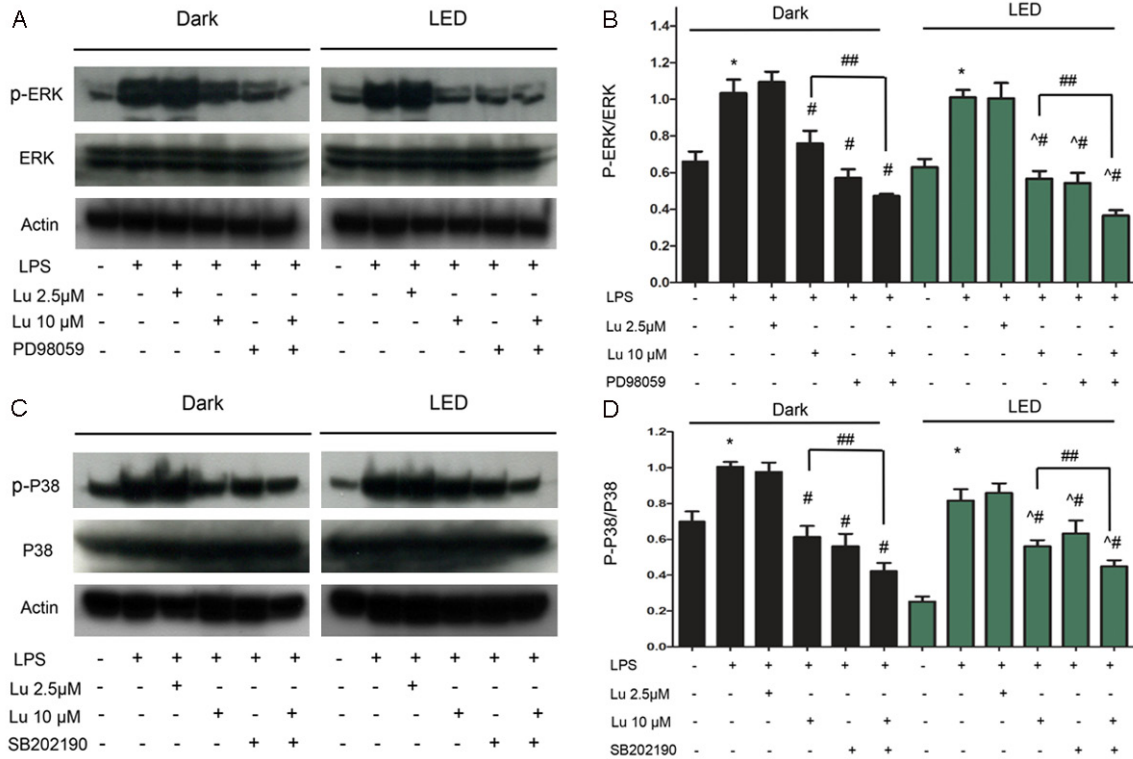
Figure 5. ERK and p38 MAPK pathways are involved in the synergistic effects of LED and luteolin. BV-2 cells were pretreated with MAPK P38/ERK inhibitors for 1 h (SB202190/PD98059, 20 µM), and then incubated with Luteolin and/or LPS for 1 h, with or without LED light. The results showed that luteolin (10 µM) and luteolin/LED combination could inhibit ERK and p38 phosphorylation levels induced by LPS, while SB202190/PD98059 could further inhibit ERK and p38 phosphorylation levels (A and C). Western Blotting was performed after collecting the cell lysis in each groups, p-ERK, ERK, p-P38 and p38 were then tested. (B and D) p-ERK/ERK ratio and p-P38/p38 ratio. Data are shown as means ± SEM.

and p38 phosphorylation levels induced by LPS, while SB202190/PD98059 could further inhibit ERK and p38 phosphorylation levels (**Figure 5**). In conclusion, ERK and p38 MAPK pathways are involved in the synergistic effect of LED and luteolin.

Discussion

Inflammation has been reported to play a central role in the initiation and/or progression of many diseases including stroke, TBI, autoimmune, psychiatric, neurodegenerative and many others diseases through activation of multiple cell signaling pathways. An extensive body of research has identified a large range of different bioactive nutrients that exhibit anti-inflammatory effects with fewer adverse side effects, offering a viable alternative approach to traditional medicine for preventive and therapeutic treatment of numerous inflammatory diseases. Moreover, a combination of multiple

bioactive components has been found to exhibit synergistic or additive anti-inflammatory effects in various model systems. Therefore, new approaches that elicit robust anti-oxidant and anti-inflammation activity in human body may provide new solution to inflammatory diseases. In this study, we investigated the additive or synergistic anti-inflammatory effects of LED light and luteolin treatment in LPS-induced inflammation in BV2 microglial cells.

Microglial cells are the most important element of the brain's immune system. Abnormal activation of microglia usually leads to inflammatory responses such as over production of pro-inflammatory cytokines, and further results in progressive neurodegenerative disorders [31]. Clinical researches show that TNFα, and IL-1β levels increase in the CSF of patients suffering from neurological diseases [32]. Increasing evidence indicates that microglia activated by various stimulants, including cytokines or bac-

terial endotoxins. As a widely used agent, LPS is the main virulence factor of gram-negative bacteria, which significantly induces the production of inflammatory mediators [33]. It has been reported that several signaling pathways including p38, ERK, NF-κB and Akt play important roles in LPS-induced inflammatory responses in BV2 microglial cells [34-36]. In accordance with these findings, the present study demonstrated the toxicity of LPS in BV2 cells, which significantly induce TNFα and IL-6 production. Moreover, phosphorylation of p38 and ERK signaling indicated the involvement of these pathways in LPS-stimulated BV2 microglial cells. Microglial activation has been demonstrated to be an early event that often triggers neuronal death in neurodegenerative diseases [37]. Attenuation of microglial activation and subsequent neuroinflammation may offer prospective clinical health benefits. As the drugs for neurodegenerative diseases in clinical process show very limited effect, many natural bioactive compounds have come to attention of researchers over the years. What interests us most is that some natural compounds possess the ability to treat neurological disorders by inhibition of microglial activation [38]. Luteolin is one of the flavonoids that suppress inflammation in the brain through down-regulation of cell signaling pathways [18, 39]. Our present study shows that luteolin at (2.5-10 μM) concentrations inhibits anti-inflammatory cytotoxicity on BV2 cells stimulated by LPS, which reduces TNFα and IL-6 production by down-regulation of p38 and ERK signaling pathways. These data indicates that luteolin suppresses LPS-stimulated inflammatory responses on BV2 microglial cells *via* down-regulation of p38 and ERK signaling pathways.

LED light therapy has been used successfully to stimulate anti-inflammatory effect to treat arthritis, reducing pain, wound-healing [23] and other types of inflammation [25] for decades. A recent study shows that violet/blue light (320-400 nm) protects against LPS-induced toxicity in THP-1 cells *via* modulation of Nrf2 inflammatory response signaling pathway [40]. In addition, blue light (450 nm) has been shown to induce microglial gene expression, such as VEGF and TGFβ in murine model [41]. Moreover, a wavelength of 780 nm light has been shown to decrease IL-6 and IL-1β gene expression in LPS-induced RAW 264.7 macrophage cell line. In addition, low-level laser therapy (632.8 nm)

has been shown to regulate microglial function through number of signaling pathways including MAPK/ERK, Src, Akt and others [42-44]. Yet, little is known about anti-inflammatory mechanism of LED therapy in microglial cell culture. Here, we found that LED light (411 to 777 nm) reversed the cytotoxicity stimulated by LPS in BV2 microglial cells through reduction of TNFα and IL-6 production. Moreover, LED enhances the anti-inflammatory effects of luteolin in LPS-stimulated BV2 microglia by reducing cytokines production and inhibiting phosphorylation of p38 and ERK. Taken together, the results indicate the anti-inflammatory synergistic or additive effect of luteolin and LED light in LPS-simulated BV2 cells.

In summary, this study demonstrates that bioflavonoid luteolin inhibits LPS-induced cytotoxicity, and reduces TNFα and IL-6 production through modulation of p38 and ERK signaling in BV2 microglial cells. Interestingly, LED light enhances the anti-inflammatory effect of luteolin in this *in vitro* model. These results indicate that a synergistic effect of luteolin and LED light could relieve neuroinflammation, which may provide a new therapeutic strategy for neuroinflammation.

Disclosure of conflict of interest

None.

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