SIRT1 and SIRT2 modulators reduce LPS-induced inflammation in HAPI microglial cells and protect SH-SY5Y neuronal cells in vitro

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Abstract

Neuroinflammation is associated with the development of depression. Deacetylases SIRT1 and SIRT2 are reported to exert neuroprotective effects in aging, neurogenesis, neurodegeneration and neuroinflammation. Therefore, this study aimed to investigate the effects of SIRT1 and SIRT2 modulators on LPS-induced neuroinflammation and neurodegeneration in vitro. To achieve this, HAPI rat microglial cells were pre-treated with the SIRT1 activator resveratrol (0.1–20 μM), the selective SIRT1 inhibitor EX527 (0.1; 1 μM), the dual SIRT1/SIRT2 inhibitor sirtinol (0.1–20 μM) and the SIRT2 inhibitor AGK2 (0.1; 1 μM), prior to exposure with LPS (5 ng/mL) for 20 h. The reference antidepressant drug fluoxetine and the nonsteroidal anti-inflammatory drug ibuprofen were also evaluated in the same paradigm, both at 1 μM. Resveratrol and sirtinol inhibited TNF-α production to a greater degree than either fluoxetine or ibuprofen. Resveratrol, sirtinol, EX527 and AGK2 significantly reduced PGE_2 production by up to 100% in microglia. Then, the supernatant was transferred to treat SH-SY5Y cells for 24 h. In all cases, SIRT modulator pretreatment significantly protected undifferentiated SH-SY5Y human neuroblastoma cells from the insult of LPS-stimulated HAPI supernatant by up to 40%. Moreover, resveratrol and sirtinol also showed significantly better neuroprotection than fluoxetine or ibuprofen by up to 83 and 69%, respectively. In differentiated SH-SY5Y cells, only sirtinol (20, 10 μM) and AGK2 (0.1 μM) pretreatment protected the cells from LPS-stimulated HAPI supernatant. This study suggests that SIRT1 and SIRT2 modulators are effective in inhibiting LPS-stimulated production of TNF-α and PGE_2 in HAPI microglial cells and protecting SH-SY5Y cells from inflammation. Thus, we provide proof of concept for further investigation of the therapeutic effect of SIRT1 and SIRT2 modulators and combination with current antidepressant medication as a treatment option.

Keywords Depression · SIRT1 · SIRT2 · Neuroinflammation · Resveratrol · Sirtinol

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Introduction

Depression is a complex phenomenon with multiple symptoms, such as depressed emotion, suicidal thoughts, insomnia, decreased appetite and fatigue. It is estimated that over 264 million people of all ages suffer from depression worldwide (World Health Organization 2020). Current antidepressant medications aim to increase levels of monoamine neurotransmitters (Penn and Tracy, 2012), but these antidepressants may have multiple side effects and toxicities which can result in poor compliance (Bockting et al. 2008; Penn and Tracy 2012). Furthermore, the NIMH-funded sequenced treatment alternatives to relieve depression study, conducted to determine the effectiveness of antidepressant treatments for major depression, found that overall remission rates were less than 30% with a single treatment, increasing to just 67% even after multiple interventions (Gaynes et al 2009).

Recent evidence has suggested a link between chronic neuroinflammation and the development of depression, with an association between inflammation and a number of depressive symptoms (Jokela et al. 2016; Miller and Raison 2016). Excessive and protracted neuroinflammation can directly result in acute neurological damage or neuronal death and accelerate long-term neurodegeneration (Lyman et al. 2014). Treatment options for neuroinflammation have been investigated, including clinical trials investigating nonsteroidal anti-inflammatory drugs (NSAIDs) and cytokine inhibitors (Zhang et al. 2012; Kostadinov et al. 2015; Köhler et al. 2016). Even so, their efficacy in neuroinflammation has not been established.

In recent years, numerous studies have reported the potential of SIRT1 and SIRT2 modulators in treating neuroinflammation and neurodegeneration (Zhang et al. 2020). The enzymes SIRT1 and SIRT2 belong to the sirtuin family, a unique class of nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases (Landry et al. 2000; Jiang et al. 2017). A series of transcription factors involved in multiple important cellular processes are substrates of SIRT1 and/or SIRT2′ deacetylation activity, such as forkhead transcription factors of class O, tumor suppressor protein p53, PPAR gamma coactivator-1α and nuclear factor-κB (Donmez and Outeiro 2013). SIRT1 activation and SIRT2 inhibition are implicated in a number of processes associated with the onset and development of depression, including aging, oxidative stress, neuronal survival, neurogenesis and neuroinflammation (Donmez and Outeiro 2013; Paraiso et al. 2013). Additionally, variation in SIRT1 and SIRT2 polymorphism or expression can influence the risk of developing depression (Porcelli et al. 2013; Liu et al. 2019; Zhang et al. 2021). This evidence suggests that investigating SIRT1 and SIRT2 may be a useful strategy for identifying potential new therapeutic interventions for depression. However, the reported effects of SIRT1 and SIRT2 activators or inhibitors on neuroinflammation and neurodegeneration are inconsistent, potentially due to the employment of different in vivo depressive-like models and other in vitro disease models (Zhang et al. 2020). Therefore, this study aimed to investigate the effects of the SIRT1 activator resveratrol, selective SIRT1 inhibitor EX527, dual SIRT1/SIRT2 inhibitor sirtinol, and the SIRT2 inhibitor AGK2, on neuroinflammation and neurodegeneration. To achieve this aim, these modulators were assessed in LPS-stimulated HAPI microglial cells and inflammation-conditioned SH-SY5Y neuronal cells.

Materials and methods

Cells and reagents

HAPI rat microglial cells and SH-SY5Y neuroblastoma cells were purchased from Sigma-Aldrich (Merck). Cells were cultured separately in ATCC-formulated DMEM (Gibco by Life Technologies) which contains 1 g/L of D-Glucose, L-glutamine, 110 mg/L of sodium pyruvate and phenol red. The medium was additionally supplemented with 1% penicillin–streptomycin (10,000 U/mL) (Gibco by Life Technologies) and 10% fetal bovine serum (FBS) (Scientifix life). Cells were cultured to 80% confluence in sterile T-75 cm^2 flasks and maintained in an incubator at 37 °C with 5% CO_2 and high humidity.

Establishment of in vitro model of neuroinflammation and subsequent neurodegeneration

HAPI cells were seeded at 2.5×10^5 cells/mL with 400 μL per well in 24-well plates and incubated at 37 °C with 5% CO_2 for 24 h. Then, media control (50 μL) or diluted solutions containing test compounds (50 μL) were added to the corresponding wells of HAPI cells and incubated for 4 h. After the incubation, LPS (*E.coli* O111: B4) solution (50 μL) was also added to the corresponding wells to induce an inflammatory response, making a final volume of 500 μL, and incubated at 37 °C with 5% CO_2 for 20 h. Thus, the final dilution factor for the drug solution and LPS solution was 0.1 and the final concentration of LPS was also 5 ng/mL. After the incubation, the supernatant was collected as samples to run enzyme-linked immunosorbent assays (ELISAs) and treat undifferentiated and differentiated SH-SY5Y neuronal cells as described below.

Undifferentiated neuronal model

SH-SY5Y cells were seeded at 10^5 cells/mL (100 μL) on 96-well plates and incubated at 37 °C with 5% CO_2 for 24 h. To generate the neuroinflammation-induced neurodegeneration, the supernatant above the SH-SY5Y cells was removed and replaced with conditioned supernatant from the HAPI cells for 24 h. The conditioned supernatant was collected using methods described above from HAPI cells in 24-well plates after 20 h LPS treatment with or without 4 h pretreatment of resveratrol, sirtinol, EX527, AGK2, fluoxetine and ibuprofen in a series of concentrations.

Differentiated neuronal model

SH-SY5Y cells were seeded at 10^5 cells/mL (100 μL) on 96-well plates and incubated at 37 °C with 5% CO_2 for 24 h. Then, cells were treated with 10 μM of retinoic acid (RA) every other day for a 5-day period in complete medium containing 10% FBS, followed with a treatment of 50 ng/mL of brain-derived neurotrophic factor (BDNF) for three consecutive days in serum-free medium. After that, the supernatant above SH-SY5Y cells was replaced with

conditioned supernatant of HAPI cells for 24 h. The conditioned supernatant was collected using methods described above from HAPI cells in 24-well plates after 20 h LPS treatment with or without 4 h pretreatment of resveratrol, sirtinol, EX527, AGK2, fluoxetine and ibuprofen in a series of concentrations.

Quantitative real-time PCR (rt-PCR)

Following the differentiation of SH-SY5Y cells or the culturing of undifferentiated cells, the mRNA expression of the mature neuronal gene markers was tested using rt-PCR. The total RNA was extracted using TRIzol™ reagent (Invitrogen). The concentration of RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Australia). cDNA was synthesized using Verso cDNA synthesis kit (Thermo Scientific) and ProFlex PCR System (Life technology). Primer sequences (Sigman Aldrich) for rt-PCR are shown in Table 1. Rt-PCR was performed using PowerUp™SYBR™ Green Master Mix kit and QuantStudioTM Real-Time PCR systems (Thermo Fisher Scientific Australia) according to manufactures' guidelines. The thermal cycling conditions were 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The dissociation curve conditions were 95 °C for 15 s and 60 °C for 1 min followed by 95 °C for 15 min. The comparative threshold cycles (CT) or $2^{-\Delta\Delta CT}$ method was used to analyse the CT values of samples of interest and control samples. All CT values were normalized against GAPDH gene.

Resazurin cell viability assay

Resazurin (alamarBlue) assays were run to detect cell viabilities of HAPI cells and SH-SY5Y cells. The oxidized non-fluorescent blue resazurin (Sigma-Aldrich) is reduced to fluorescent dye resorufin by the mitochondrial respiratory chain of living cells (Anoopkumar-Dukie et al. 2005). After the treatments (previously described), the supernatant above the cells was removed and replaced with media

Table 1 List of oligonucleotides for gene markers used in rt-PCR of neuronal differentiation

Primer	Sequence
SYP (human)	Forward: 5′-TCCTCGTCAGCCGAATTCTTT-3'
	Reverse: 5′-CTCGCTACTTGTTCTGCAGGAA-3'
ENO2 (human)	Forward: 5′-CGGGAACTGCCCCTGTATC-3'
	Reverse: 5′-CATGAGAGCCACCATTGATCA-3'
GAPDH (human)	Forward: 5′-TGCACCACCAACTGCTTAGC-3'
	Reverse: 5′-GGCATGGACTGTGGTCATGAG-3'

containing diluted resazurin (44 μM) and incubated in the dark at 37 °C with 5% CO_2 for 3 h. Then, fluorescence intensity (excitation: 530 nm and emission: 590 nm) was determined and recorded using a Tecan Infinite 200 Pro microplate reader (Tecan, Australia).

Measurement of the release of PGE₂, TNF-α, IL-1β and IL-10

To investigate the effect of resveratrol, sirtinol, EX527, AGK2 and reference drugs on the production of inflammatory mediators, as previously described, HAPI cells were seeded at 2.5×10^5 cells/well on 24-well plates and incubated at 37 °C with 5% CO_2 for 20 h. The test solution was added to HAPI cells and incubated for 4 h. After the incubation, LPS solution was added to the cells (final concentration: 5 ng/mL) and incubated at 37 °C with 5% CO_2 for 20 h. For measurement of PGE_2 production in HAPI cells after LPS challenge, prostaglandin E_2 (PGE_2) express ELISA kit (Cayman 500141, Australia) was used to conduct the assay. For measurement of cytokine production of HAPI cells after LPS stimulation, rat TNF-α ELISA kit (Biosensis BEK-2101, Australia), rat IL-1β ELISA kit (Biosensis BEK-2309, Australia) and rat IL-10 ELISA kit (Biosensis BEK-2047, Australia) were used in line with the manufacturer's instructions. Absorbance was measured using a Tecan infinite 200 microplate reader.

Measurement of intracellular oxidative stress production

HAPI cells were seeded at 2×10^4 cells/well on 96-well plates and incubated at 37 °C with 5% CO_2 for 20 h. Then, the pretreatment solution was added to the HAPI cells and incubated for 4 h. After the incubation, the LPS solution was added to each well to challenge the HAPI cells and incubated at 37 °C with 5% CO_2 for 20 h. After that, reactive oxygen species (ROS) production was measured by conducting a 2,7-dichlorofluorescin diacetate (DCFH-DA) assay, whereby ROS oxidizes DCFH-DA into the highly fluorescent compound dichlorofluorescein (DCF). The supernatant above the HAPI cells was removed and discarded. The diluted DCFH-DA solution (10 μM) was added to respective wells and incubated at 37 °C with 5% CO_2 for 1 h and protected from the light. Then, the solution above the cells was replaced with 200 μL of PBS and washed out. Following this, PBS (100 μL) was added into each well. Fluorescence (excitation: 485 nm and emission: 535 nm) was measured and recorded using a Tecan Infinite 200 Pro microplate reader (Tecan, Australia).

Measurement of caspase 3/7 activity

SH-SY5Y cells were seeded at 10^5 cells/mL (100 μL) on 96-well plates and incubated at 37 °C with 5% CO_2 for 24 h. For the undifferentiated cells, the supernatant of HAPI cells after the LPS challenge was transferred onto the cells in the 96-well plates and incubated for 24 h. For the differentiated model, SH-SY5Y cells were induced to differentiate after seeding for an 8-day period, and then underwent the same treatment as for the undifferentiated cells. After that, caspase 3/7 assays (Cayman Chemical, Australia) were conducted to measure the apoptosis level of cells. The protocol was followed according to the manufacturer's instructions. The fluorescence (excitation: 485 nm and emission: 535 nm) intensity value was read and recorded using a Tecan Infinite 200 Pro microplate reader (Tecan, Australia).

Statistical analysis

All data were analyzed by GraphPad Prism 5 and presented as mean + SD of three independent experiments. One-way ANOVA followed by Turkey's multiple comparison test was applied. Significance levels were shown as $p < 0.05$ (*, #), $p < 0.01$ (**, ##) and $p < 0.001$ (***, ###).

Results

SIRT1 and SIRT2 modulators induced cytotoxicity in HAPI and SH-SY5Y cells in a dose-dependent way

In HAPI cells, resveratrol and sirtinol had high cytotoxicity at the concentrations of 100 and 50 μM and mild cytotoxicity at 25 μM (Fig. 1a, b). EX527 and fluoxetine had high cytotoxicity at concentrations of 100, 50, and 25 μM and mild cytotoxicity at 10 μM (Fig. 1c, e). AGK2 demonstrated high cytotoxicity at 100, 50, 25, and 10 μM (Fig. 1d). Ibuprofen had only mild cytotoxicity at 100 μM (Fig. 1f). Dimethyl sulfoxide (DMSO) as the co-solvent of drug solution in each well was below < 0.01% and did not induce cytotoxicity in HAPI cells (data not shown). Non-toxic concentrations of the compounds were used in the following cellular assays.

In undifferentiated SH-SY5Y cells, all SIRT1 and SIRT2 modulators were shown to have dose-dependent cytotoxicity (Fig. 2a–d). Fluoxetine and ibuprofen did not exert marked cytotoxicity in undifferentiated SH-SY5Y cells at 10 and 1 μM (Fig. 2e, f). DMSO (< 0.01%) as the solvent of drug solution and LPS (5 ng/mL) did not induce cytotoxicity in SH-SY5Y cells (Data not shown).

SIRT1 and SIRT2 modulators inhibit LPS-induced inflammation in HAPI microglial cells

All SIRT modulators, fluoxetine and ibuprofen were shown to significantly reduce PGE_2 production by HAPI cells after LPS (5 ng/mL) challenge for 20 h ($p < 0.05$) (Fig. 3a–d). Resveratrol (20 μM), sirtinol (1–20 μM), EX527 (0.1 μM) and AGK2 (0.1 μM) strongly reduced PGE_2 production of HAPI cells after LPS stimulation to different degrees. Among these, the treatment of resveratrol at 20 μM and sirtinol at 1 μM induced 97 and 100% reductions, respectively. The antidepressant fluoxetine (1 μM) and the NSAID ibuprofen (1 μM), used as reference compounds, also were shown to have significant effects on the reduction of PGE_2 production of HAPI cells.

Resveratrol (0.1–20 μM) decreased TNF-α production by HAPI cells after LPS (5 ng/mL) challenge for 20 h ($p < 0.001$) with concentrations of 20 and 10 μM showing 95 and 90% reductions, respectively (Fig. 4a). Compared to fluoxetine (1 μM) and ibuprofen (1 μM), resveratrol (0.1–20 μM) had a more potent inhibiting effect ($p < 0.05$). Sirtinol with concentrations of 20 and 10 μM also significantly reduced TNF-α production by approximately 93% and 85%, respectively (Fig. 4b). EX527 (Fig. 4c), AGK2 (Fig. 4d), and ibuprofen had no significant effect on TNF-α production of HAPI cells after LPS treatment. However, fluoxetine at 1 μM was shown to enhance TNF-α release after LPS stimulation in HAPI cells by approximately 79% ($p < 0.05$).

IL-1β and IL-10 were not detectable in the supernatant of LPS (5 ng/mL)-activated HAPI cells. SIRT1 and SIRT2 modulators including resveratrol (0.1–20 μM), sirtinol (0.1–20 μM), EX527 (0.1, 1 μM), AGK2 (0.1, 1 μM) and fluoxetine and ibuprofen (01 μM) also did not stimulate the production of IL-1β or IL-10 in LPS (5 ng/mL)-induced HAPI cells.

SIRT1 and SIRT2 modulators affect the production of LPS-induced reactive oxidative species in HAPI cells

DCF assays were conducted to determine whether SIRT1 and SIRT2 modulators affect the production of reactive oxidative species (ROS) in LPS-challenged (5 ng/mL) HAPI cells. Resveratrol (0.1–20 μM) significantly reduced ROS production of LPS-challenged HAPI cells ($p < 0.01$), which at 20 μM was more marked than seen with fluoxetine (1 μM) or ibuprofen (1 μM) ($p < 0.001$) (Fig. 5a). In contrast, sirtinol pretreatment led to significantly higher ROS levels in LPS-challenged HAPI cells at 20 and 10 μM ($p < 0.001$) (Fig. 5b). EX527 (1 μM) significantly decreased ROS production ($p < 0.05$) (Fig. 5c), however, AGK2 was not shown to affect the ROS production of LPS-challenged HAPI cells (Fig. 5d). Fluoxetine (1 μM) and ibuprofen (1 μM) significantly reduced the ROS production of LPS-challenged HAPI cells ($p < 0.001$).
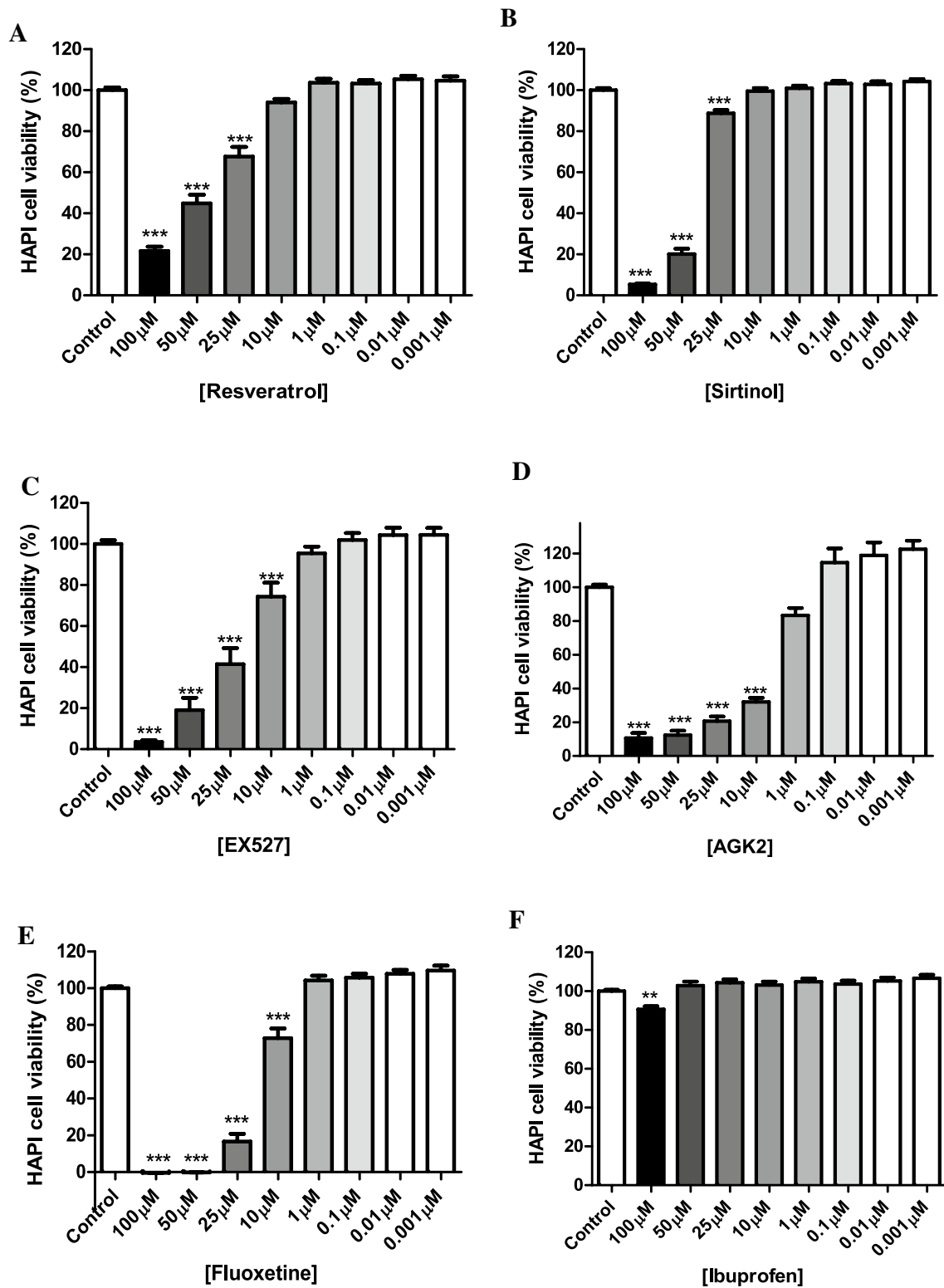
Fig. 1 SIRT1 and SIRT2 drugs induced cytotoxicity in undifferentiated SH-SY5Y cells in a dose-dependent way. Undifferentiated SH-SY5Y cells were treated with resveratrol (**a**), sirtinol (**b**), EX527 (**c**), AGK2 (**d**), fluoxetine (**e**) and ibuprofen (**f**) for 24 h and assessed for cell viability using resazurin assay

Fig. 2 SIRT1 and SIRT2 drugs induce cytotoxicity in HAPI cells in a dose-dependent way. HAPI cells were treated with resveratrol (**a**), sirtinol (**b**), EX527 (**c**), AGK2 (**d**), fluoxetine (**e**) and ibuprofen (**f**) for 24 h and assessed for cell viability using resazurin assay
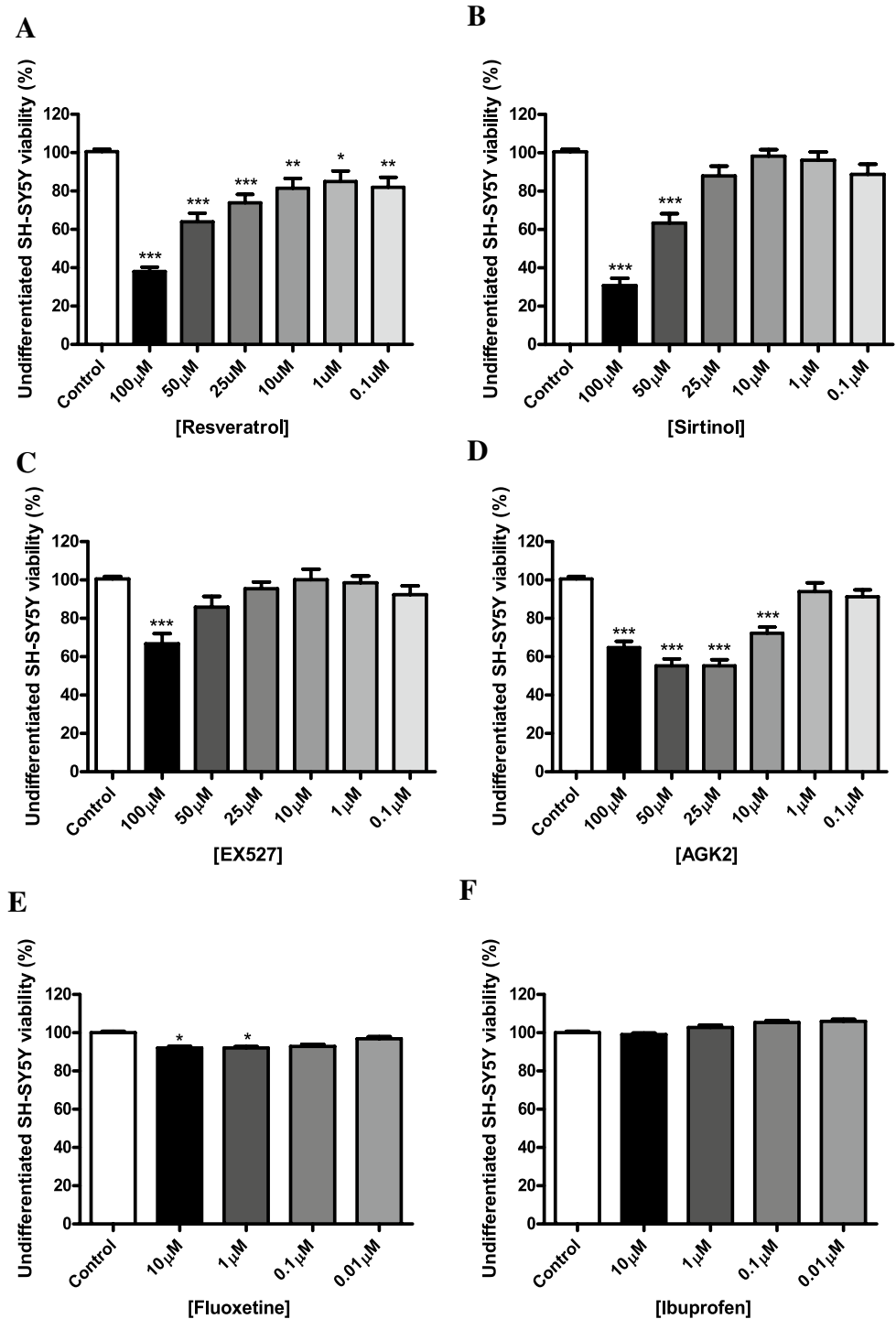
SIRT1 and SIRT2 modulators protected undifferentiated SH-SY5Y cells from death induced by HAPI-conditioned supernatant

To test whether SIRT1 and SIRT2 modulators protect SH-SY5Y neuronal cells from subsequent neurodegeneration, undifferentiated SH-SY5Y cells were incubated for 24 h with supernatant collected from HAPI cells after incubation with LPS (5 ng/mL) and the test compounds as described in the methods. Then, a resazurin assay was conducted to measure cell viability. Resveratrol pretreatment at 0.1–20 µM on HAPI cells protected undifferentiated SH-SY5Y cells against the LPS-challenged of HAPI supernatant, increasing viability by 11–21% ($p < 0.05$) (Fig. 6a). Sirtinol (0.1–20 µM) also

Fig. 3 SIRT1 and SIRT2 drugs repress LPS-induced PGE2 production in HAPI cells. HAPI cells were treated with resveratrol (**a**), sirtinol (**b**), EX527 (**c**) and AGK2 (**d**) at a series of concentrations 4 h prior to LPS (5 ng/mL) challenge for 20 h and assessed for PGE2 production using ELISA. # vs untreated media control only, * vs LPS only
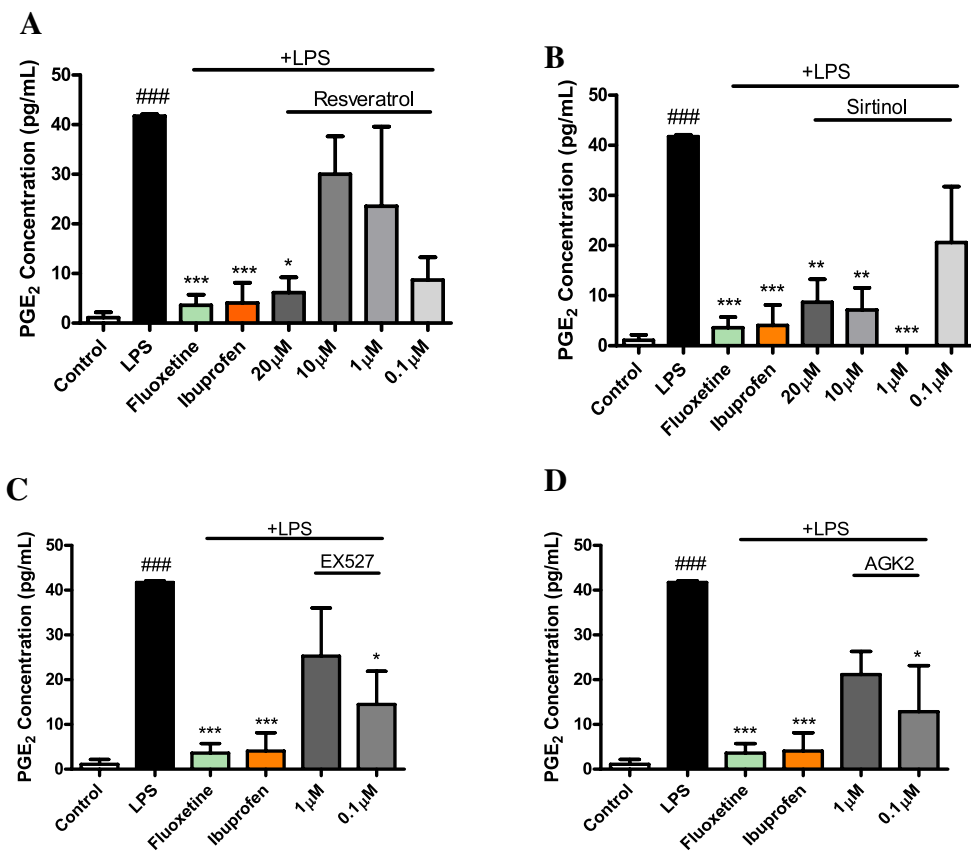

Fig. 4 SIRT1 and SIRT2 drugs repress LPS-induced TNF-α production in HAPI cells. HAPI cells were treated with resveratrol (**a**), sirtinol (**b**), EX527 (**c**) and AGK2 (**d**) at a series of concentrations 4 h prior to LPS (5 ng/mL) challenge for 20 h and assessed for TNF-α production using ELISA. # vs untreated media control only, *vs LPS only
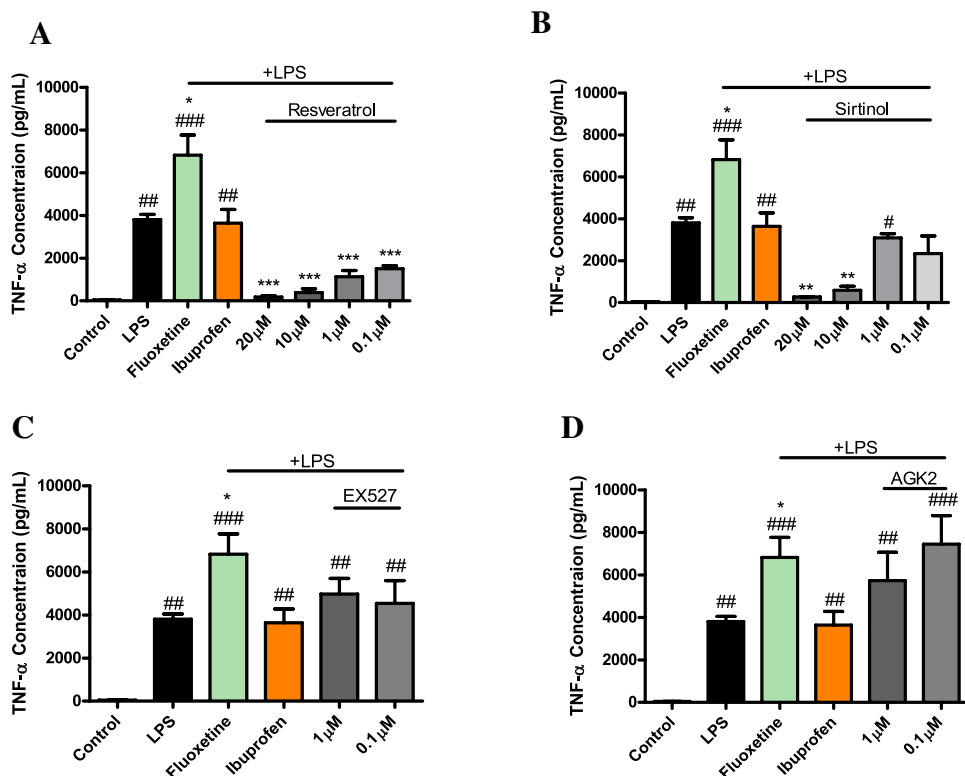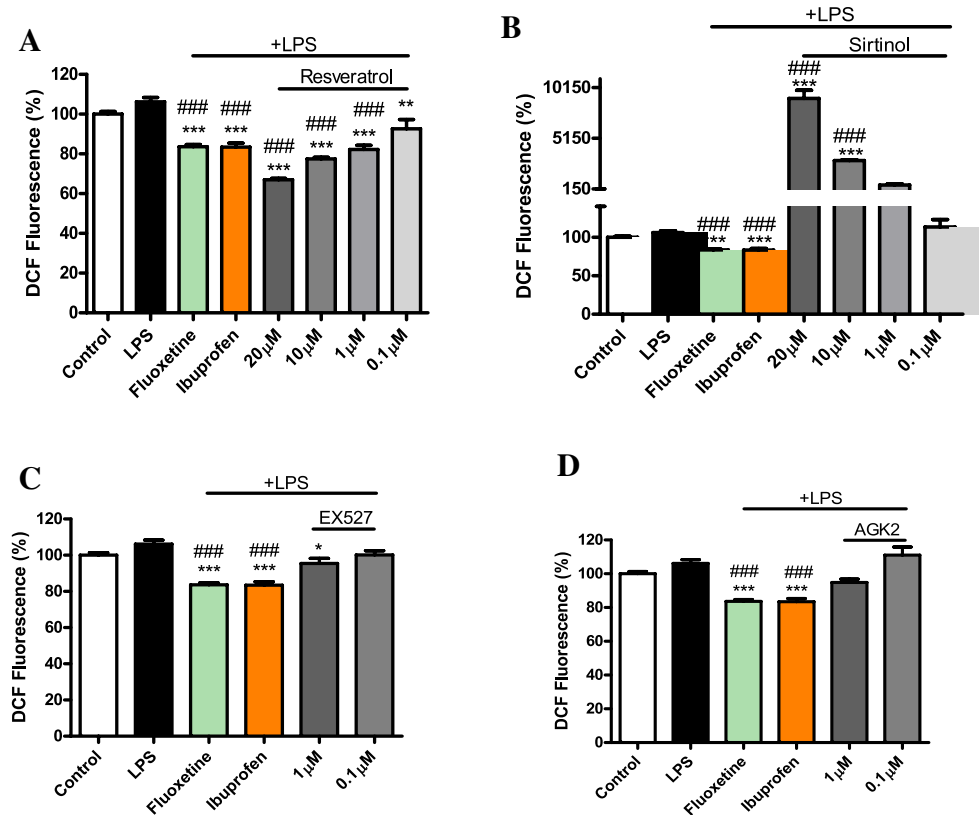
Fig. 5 SIRT1 and SIRT2 drugs affect LPS-induced oxidative stress production in HAPI cells. HAPI cells were treated with resveratrol (**a**), sirtinol (**b**), EX527 (**c**) and AGK2 (**d**) at a series of concentrations 4 h prior to LPS (5 ng/mL) challenge for 20 h and assessed for oxidative stress production using DCF assay. # vs untreated media control only, *vs LPS only

significantly improved the viability of undifferentiated SH-SY5Y cells by 15–40% ($p < 0.001$) (Fig. 6b), as did EX527 (0.1, 1 μM) and AGK_2 (0.1 μM) by 8 to 9% ($p < 0.01$) and 10% ($p < 0.001$), respectively (Fig. 6c, d). Ibuprofen (1 μM)-conditioned supernatant ($p < 0.01$) significantly decreased the toxicity to undifferentiated cells.

Sirtinol and AGK2 protected differentiated SH-SY5Y cells from death induced by HAPI-conditioned supernatant

Differentiated SH-SY5Y cells were treated with HAPI-conditioned supernatant in the same way, and resazurin assay conducted to measure the cell viability. Sirtinol (10, 20 μM) and AGK2 (0.1 μM) pretreatment on HAPI cells protected differentiated SH-SY5Y cells against the toxicity of LPS-conditioned HAPI supernatant challenge, improving cell viability by 19 to 30% ($p < 0.01$) and 20% ($p < 0.05$), respectively (Fig. 7b, d). Sirtinol at 20 μM also showed significantly greater viability protection than fluoxetine (1 μM) ($p < 0.001$) but not ibuprofen (1 μM). Resveratrol and EX527 had no significant effect on the viabilities of SH-SY5Y cells after the conditioned-supernatant challenge (Fig. 7a, c). Fluoxetine was not shown to protect the SH-SY5Y cells

from toxicity after the conditioned-supernatant challenge, whereas ibuprofen (1 μM) significantly protected differentiated SH-SY5Y cells after the conditioned-supernatant challenge ($p < 0.001$).

Resveratrol pretreatment decreased apoptosis in inflammation-challenged undifferentiated SH-SY5Y cells by attenuating caspase 3/7 activity

In undifferentiated SH-SY5Y cells, LPS (5 ng/mL)-conditioned supernatant collected from HAPI cells significantly increased the level of caspase 3/7 (Fig. 8). Resveratrol (10, 20 μM) pretreatment in HAPI cells significantly decreased the elevation of caspase 3/7 activity induced by the treatment of LPS-conditioned supernatant ($p < 0.001$) (Fig. 8a). Sirtinol, EX527, AGK2, fluoxetine (1 μM) and ibuprofen (1 μM) had no effect on reducing caspase 3/7 activities compared to LPS stimulation-conditioned supernatant (Fig. 8b–d).

In differentiated SH-SY5Y cells (Fig. 9), none of these modulators were shown to reduce caspase 3/7 activity. Indeed, EX527 (0.1, 1 μM) and AGK2 (0.1, 1 μM) significantly increased caspase 3/7 activities compared to LPS stimulation-conditioned supernatant ($p < 0.05$) (Fig. 9b, c).

Fig. 6 SIRT1 and SIRT2 drugs protected undifferentiated SH-SY5Y cells from death induced by supernatant of LPS-challenged HAPI cells. Undifferentiated SH-SY5Y cells were treated with supernatant produced by HAPI cells after treatment of resveratrol (**a**), sirtinol (**b**), EX527 (**c**) and AGK2 (**d**) at a series of concentrations 4 h prior to LPS (5 ng/mL) challenge for 20 h. # vs untreated media control only, *vs LPS only
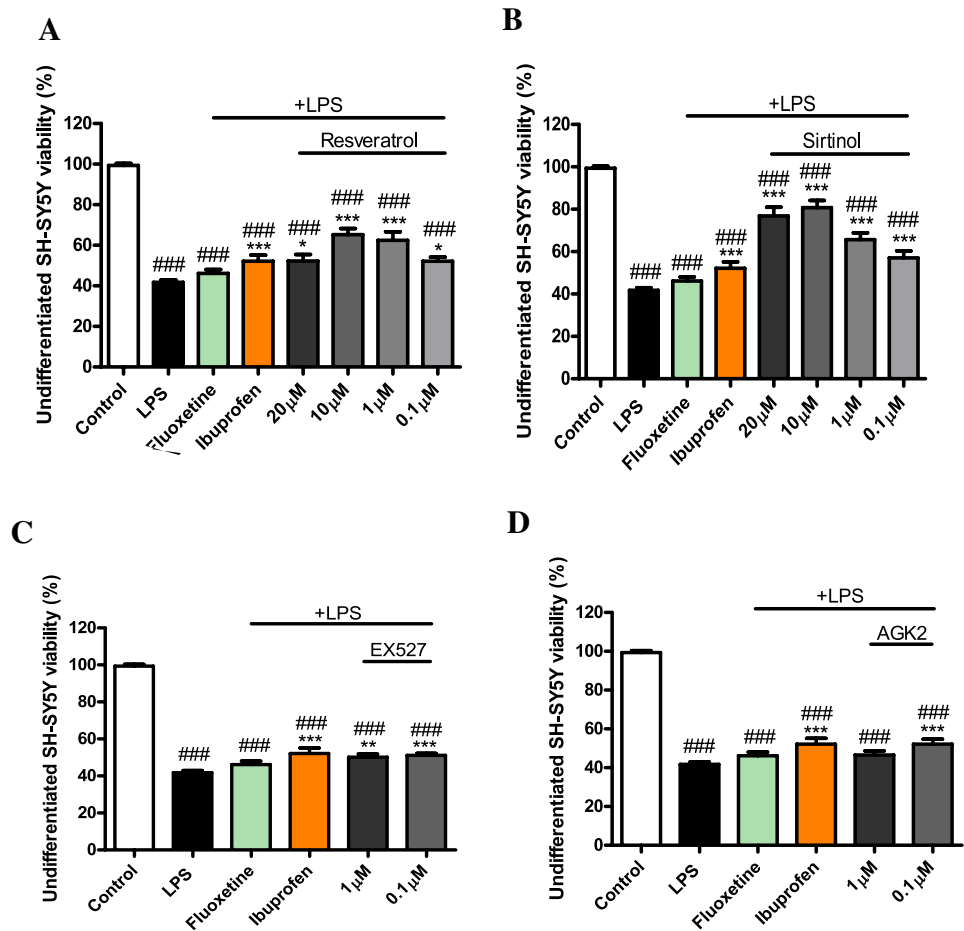
Discussion

This study first investigated and compared the effects of SIRT1 and SIRT2 modulators on the release of inflammatory mediators in LPS (5 ng/mL)-treated HAPI microglial cells. The antidepressant fluoxetine and the NSAID ibuprofen were used as controls. Resveratrol, EX527, sirtinol and AGK2 were shown to reduce the expression of PGE_2 production. However, for TNF-α production, only resveratrol and sirtinol showed significant inhibiting effects. Resveratrol's anti-inflammatory effects in HAPI cells are consistent with numerous other studies. Its protective effect against neuroinflammation has been found in different in vitro models, such as murine macrophages, mouse microglial N9 and BV-2 cell lines, working through different mechanisms including down-regulating NF-κB activation or stimulating PGC-1α expression (Bi et al. 2005; de Sá Coutinho et al. 2018).

In previous neurodegeneration studies, sirtinol was often used as a SIRT1 inhibitor and shown to reverse the protective effects of SIRT1 (Albani et al. 2009; Abe-Higuchi et al. 2016). However, in this study, it was found that sirtinol exerts significant anti-inflammatory effects in HAPI

microglial cells. EX527 is another frequently used SIRT1 inhibitor that can reverse SIRT1 activator resveratrol's protection in neurodegeneration studies (Diaz-Ruiz et al. 2015; Guo et al. 2016). However, our results showed that EX527 had a similar inhibiting effect on PGE_2 production to resveratrol. AGK2 as a SIRT2 inhibitor did not have a very promising effect on inhibiting LPS-induced inflammation. In contrast, AGK2 was found to be potentially beneficial in antagonizing neuroinflammation and neurodegeneration in previous studies. In these studies, AGK2 was reported to block LPS-induced gene expression of TNF-α and IL-6 in a neuroinflammation mouse model and inhibit astrocyte activation and production of iNOS in Aβ-induced primary astrocytes (Scuderi et al. 2014; Wang et al. 2016). Therefore, different cell lines, experimental systems, types of stimuli and animal experiments can result in different findings.

It has been reported that fluoxetine can inhibit the production of TNF-α, PGE_2 and IL-6 in the LPS-stimulated BV-2 microglial cells (Liu et al. 2011), which is in contrast to our results in HAPI cells. In our study, TNF-α production is exaggerated by fluoxetine after LPS-stimulation. It is apparent that fluoxetine can have different effects in different experimental models. For example, fluoxetine reduced

Fig. 7 Effects of SIRT1 and SIRT2 drugs on neuroinflammation-induced neurodegeneration in differentiated SH-SY5Y cells. Differentiated SH-SY5Y cells were treated with supernatant collected from HAPI cells treated with resveratrol (**a**), sirtinol (**b**), EX527 (**c**) and AGK2 (**d**) combining LPS (5 ng/mL) challenge for 24 h and assessed for cell viability using resazurin assay. #vs untreated media control only, *vs LPS only
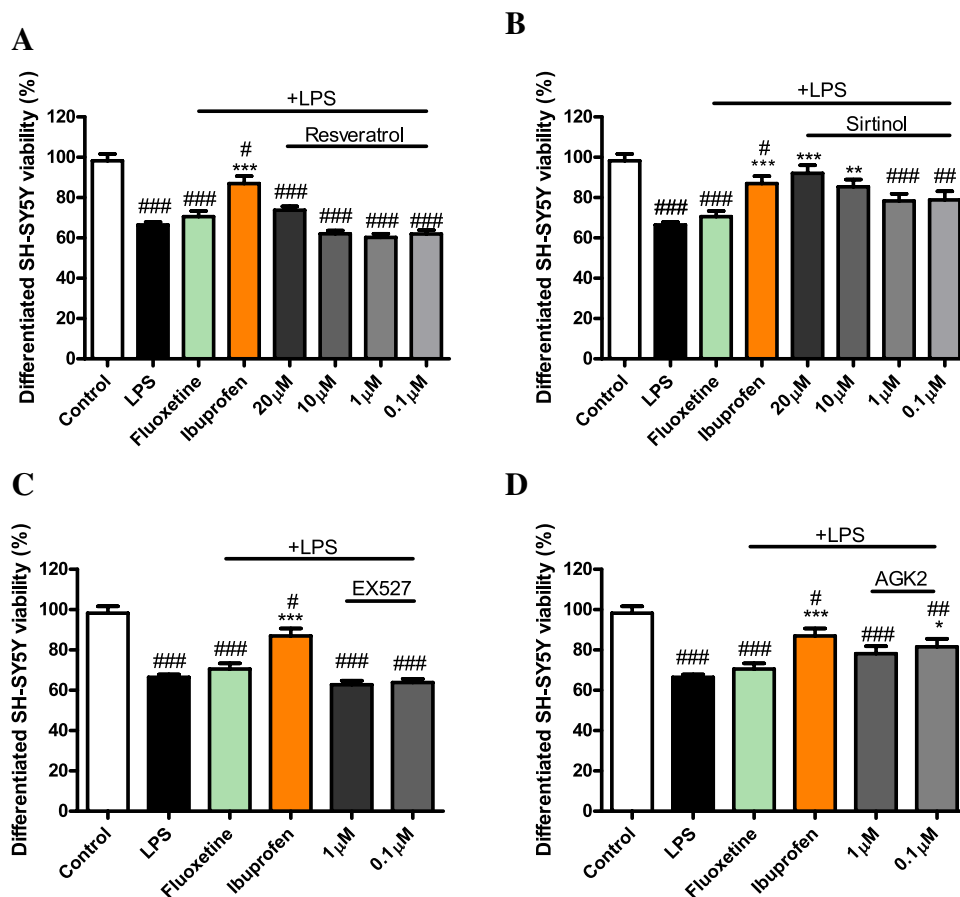

TNF-α production in LPS-induced inflammation in rats, and also had an inhibitory effect in carrageenan-induced inflammation following multiple doses, but enhanced TNF-α production in the same model if only given as a single dose (Kostadinov et al. 2015). Hence, it is apparent that the dose, frequency/duration of administration and model of inflammation will lead to contrasting observations in relation to the effects of fluoxetine on TNF-α. There are several hypotheses that have been suggested regarding the influence of fluoxetine on TNF-α in different models of inflammation including acting via the immunosuppressant properties of serotonin, inhibition of the transcription factor NF-κB and inhibition of the phosphorylation of p38 MAPK, or potentially acting as a serotonin antagonist on 5-HT$_{2A}$ receptors (Kostadinov et al. 2015). The present study does not provide data to enable the elucidation of the role of serotonin or the other mentioned pathways' participation in our model. Besides, the subtypes and numbers of 5-HT receptors expressed on microglia have not been identified (Krabbe et al. 2012). Therefore, the extent of fluoxetine's interaction with serotonin 5-HT receptors is also unknown in our study. Furthermore, fluoxetine has been shown to increase the expression of pro-inflammatory markers in experimental mice living in an enriched environment, but had an anti-inflammatory effect in mice living in a stressful condition, which further indicates that there are complex mechanisms involved (Alboni et al. 2016). The data provided by limited clinical studies are also highly variable and contradictory. Therefore, it cannot yet be concluded that the antidepressant therapeutic effect of fluoxetine is partially attributed to its anti-inflammatory effect. However, the inhibition of neuroinflammation can help to prevent the imbalanced metabolism of monoamine neurotransmitters (Miller and Raison 2016). Thus, combination treatment with antidepressant and SIRT modulators might be worth investigating in animal models and clinical studies to identify whether there are potential synergistic effects in the treatment of depression.

The subsequent neurodegeneration was assessed by treating both undifferentiated and differentiated SH-SY5Y cells with HAPI supernatant after LPS challenge with/without drug pretreatment. The SH-SY5Y cell line has neuron-like properties and has been used as a neuronal cell model in many other neurological studies (Cheung et al. 2009). The differentiation of SH-SY5Y cells aims to cease progressive proliferation and induce the expression of mature neuronal genes. However, many differentiation methods, including the one used in this study, have limitations. Therefore, we believe that using both undifferentiated and differentiated

Fig. 8 Effects of SIRT1 and SIRT2 drugs on caspase 3/7 activation in neuroinflammation-induced neurodegeneration in undifferentiated SH-SY5Y cells. Undifferentiated SH-SY5Y cells were treated with supernatant collected from HAPI cells treated with resveratrol (**a**), sirtinol (**b**), EX527 (**c**) and AGK2 (**d**) combining LPS (5 ng/mL) challenge for 24 h and assessed for caspase 3/7 activation. # vs untreated media control only, *vs LPS only
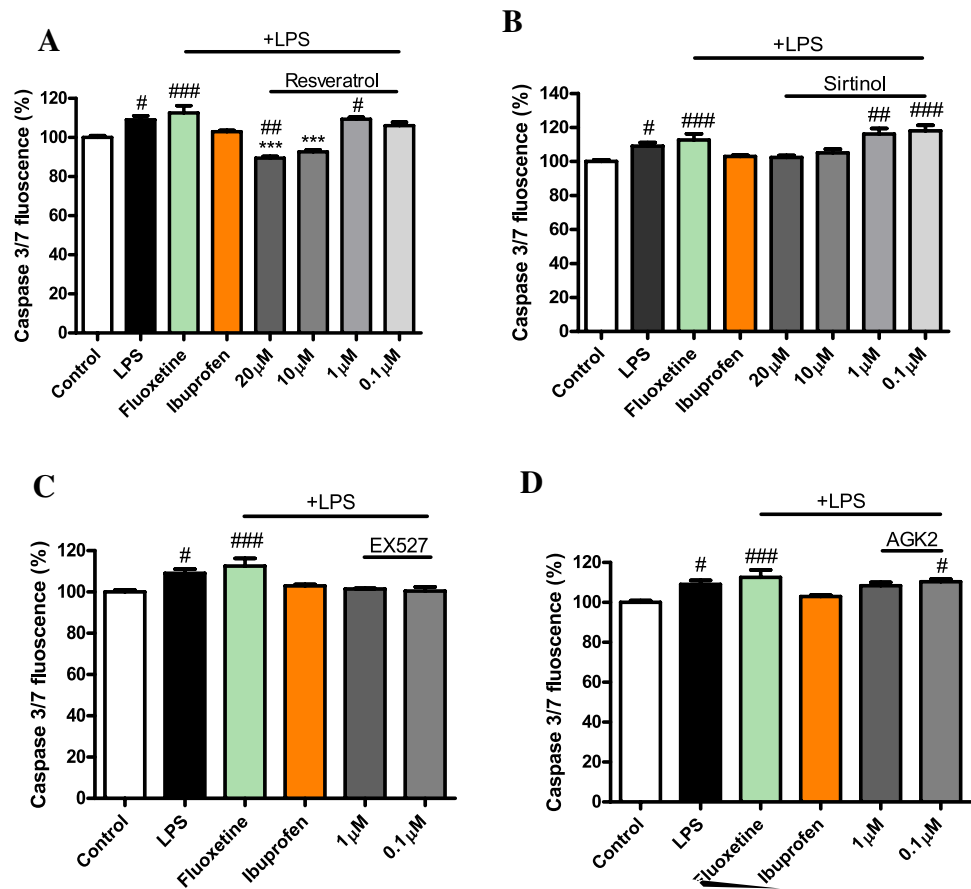

SH-SY5Y cells, we are able to present a more comprehensive data-set that will inform future work regardless of which model others are working with. It was found that undifferentiated SH-SY5Y cells are protected against the supernatant from LPS-challenged microglia by resveratrol, sirtinol, EX527 and AGK2 pretreatment. Whereas, in differentiated SH-SY5Y cells, only sirtinol and AGK2-pre-treated supernatant were shown to exert neuroprotection against LPS-induced inflammation. This indicates the importance of the differentiation of SH-SY5Y cells. Even though SH-SY5Y cells have neuronal-like traits, they still maintain the characteristics of neuroblastomas. The reduction of cell viability induced by the treatment of LPS-conditioned supernatant in undifferentiated SH-SY5Y cells may be partially due to the inhibition of aggressive cell proliferation. Therefore, it was appropriate to also test the effects of LPS-conditioned supernatant on neuronally differentiated SH-SY5Y cells. There was a difference in apoptosis activity observed in differentiated vs undifferentiated cells. LPS (5 ng/mL)-conditioned supernatant collected from HAPI cells significantly increased caspase 3/7 activity in undifferentiated SH-SY5Ycells but not the differentiated cells. This work indicates that apoptosis might not be the pathway

leading to neuronal death in a microenvironment with a mild level of neuroinflammation.

From these results, resveratrol and sirtinol exert significant inhibitory effects on the production of TNF-α and PGE$_2$ in HAPI cells, which corresponds with the result that SH-SY5Y cells are most protected by resveratrol and sirtinol pre-treated supernatant. TNF-α can induce cell death by binding with TNF receptors (Wajant et al. 2003). Thus, the reduction of TNF-α after the treatment with resveratrol and sirtinol might account for the decrease in cell death of SH-SY5Y cells. Prostaglandins have been demonstrated to play an important role in the development of chronic inflammation (Honda et al. 2006; Narumiya 2009). They also contribute to prolonging acute inflammatory responses by activating chronic gene expression through inhibiting the differentiation of Th2 cells which produce the anti-inflammatory cytokine IL-10 (Leonard 2018). Therefore, SIRT modulators' inhibiting effects on PGE$_2$ might exert potential benefits via the enhanced production of anti-inflammatory mediators.

Resveratrol, EX527, fluoxetine and ibuprofen were shown to have significant inhibitory effects on oxidative stress in HAPI cells exposed to LPS. In contrast, sirtinol caused a

Fig. 9 Effects of SIRT1 and SIRT2 drugs on caspase 3/7 activation in neuroinflammation-induced neurodegeneration in differentiated SH-SY5Y cells. Differentiated SH-SY5Y cells were treated with supernatant collected from HAPI cells treated with resveratrol (**a**), sirtinol (**b**), EX527 (**c**) and AGK2 (**d**) combining LPS (5 ng/mL) challenge for 24 h and assessed for caspase 3/7 activation. # vs untreated media control only, *vs LPS only
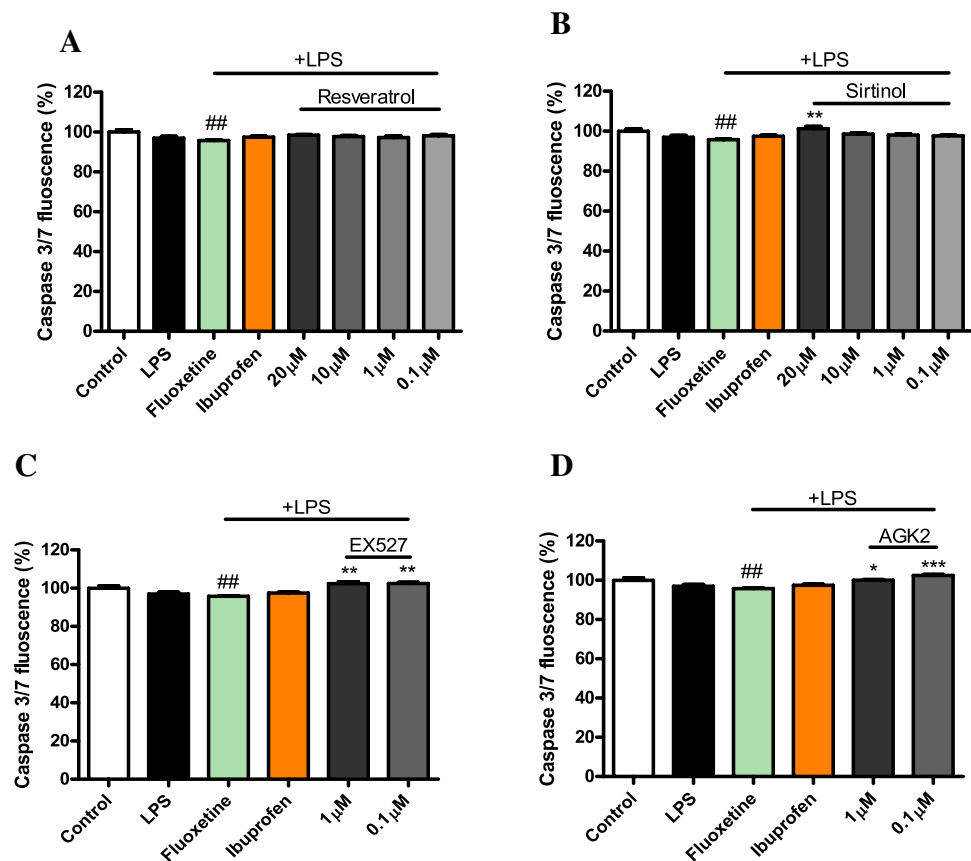
significant increase in ROS production. This result indicates that sirtinol could potentially cause damage to tissues and organ systems. Other compounds including resveratrol, EX527, AGK2, fluoxetine and ibuprofen would be expected to exert less damage to the target tissue in terms of oxidative stress.

The production of IL-1β was not detectable in LPS-activated HAPI microglial cells. The mechanism of release of LPS-induced secretion of IL-1β is unclear as it does not follow the conventional endoplasmic reticulum (ER)-Golgi route (Lopez-Castejon and Brough 2011). Under the stimulation of LPS, IL-1β is first generated as inactive precursor pro-IL-1β which is subsequently cleaved by IL-1 converting enzyme (ICE), a member of caspase-1. Caspase-1 also needs to be activated by the formation of inflammasomes that contain pro-caspase-1 (Lopez-Castejon and Brough 2011). Also, the assembly of inflammasomes is dependent upon the activation of P2X7 receptors on the cell membrane by extracellular adenosine triphosphate (ATP) (Stoffels et al. 2015). Therefore, this might be a reason why IL-1β was not detected through ELISA measurement in this LPS-induced neuroinflammation model. In this model, LPS did not result in acute cell death of HAPI cells, which suggests that there is not a large amount of ATP released following cell membrane damage to activate the P2X7 receptors on

the remaining HAPI cells leading to maturation and release of IL-1β (Sanz and Di Virgilio 2000). A different stimulus or a higher concentration of LPS would induce a significant increase in ROS, IL-1 and IL-10. However, this study was designed to mimic the mild inflammatory microenvironment of depression, not the level of inflammation that might be induced in response to a bacterial or viral infection. This is an important differentiation for researchers investigating the relationship between neuroinflammation and depression, rather than more pronounced models of inflammation.

Conclusion

This study has shown the effectiveness of SIRT1 and SIRT2 modulators and how they compare to fluoxetine and ibuprofen in inhibiting LPS-stimulated production of TNF-α and PGE$_2$ in HAPI microglial cells, and protecting SH-SY5Y cells from inflammatory insult. Besides the validation for resveratrol's potent anti-inflammatory effects in this in vitro model, dual SIRT1/SIRT2 inhibitor sirtinol's protective effects also need to be considered in future neuroinflammation studies. These findings highlight the importance of further investigating the potential therapeutic effect of SIRT1 and SIRT2 modulators and combination treatments

with current antidepressant medication in the animal studies of depression. However, further assessment of the modulators' efficacy in SIRT1 and SIRT2 activity modulation and broader screening scope of cell lines will provide additional supporting data to guide researchers to choose more effective and selective SIRT1 and SIRT2 modulators. Furthermore, other potential mechanisms besides apoptosis need to be further investigated to warrant SIRT1 and SIRT2 modulators' protective effects.

Authors' contributions YZ, SA-D, and AKD designed and conducted this study and prepared the final manuscript. SBM contributed to the establishment of the experiment model.

Declarations

Conflict of interest The authors declare no conflict of interest.

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