Luteolin blocks dopaminergic neuron degeneration and shifts microglial M1/M2 polarization by inhibiting Toll like receptor 4

Luo yan[1], Yangzhi Xie[1], Liang Chen[1], and Jiacheng Chen[1]

[1]University of South China Affiliated Nanhua Hospital

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Abstract

Background: Luteolin is a natural flavonoid, and its neuroprotective and anti-inflammatory effects have been confirmed to mitigate neurodegeneration. Despite these findings, the underlying mechanisms responsible for these effects remain unclear. Toll-like receptor 4 (TLR4) is widely expressed in microglia and plays a pivotal role in neuroinflammation and neurodegeneration. Here we outline studies aimed at determining the mechanisms responsible for the neuroprotective and anti-inflammatory effects of luteolin using a mouse model of Parkinson's disease (PD), and specifically focusing on the role of TLR4 in this process. Methods: The mouse model of PD used in this study was established through a single injection of LPS. Mice were subsequently randomly assigned to either the luteolin or vehicle-treated group, then motor performance and dopaminergic neuronal injury were evaluated. BV2 microglial cells were treated with luteolin or vehicle saline prior to LPS challenge. mRNA expression of microglial marker IBA-1 and M1/M2 polarization markers, as well as the levels of pro-inflammatory cytokines in the mesencephalic tissue and BV2 were quantified by RT-PCR and ELISA, respectively. Apoptosis and cell viability of SH-SY5Y cells co-cultured with BV2 were examined. TLR4 RNA transcript and protein abundance in mesencephalic tissue and BV2 cells were detected. NFkB p65 subunit phosphorylation both in vivo and in vitro was evaluated by immunoblotting. Results: Luteolin treatment induced functional improvements and alleviated dopaminergic neuronal loss in our PD model. Luteolin inhibited apoptosis and promoted cell survival in SH-SY5Y cells. Luteolin treatment shifted microglial M1/M2 polarization towards the anti-inflammatory M2 phenotype both in vivo and in vitro. Finally, we found that luteolin treatment significantly downregulated both TLR4 mRNA and protein expression as well as restraining NFkB p65 subunit phosphorylation. Conclusions: Luteolin promoted dopaminergic neuronal survival in vivo and in vitro by blocking TLR4-mediated neuroinflammation.

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Running title:

Yangzhi Xie[1,*], Liang Chen[1,*], Jiacheng Chen[2], Yan Luo[1]

[1]Department of Neurology, The affiliated Nanhua Hospital, Hengyang Medical School, University of South China, 421001 Hengyang, China; [2]Department of Intensive Care Unit, The affiliated Nanhua Hospital, Hengyang Medical School, University of South China, 421001 Hengyang, China. *These authors contributed equally to this work.

Correspondence: Yan Luo, Email: luoyan820226@163.com. ORCID: https://orcid.org/0000-0002-5786-8244.

Author detail:

Yangzhi Xie, Department of Neurology, Email: yangtzexie_usc@126.com. ORCID: https://orcid.org/0000-0003-2248-9719.

Liang Chen, Email: liang20131214@sina.com. ORCID: https://orcid.org/0000-0003-2895-5127.

Jiacheng Chen: Email: jiachengchen_usc@126.com. ORCID: https://orcid.org/0000-0001-7583-9001.

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Abstract

Background: Luteolin is a natural flavonoid, and its neuroprotective and anti-inflammatory effects have been confirmed to mitigate neurodegeneration. Despite these findings, the underlying mechanisms responsible for these effects remain unclear. Toll-like receptor 4 (TLR4) is widely expressed in microglia and plays a pivotal role in neuroinflammation and neurodegeneration. Here we outline studies aimed at determining the mechanisms responsible for the neuroprotective and anti-inflammatory effects of luteolin using a mouse model of Parkinson's disease (PD), and specifically focusing on the role of TLR4 in this process.

Methods: The mouse model of PD used in this study was established through a single injection of LPS. Mice were subsequently randomly assigned to either the luteolin or vehicle-treated group, then motor performance and dopaminergic neuronal injury were evaluated. BV2 microglial cells were treated with luteolin or vehicle saline prior to LPS challenge. mRNA expression of microglial marker IBA-1 and M1/M2 polarization markers, as well as the levels of pro-inflammatory cytokines in the mesencephalic tissue and BV2 were quantified by RT-PCR and ELISA, respectively. Apoptosis and cell viability of SH-SY5Y cells co-cultured with BV2 were examined. TLR4 RNA transcript and protein abundance in mesencephalic tissue and BV2 cells were detected. NFkB p65 subunit phosphorylation both*in vivo* and *in vitro* was evaluated by immunoblotting.

Results: Luteolin treatment induced functional improvements and alleviated dopaminergic neuronal loss in our PD model. Luteolin inhibited apoptosis and promoted cell survival in SH-SY5Y cells. Luteolin treatment shifted microglial M1/M2 polarization towards the anti-inflammatory M2 phenotype both *in vivo* and *in vitro* . Finally, we found that luteolin treatment significantly downregulated both TLR4 mRNA and protein expression as well as restraining NFkB p65 subunit phosphorylation.

Conclusions: Luteolin promoted dopaminergic neuronal survival*in vivo* and *in vitro* by blocking TLR4-mediated neuroinflammation.

Keywords:

Parkinson's disease, Luteolin, M1/M2 polarization, neuroinflammation, TLR4

Introduction

Parkinson's disease (PD) is a movement disorder clinically characterized by rest tremor, rigidity, bradykinesia, and disturbance in balance. The pathological hallmark of PD is a progressive loss of dopaminergic neurons within the substantia nigra. The current therapeutic cornerstone of PD is dopamine (DA) replacement therapy. However, DA replacement therapy is only effective in temporarily attenuating disease symptoms and symptoms eventually worsen. Moreover, extended dopamine use is associated with several side effects [1]. Therefore, emerging PD studies generally focus on phytochemicals for long-term disease symptom modification.

Luteolin is a natural polyphenolic flavonoid compound present in many fruits and vegetables, such as chrysanthemum, *Perilla* species, beets, and carrots [2]. The neuroprotective effect of luteolin has been demonstrated

in a variety of neurological diseases and previous studies have shown that luteolin can reduce cerebral edema and neuronal apoptosis in a rodent model of traumatic brain injury [3]. Luteolin also functions in a neuroprotective role in the cognitive dysfunction displayed in an experimental model of epilepsy by inhibiting inflammation and reducing oxidative stress [4]. Depressive-like mice treated with luteolin also show improvement in anxiety behavior [5]. Despite this body of evidence, the exact role and mechanism of luteolin's actions in PD remain unclear.

Microglia are the major immune effector cells in the central nervous system (CNS). This cell type plays a vital role in CNS homeostasis, including immune regulation, debris removal, and damage repair [6]. Microglia are most dense in the substantia nigra and this specific distribution lays an anatomical foundation for microglia as an important player in PD pathogenesis. Microglia are subdivided into pro-inflammatory M1 phenotype and anti-inflammatory M2 phenotype. Upon stimulation in response to foreign bodies, infection, trauma, or other harmful stimuli, microglia are rapidly activated into the M1 type, and their morphology changes with branch protrusions becoming thicker and cell bodies becoming larger. Coordinately, microglia release a large number of inflammatory factors, reactive oxygen species, nitric oxide, and superoxide glutamate to both kill pathogenic microorganisms and recruit additional microglia to the lesion site. This activity causes an inflammatory reaction and, therefore, the activation of M1-type microglia is viewed as a protective response for the brain; however, overactivation of microglia causes neuronal damage [7].

Activation of microglia may be an initiating factor for Parkinson's disease. TLR4 is widely expressed in the CNS, mostly in astrocytes and microglia. TLR4 is the most important pattern recognition receptor expressed in microglia and plays an important role in regulating innate immune and inflammatory responses [8]. TLR4 can recognize a variety of damage-associated molecular patterns such as HSP90 and HMGB1, and this both activates microglia and triggers an immune inflammatory cascade [9]. Several pre-clinical studies have demonstrated that TLR4 activity is associated with neuroinflammation and neuronal loss [10]. Clinical data also confirmed that expression of TLR4 in brain tissue of PD patients is significantly increased, and that this effect is closely related to PD progression [11].

In this study, we focused efforts on investigating the therapeutic effects of luteolin in a mouse model of PD as well as cultured microglial cells. Based on our observations, we speculate that the neuroprotective and anti-inflammatory effects of luteolin are associated with its potential to limit TLR4 signaling.

Materials and Methods

Animals and treatments

All animal procedures were approved by the Ethics Committee of The Affiliated Nanhua Hospital, University of South China. 6-8 weeks old C57BL/6 mice (20-22g) were housed with free access to food and water. Mice were trained to adjust to a behavioral device for 5 consecutive days, and mice that exhibited poor motor performance in the behavioral tests were not retained in this study. A total of 40 animals were enrolled in this study, animals were randomly assigned to each group.

On day 6, mice were anesthetized with a mixture of ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Following this, mice were injected with LPS (5 μg dissolved in 2 μl PBS) or saline vehicle following stereotaxic coordinates measured from the bregma (Lateral: 1.3 mm; Posterior: 2.8 mm; Ventral to the surface of the dura mater: 4.5 mm). The needle was retained in the left side of the substantia nigra for a period of 10 minutes. The day after LPS injection, mice were intra-peritoneally injected with 40 mg/kg/d luteolin (Catalog: L409168; Aladdin, China,) or saline for consecutive 9 days. Subsequently, mice were subjected to behavioral tests to evaluate motor performance on days 16 and 17. Afterward, mice were sacrificed by decapitation, and samples were collected for further analysis. The experimental time schedule is given in Fig. 1.

Cell cultures and treatment

BV2 microglia or SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin. BV2 and SH-SY5Y were

both cultured in a 5% CO2 incubator at 37°C. BV2 were pretreated with luteolin (40 μM) or saline solution for 3h after which BV2 were treated with either LPS (1 μg/ml) or saline (control) for 12h. For establishing the co-culture system, SH-SY5Y was incubated in a 24-well plate for 48h, BV2 was then transferred to a 0.4 μm pore-sized Transwell insert and co-cultured with SH-SY5Y (SH-SY5Y: BV2 = 2:1) for another 48h.

Behavioral tests

The rotarod test was applied to examine animal strength and coordination. Each mouse was placed on an accelerated rotating rod (30 revolutions per minute) and the latency period to fall from the rod was recorded. If the subject did not fall from the rod within 300 seconds, a maximum of 300 seconds was recorded. Each experiment was repeated three times and the mean value was calculated.

A ball wrapped with gauze was fixed on a rough rod (diameter: 0.8cm; Height: 60 cm). Mice were placed on the ball with its head vertically up. The time it took to turn its head completely downward was recorded as T-turn, and the time it took to climb down to the ground was recorded as T-D. If the mouse fell off the pole, the data was not recorded. When mice stayed on the pole for more than 120 seconds, a maximum value of 120 seconds was recorded.

Immunohistochemistry and immunofluorescence

Mice were transcardially perfused with saline followed by infusion of 4% paraformaldehyde through the left ventricle. Following this, the brain was dissected from the skull, and tissue was placed in 4% PFA and left overnight at 4°C, after which the brain tissues were preserved in 30% sucrose solution until the tissue sank. The fixed tissue was sliced using a sliding microtome and brain sections were rinsed and incubated overnight with primary antibodies. For immunohistochemical staining, sections were incubated with anti-tyrosine hydroxylase (1:400; Abcam, USA, cat: ab75875), followed by enzyme-conjugate IgG (1:50; Beyotime, China) secondary antibody.

For immunofluorescence staining, BV2 was incubated with primary antibody against TLR4 (1:100; Santa Cruz, USA, cat: sc-293072). After several washings, cells were incubated with goat anti-mouse secondary antibody CY3 (1:8000; Abcam, UK) in a dark room. The images were taken using a confocal fluorescence microscope (Leica, Germany). IHC stained cells were examined using a brightfield microscope (Leica Germany).

Liquid Chromatography tandem mass spectrometry (LC-MS/MS)

Appropriate amounts of tissue were exhaustively chopped and homogenized. Following this, tissues were transferred into a centrifuge tube, and 3 mL of 10% sodium carbonate solution and 10 mL ethyl acetate were added to the tube. Tissues were subsequently homogenized by shaking for 10 min at 4°C. After centrifugation (6000 r/min for 10 min), the upper layer organic phase was transferred into a pear-shaped bottle and then subjected to rotary evaporation to dry at 40°C. The residue was dissolved in 1 mL 50% acetonitrile solution and this solution was cooled for 30 min and centrifuged at 16,000 r/min for 5 min. An appropriate amount of the supernatant was filtered through a 0.22μm membrane and then analyzed by LC-MS/MS. The devices used in this study are a Waters Acuity UPLC liquid chromatography (Waters, USA), an AB SCIEX 5500 Qtrap-MS mass spectrography (AB SCIEX, USA), and an Acquity UPLC HSS T3 chromatographic column (1.8 μm x 2.1 mm x 100 mm; Waters, USA).

Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from BV2 microglial cells or mesencephalic tissue using Trizol reagent (Sigma-Aldrich, USA). The RNA was subsequently reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Japan). RT-qPCR was conducted using a 7300 Plus Real-time PCR System (Thermo Fisher, USA) and an SYBR Green kit (Takara, Japan). Thermocycling was conducted under the following conditions: Denaturation at 95*C for 15 s, 40 cycles at 95*C for 10 s, 60*C for 30 s. The comparative threshold (Ct) method was used to analyze the data. Each 20μl reaction mixture contained 10μl PCR mixture, 1μl cDNA

template, 5 pmol primer, and a proper amount of water. Primers used for RT-qPCR are outlined in Table 1.

Table 1. Primers used for RT-qPCR

Target gene	Forward primer sequence	Reverse primer sequence
iNOS	GCAGAATGTGACCATCATGG	ACAACCTTGGTGTTGAAGGC
CD32	AATCCTGCCGTTCCTACTGATC	GTGTCACCGTGTCTTCCTTGAG
TNF-α	GTAGCCCACGTCGTAGCAAA	CCCTTCTCCAGCTGGGAGAC
Arg-1	TCACCTGAGCTTTGATGTCG	TTCCCAAGAGTTGGGTTCAC
CD206	AGTTGGGTTCTCCTGTAGCCCAA	ACTACTACCTGAGCCCACACCTGCT
IL-10	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG
TLR4	AGTTGATCTACCAAGCCTTGAGT	GCTGGTTGTCCCAAAATCACTTT
IBA-1	CGGGATCCGAGCTATGAGCCAGAGCAAG	GGAATTCCCCACCGTGTTATATCCACC
GAPDH	GTTTGTGATGGGTGTGAACC	TCTTCTGAGTGGCAGTGATG

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1β, TNF-α, and IL-6 proteins in the mesencephalic tissue and BV2 were determined using ELISA according to the manufacturer's instructions (Beyotime, China).

Flow cytometry

SH-SY5Y apoptosis when co-cultured with BV2 cells was determined by flow cytometry. Specifically, SH-SY5Y cells were stained with Annexin and propidium iodide (PI; Multi Sciences, AP101, China) according to the manufacturer's manual. Stained cell preparations were run and analyzed on a flow cytometer (CytoFLEX, Beckman, USA).

Cell viability

Cells were inoculated into a 96-well plate at 1x10^4cells per well. 10% volume of Cell Counting kit-8 (CCK-8; Beyotime, C0039, China) buffer was added to each well, and cells were incubated at 37°C for 2 hours. Absorbance at 450 nm was analyzed using a microplate reader.

Western blotting

Tissues and cells were homogenized with RIPA buffer containing PMSF. Proteins were resolved on 10% SDS-PAGE and transferred to the PVDF membrane. After blocking for 1 hr, membranes were incubated with primary antibody TLR4 (1:100; Santa Cruz, USA, cat: sc-293072), phospho-NFκB p65 (1:800; CST, USA, cat: #3033), β-actin (1:1000; HuaBio, China, cat: ET1701-80), and TATA-binding protein (TBP) (1:1000; HuaBio, China, cat: HA500518). Membranes were subsequently incubated with peroxide-labeled secondary antibodies. Immune complexes were detected using enhanced chemiluminescence (Millipore, USA), and results were recorded using a chemiluminescence imaging system (Biorad, USA).

Statistical analysis

All data presented are expressed as mean ± SEM. Data were analyzed using one-way ANOVA with Tukey's post-test. Data were analyzed using SPSS 22 software.$p < 0.05$ is considered statistically significant.

Results

Luteolin treatment protects against LPS-induced dopaminergic neuronal loss in a PD animal model

To evaluate the potential neuroprotective effects of luteolin in the outlined PD model, we measured Th-positive cells in the Substantia Nigra using immunohistochemical staining (Fig. 2A). We observed that LPS injection resulted in a remarkable decrease of dopaminergic neurons in the SN ($p < 0.001$, Fig 2B), and that luteolin treatment attenuated LPS-induced dopaminergic neuronal loss when LPS-only treated mice

are compared to the LPS+luteolin group ($p = 0.02$; Fig. 2B). We also measured the content of dopamine (DA) and its metabolites in the striatum using LC-MS/MS. As shown in Figure 2C, when compared to the sham group, LPS injection resulted in decreased DA ($p < 0.001$), DOPAC ($p < 0.001$), and HVA ($p = 0.0016$) abundance within the striatum. In contrast, treatment with luteolin effectively prevented DA and metabolite loss in the PD model (DA:$p = 0.007$; DOPAC: $p = 0.02$; HVA: $p = 0.016$; Fig. 2C). These data indicated that luteolin alleviates LPS-induced dopaminergic neuronal injury *in vivo* .

Luteolin treatment results in functional improvement in the PD model

Mice were subjected to pole and rotarod tests to assess grip strength and coordination, respectfully. Mice injected with LPS exhibited a poorer performance in the pole test (T-turn: $p < 0.001$; T-D: $p < 0.001$, Fig. 3A) and rotarod test ($p< 0.001$, Fig. 3B) when compared to sham control animals. Luteolin treatment significantly improved motor performance when compared to LPS-only injected animals in both the pole (T-turn: $p= 0.025$; T-D: $p = 0.007$, Fig. 3A) and rotarod test ($p = 0.003$, Fig. 3B). These data suggest that luteolin treatment can improve motor function in our PD mouse model.

Luteolin treatment shifted microglial M1/M2 polarization and restrained pro-inflammatory cytokine release in the PD model

Microglial polarization is vital for neuroinflammation and neuronal degeneration in PD. Thus, we measured relative mRNA levels of several M1/M2 polarization markers in the midbrain using RT-qPCR. LPS injection reduced anti-inflammatory M2 phenotype markers such as Arg-1 ($p< 0.001$), CD206 ($p < 0.001$), and IL-10 ($p < 0.001$) when sham control animals were compared to LPS-injected mice (Fig. 4A). In contrast, LPS promoted expression of pro-inflammatory M1 markers such as CD32 ($p < 0.001$), iNOS ($p < 0.001$), and TNF-α ($p < 0.001$) (Fig. 4B). Luteolin treatment significantly increased the expression of anti-inflammatory M2 markers Arg-1 ($p = 0.006$), CD206 ($p = 0.005$), IL-10 ($p = 0.029$), when luteolin+LPS mice were compared LPS-only injected mice (Fig. 4A), and reduced the expression of M1 markers CD32 ($p = 0.04$), iNOS ($p = 0.006$), and TNF-α ($p = 0.012$) (Fig. 4B). Luteolin treatment in this PD model also inhibited microglial activation, as evidenced by lower IBA-1 mRNA expression when compared to LPS-only group. ($p = 0.007$, Fig. 4C).

We next measured the abundance of pro-inflammatory cytokines in the midbrain using ELISA. LPS injection resulted in an elevated level of pro-inflammatory cytokine release in the midbrain; specifically, TNF-α: ($p < 0.001$), IL-1β ($p < 0.001$), and IL-6 ($p < 0.001$) abundance increased in response to LPS injection (Fig. 4D). This rise in inflammatory cytokines was significantly blunted in response to luteolin treatment (TNF-α: $p= 0.009$; IL-1β: $p < 0.001$; IL-6: $p = 0.009$) when LPS injected mice were compared to those receiving both luteolin and LPS (Fig. 4D). These findings support a potential anti-inflammatory effect for luteolin in this LPS-induced PD mouse model.

Luteolin treatment shifts microglial M1/M2 polarization and restrains pro-inflammatory cytokine release in BV2 microglial cells challenged with LPS

We next measured relative mRNA levels of M1/M2 phenotypic markers in BV2 microglial cells. As expected, following LPS challenge, BV2 cells exhibited decreased expression of the M2 markers Arg-1 ($p< 0.001$), CD206 ($p < 0.001$), and IL-10 ($p < 0.001$) when compared to the control group (Fig. 5A). Coordinately, we measured increased expression of M1 markers CD32 ($p < 0.001$), iNOS ($p < 0.001$), and TNF-α ($p < 0.001$) following LPS challenge (Fig. 5B). Luteolin pretreatment significantly increased expression of M2 markers Arg-1 ($p = 0.035$), CD206 ($p = 0.009$), and IL-10 ($p= 0.025$) (Fig. 5A), and inhibited expression of M1 markers CD32 ($p = 0.002$), iNOS ($p = 0.031$), and TNF-α ($p = 0.02$) compared to mice injected with LPS alone (Fig. 5B). Luteolin pretreatment prior to LPS challenging significantly inhibited microglial activation, as evidenced by lower IBA-1 mRNA expression when compared to LPS-only group. ($p = 0.027$, Fig. 5C). In addition, we observed that luteolin pretreatment reduced LPS-induced pro-inflammatory cytokines release in protein extracts of BV2. Specifically, we measured lower TNF-α ($p = 0.028$), IL-1β ($p = 0.003$), and IL-6 ($p = 0.038$) levels in BV2 cells following both luteolin and LPS administration (Fig. 5D). These data clearly support an anti-inflammatory effect of luteolin in BV2 cells challenged with LPS.

Luteolin treatment in BV2 attenuated neuronal injury in the co-culture system

We established a microglia/neuron co-culture system to explore the potential indirect neuroprotective effects of luteolin-treated microglia on dopaminergic neurons (Fig. 6A). A 0.4 μm pore-sized membrane was used to separate BV2 and SH-SY5Y cells. After treatment with LPS, luteolin, or LPS combined with luteolin, BV2 cells were transferred to the co-culture system and co-cultured with SH-SY5Y for an additional 48 h. After this, SH-SY5Y apoptosis was measured by flow cytometry (Fig 6B), and cell survival was measured using a CCK8 assay. In the analysis of apoptosis, pretreatment of BV2 cells with luteolin significantly reduced the apoptotic response of SH-SY5Y cells ($p = 0.002$) compared to BV2 cells treated with LPS-only (Fig. 6C). In the CCK8 cell viability analysis, pretreatment with luteolin in BV2 prior to LPS administration remarkably increased cell viability of SH-SY5Y ($p = 0.027$) when compared to LPS-only cells (Fig. 6D). These *in vitro* results suggest that luteolin treatment of BV2 cells robustly ameliorates inflammation-induced neuronal injury.

Luteolin treatment reduces LPS-induced activation of TLR4/ NFkB signaling in the PD model and BV2 cells.

TLR4/NFkB signaling plays a pivotal role in microglial-mediated neuroinflammation. Thus, we next evaluated whether the anti-inflammatory potential of luteolin was associated with TLR4/NFkB signaling in both*in vivo* and *in vitro* experimental systems. Increased relative TLR4 mRNA abundance was observed upon LPS challenge in mesencephalic tissue ($p < 0.001$; Fig. 6A) dissected from LPS-treated PD model mice, and in LPS-treated cultured BV2 cells ($p < 0.001$; Fig. 6B). This rise in TLR4 expression was inhibited by luteolin treatment both *in vivo* ($p = 0.016$, Fig. 6A) and *in vitro* ($p = 0.004$; Fig. 6B). We also examined the intensity of TLR4 staining in BV2 cells using immunofluorescence microscopy (Fig. 6C). Compared to the control group, BV2 cells challenged with LPS resulted in a higher intensity of TLR4 staining ($p < 0.001$; Fig. 6D). In contrast, luteolin pretreatment prior to LPS challenge decreased the fluorescence intensity of TLR4 ($p = 0.002$; Fig. 6D). Immunoblot analysis also indicated that luteolin treatment significantly decreased LPS-induced increases in TLR4 within dissected mesencephalic tissue ($p = 0.029$; Fig. 6E) and cultured BV2 cells ($p = 0.016$; Fig. 6F).

LPS treatment was also observed to significantly increase the abundance of phosphorylated (activated) NFkB subunit p65 both *in vivo* ($p < 0.001$; Fig. 6G) and *in vitro* ($p < 0.001$; Fig. 6H). Luteolin treatment significantly downregulated LPS-induced, phosphorylated p65 abundance in the PD mouse model ($p = 0.024$; Fig. 6G) and cultured BV2 cells ($p = 0.0035$; Fig. 6H). These data provide evidence that the anti-inflammatory activity of luteolin observed in both the *in vivo* PD model, and *in vitro*cultured microglial cells is likely associated, in part, with diminished TLR4/NFkB signaling.

Discussion

In this study, we explored the neuroprotective effects of luteolin during inflammation-induced dopaminergic injury using both *in vivo* and *in vitro* model systems. The results showed that luteolin treatment induced a functional improvement in this response, and protected against dopaminergic neuronal loss. Luteolin treatment also promoted dopaminergic neuronal survival when SH-SY5Y neuronal cells were co-cultured with BV2 microglia challenged with LPS-only. Moreover, luteolin treatment shifted microglial M1/M2 polarization towards an anti-inflammatory M2 phenotype and blunted pro-inflammatory cytokine release in both *in vivo* and *in vitro* model systems. Mechanistically, our findings indicate that luteolin treatment deactivated TLR4 and downstream NFkB signaling. The effect of this resulted in an improved inflammatory microenvironment and reduced neuronal loss.

Imbalances in microglial polarization status are a key factor in the initiation of neurodegenerative diseases. Moreover, there is the possibility of mutual transformation between M1 and M2 microglial phenotypes corresponding to specific treatments. Therefore, we recognize the potential therapeutic value in agents that, in situations where phenotypical M1 microglia are over-activated, promote the transformation of M1-type microglia into M2 type and maintain a relative balance of M1/M2 cells. Luteolin has a wide range of effects *in vivo* , including anti-inflammatory effects, antioxidant action, and estrogen-like effects [12-14]. In

accordance with our findings, other studies indicate that luteolin functions in a neuroprotective capacity during response to dopaminergic injury by limiting inflammatory response [15]. However, the mechanism(s) that govern such response remain unclear. We found that in LPS-treated PD mouse model and cultured BV2 cells, luteolin intervention inhibited the activation of M1-type microglia, reduced the production and release of pro-inflammatory cytokines, and promoted the activation of anti-inflammatory M2-type microglia resulting in a restoration of the M1/M2 ratio. These effects reduced the neuronal damage that occurs through inflammatory response. In our animal model, we further demonstrated that luteolin significantly blunts both dopaminergic neuronal injury and motor function in inflammation-induced PD mice. Because of limitations stemming from the blood-brain barrier, classic anti-inflammatory drugs are of limited benefit in the treatment of neurological diseases. Luteolin is a highly active natural polyphenol that crosses the blood-brain barrier, thus luteolin is potentially a more effective drug for the prevention and treatment of inflammatory responses mounted within the central nervous system [16].

Although the anti-inflammatory effects of luteolin have been extensively studied, its downstream effector targets remain unclear. Consistent with our results, previous studies have determined that LPS triggers the activation of TLR4 and downstream NFκB signaling [17, 18]. Using TLR4 KO mice, prior studies indicate that damage-associated response does not stimulate microglia through the TLR4 pathway [19, 20]. This implies that TLR4 deficiency may shape microglia polarization towards the M2 phenotype while inhibiting the M1 phenotype. TLR4 is a type of pattern recognition receptor and once activated, can prompt NFkB activation through nuclear translocation [21]. Activated NFkB subsequently promotes the expression of a variety of pro-inflammatory factors, activates anti-apoptotic genes, and promotes the activation of cell proliferation. Specifically, active NFkB can promote the expression of IL-1β, IL-6, TNF-α, iNOS, and other inflammatory factors, and regulate microglial polarization [22]. In addition, NFkB binds to the TLR4 gene promoter and promotes its expression [23]. Thus, harnessing TLR4/NFkB signaling is a crucial mechanism during the induction of a neuroinflammatory response. In this study, we found that luteolin can decrease TLR4 and p65 phosphorylation, indicating that TLR4/NFκB pathway is, at least in part, a target for luteolin in limiting neuroinflammation and dopaminergic neuron degeneration.

We note some limitations in this study. For example, the concentration of luteolin in mouse brain tissue is unclear. More reliable methods for judging drug distribution are needed to better understand how luteolin reaches the brain. Tissue distribution of luteolin may be considered using *in vivo* imaging of animals or through HPLC approaches. In addition, the anti-inflammatory effect of luteolin in TLR4-overexpressing microglial cells requires further examination.

Conclusion

Our results show that luteolin protects against inflammation-induced dopaminergic neuron loss. Mechanistically, luteolin deactivated TLR4 signaling in microglia, thus improving the inflammatory niche and subsequently attenuating dopaminergic neuronal loss both *in vivo*and *in vitro* . Luteolin has limited adverse reactions and displays low toxicity, and thus has broad application prospects as a PD therapeutic.

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Figure legends

Figure 1. The schedule used in this study.

Figure 2. Luteolin protects against LPS-induced neuronal injury in the nigrostriatal system of the PD mouse model. (A) Representative immunohistochemical staining for tyrosine hydroxylase in the SN. Scale bar = 100μm. (B) Number of Th-positive cells within the SN was counted. (B) Abundance

of DA, DOPAC, and HVA in the striatum measured by LC-MS/MS. N = 5 per group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the sham group. $\#p < 0.05$, $\#\#p < 0.01$ compared to the LPS group.

Figure 3. Luteolin improved motor performance in the PD model.(A) In the pole test, the time for each mouse to completely turn its head downward (T-turn) and climb down to the ground (T-D) was recorded. (B) Using a rotarod test, the latency to fall off the rotating rod was recorded. N = 10 per group. $^{***}p < 0.001$ compared to the sham group, $\#p < 0.05$, $\#\#p < 0.01$ compared to the LPS group.

Figure 4. Luteolin shifted microglial M1/M2 polarization and inhibited pro-inflammatory cytokine release in the PD model. (A) Relative mRNA expression of microglial M2 polarization phenotypic markers Arg-1, CD206, and IL-10. (B) Relative mRNA expression of microglial M1 polarization markers CD32, iNOS, and TNF-α. (C) Relative mRNA expression of IBA-1. (D) Pro-inflammatory cytokine levels in the mesencephalic tissue were measured using ELISA. N = 5 per group. $^{***}p < 0.001$ compared to the sham group; $\# p < 0.05$, $\#\#p < 0.01$ compared to the LPS-only group.

Figure 5. Luteolin shifts microglial M1/M2 polarization and inhibited pro-inflammatory cytokine release in BV2 microglia cells challenged with LPS. (A) Relative mRNA expression of microglial M2 phenotypic markers Arg-1, CD206, and IL-10. (B) Relative mRNA expression of microglial M1 phenotypic markers CD32, iNOS, and TNF-α. (C) Relative mRNA expression of IBA-1. (D) Pro-inflammatory cytokines levels in BV2 cells were measured using ELISA. N = 5 per group. $^{***}p < 0.001$, $^{**}p < 0.01$ compared to the Control (sham) group, $\#p < 0.05$, $\#\#p < 0.01$ compared to the LPS-only group.

Figure 6. Luteolin pretreatment before LPS challenging in BV2 rescued neuronal injury in the co-cultured system. (A) A sketch of the co-culture system used. (B) Apoptosis detected by flow cytometry. (C) Bar graph showing the cellular apoptosis. (D) Cell viability was measured by CCK8. N = 4 per group. $^{***}p < 0.001$ compared to the control group, $\#p < 0.05$, $\#\#p < 0.01$ compared to the LPS-only group.

Figure 7. Luteolin treatment reduces LPS-induced TLR4/NFkB signaling *in vivo* and *in vitro* model systems. (A) Relative TLR4 mRNA expression in mesencephalic tissue dissected from mice in the indicated treatment groups. N = 5 measurements per group. (B) Relative TLR4 mRNA abundance in cultured BV2. N = 5 measurements per group. (C) Representative immunofluorescent confocal images of TLR4 staining in BV2 cells. Scale bar = 20 μm. (D) Mean TLR4 fluorescence intensity in treated and untreated BV2 cells. N = 5 measurements per group. (E) Immunoblots (*top*) and bar graph of immunoblot quantification (*bottom*) for TLR4 in mesencephalic tissue. N = 3 per group. (F) Immunoblots (*top*) and bar graph of immunoblot quantification (*bottom*) for TLR4 in treated and untreated BV2 cells. N = 3 per group. (G) Immunoblots (*top*) and bar graph of immunoblot quantification (*bottom*) for phospho-p65 in mesencephalic tissue. N = 3 per group. (H) Immunoblots (*top*) and bar graph of immunoblot quantification (*bottom*) for phospho-p65 in treated and untreated BV2 cells. N = 3 per group. $^{***}p < 0.001$ compared to the control group, $\#p < 0.05$, $\#\#p < 0.01$ compared to the LPS-only group.