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Fucosterol inhibits the cholinesterase activities and reduces the release of pro-inflammatory mediators in lipopolysaccharide and amyloid-induced microglial cells

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Abstract

According to the cholinergic hypothesis, memory impairment in patients with Alzheimer's disease (AD) is associated with the deficit of cholinergic function in the brain. In addition, microglial activation plays an important role in AD by producing pro-inflammatory cytokines, nitric oxide (NO), and prostaglandin E_2 (PGE_2). It was noted that lipopolysaccharide (LPS) and β-amyloid (Aβ) induced microglial activation leading to neuroinflammation and ultimately neuronal cell death. Fucosterol, a plant sterol found in brown algae, has been reported to exhibit several bioactivities. This study aimed to investigate the anti-cholinesterase activities of fucosterol and its effects on the release of pro-inflammatory mediators by LPS- and Aβ-induced microglial cells. Cholinesterase inhibition was determined using the modified Ellman colorimetric method. Expression of pro-inflammatory mediators was determined using RT-PCR and ELISA. The NO content was determined using the Griess test. Fucosterol exhibited dose-dependent inhibitory activities against both acetylcholinesterase and butyrylcholinesterase. It significantly inhibited the production of cytokines, namely interleukins (IL-6, IL-1β), tumor necrosis factor-α (TNF-α), NO, and PGE_2 in LPS- or Aβ-induced microglial cells. Fucosterol provided protective effects against Aβ-mediated neuroinflammation by inhibiting the production of pro-inflammatory mediators. These findings provided insights into the development of fucosterol as a potential drug candidate for AD, a multifactorial neurodegenerative disorder.

Keywords Alzheimer · Anti-inflammatory · Brown algae · *Padina* · Neurodegeneration · Neurotoxicity · Pro-inflammatory cytokines

Introduction

Alzheimer's disease (AD) is an irreversible neurodegenerative disease that is characterized by progressive cognitive decline that begins with failure of forming recent memories, thus inexorably affecting all intellectual and bodily functions, leading to complete reliance on caregivers for basic daily functions and ultimately death (Alloul et al. 1998). As suggested by the cholinergic hypothesis, memory impairment in AD patients was associated with the deficiency of brain neurotransmitter acetylcholine (ACh) (Francis et al. 1999; Terry and Buccafusco 2003; Craig et al. 2011; Hampel et al. 2017). This has led to the development of cholinesterase inhibitors such as tacrine, donepezil, rivastigmine, and galantamine for the treatment of AD (Amenta et al. 2001; Gauthier 2002; Terry and Buccafusco 2003; Williams et al. 2003; Tan et al. 2014). These drugs inhibit cholinesterase enzymes that breakdown ACh and preserve the presence of ACh in the synaptic cleft, thus allow greater diffusion and half-life of ACh which result in improved cholinergic neurotransmission.

In addition, the amyloid cascade hypothesis proposes that the dysregulation in amyloid precursor protein (APP) results

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in excessive accumulation of β-amyloid (Aβ) peptides in the brain of AD patients. This leads to the deposition of senile plaques and eventually neuronal death (Selkoe 1999; Selkoe and Hardy 2016). Neurotoxicity could also be due to presence of soluble amyloid oligomers (Lambert et al. 1998; Lesné et al. 2006; Ferreira et al. 2015; Yang et al. 2017). Aβ toxicity subsequently resulted in the generation of reactive oxygen species (Butterfield et al. 2013; Cheignon et al. 2018) and loss of synapses (Dumery et al. 2001; Spires-Jones and Hyman 2014). Not only its direct toxic effects on neuronal cells, Aβ could also activate microglia cells and induce neuroinflammation (Yates et al. 2000; Rodríguez et al. 2010; Selkoe and Hardy 2016). Aβ was found to induce inflammation via the activation of the p38MAPK pathway (Giovannini et al. 2002). Chronic microglia activation by Aβ and lipopolysaccharides (LPS) triggered the generation of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukins (IL-1β, IL-6), prostaglandin E_2 (PGE_2), and nitric oxide (NO) which could lead to neuroinflammation and ultimately neuronal cell death (Qin et al. 2007; Heneka et al. 2015; Lukiw 2016).

Several studies have revealed the potential application of marine algae in the development of drugs for neurodegenerative disorders (Alghazwi et al. 2016). Algal extracts and compounds exhibited anti-oxidative (Ye et al. 2009; Sevevirathne et al. 2012; Farasat et al. 2014), anti-inflammatory (de Souza et al. 2009; Kim et al. 2013; Kim et al. 2014), anti-cholinesterase (Stirk et al. 2007), and neuroprotective properties (Shimizu et al. 2015; Tirtawijaya et al. 2016; Mohibbullah et al. 2018). To date, various studies have investigated the bioactivities of *Padina australis*, a brown alga of the Dictyotaceae family. Its extracts exhibited anti-bacterial, anti-neuroinflammatory, anti-oxidative, anti-radical, and anti-cholinesterase properties (Gany et al. 2014; Murugan et al. 2015; Zailanie 2016). Fucosterol, which is mostly found in brown algae including *P. australis*, has been reported to exhibit several biological activities such as anti-cholinesterase (Yoon et al. 2008), anti-oxidative (Lee et al. 2003), anti-diabetic (Lee et al. 2004), and anti-inflammatory (Yoo et al. 2012; Jung et al. 2013). Therefore, this study aimed to assess the cholinesterase inhibitory properties of fucosterol as well as its effects on the release of pro-inflammatory mediators by LPS- or Aβ-induced microglial cells. This study provides an insight into the potential neuroprotective effects of fucosterol against neuroinflammation which is associated with neurodegenerative disorders such as AD.

Materials and methods

Materials

Acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel), butyrylcholinesterase (BuChE) from equine serum, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, butyrylthiocholine iodide, galanthamine hydrobromide, LPS, fucosterol, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA). Human amyloid-β ($Aβ_{1-42}$) was provided by GenScript (USA). Extraction solvents (hexane, dichloromethane, ethyl acetate, methanol), silica gel 60 (mesh size 0.063–0.200 mm), and thin layer chromatography (TLC) were from Merck, USA. Griess reagent assay kit and enzyme-linked immunosorbent assay kits (ELISA) for IL-1β, IL-6, TNF-α, and PGE_2 were purchased from R&D systems (USA) and Qiagen (USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and penicillin/streptomycin were provided by Lonza (USA). C8-B4 microglia cell line (ATCC CRL-2540) was from ATCC, USA while murine microglia BV-2 cell line (C57BL/6, ATLO3001) was from Interlab Cell Line Collection, Italy. RNeasy plus mini kit, QuantiNova reverse transcription kit, Quantitect primer assay and QuantiNova SYBR green polymerase chain reaction (PCR) kit were supplied by Qiagen (USA).

Isolation of fucosterol from *P. australis*

Fresh *Panida. australis* was collected from Semporna, Sabah, in October 2012. The samples were authenticated by Professor Phang Siew Moi of University Malaya, Malaysia. Voucher specimen (PSM12861) was deposited at the University of Malaya. The fresh samples were cleaned, dried, and ground to fine powder (680 g). The algal powder was subjected to dichloromethane extraction yielding 7 g of crude extract. The crude extract was subjected to silica gel column chromatography, eluted with hexane/ethyl acetate (8:2, 7:3, 6:4, and 5:5 *v*/*v*), and exhausted with ethyl acetate yielding 138 fractions (30 mL each). These fractions were monitored by thin layer chromatography (TLC) and fractions 37–40 gave white crystalline solids identified as fucosterol (41 mg) using NMR (^{1}H and ^{13}C) and GC/MS. The GC-MS spectrum yielded an [M^+] ion at $m/z = 412.37$ corresponding to a molecular formula of $C_{29}H_{48}O$. In addition, data obtained from ^{1}H NMR and ^{13}C NMR were consistent with the report by Hwang et al. (2012), thus confirming the structure of fucosterol (Fig. 1).

Inhibitory effects of fucosterol from *P. australis* on cholinesterase enzymes

Cholinesterase enzyme inhibitory activities of fucosterol were determined using the Ellman's colorimetric method with modifications (Ellman et al. 1961). The reaction mixture contained 140 μL of 0.1 M PBS (pH 8.0), 20 μL of

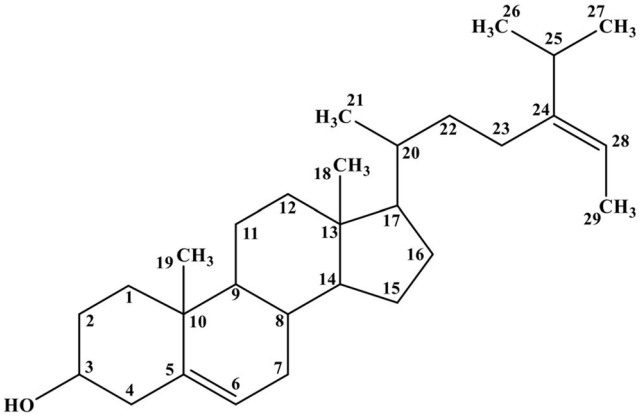

Fig. 1 Fucosterol: ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H (ppm): 5.33 (1H, br. d, J = 5.2 Hz, H-6), 5.20 (1H, q, J = 6.7 Hz, H-28), 3.54 (1H, m, H-3), 1.59 (3H, d, J = 6.7 Hz, H-29), 1.04 (3H, s, H-19), 1.01 (3H, d, H-21), 1.01(3H, d, J = 1.2 Hz, H-27), 1.00 (3H, d, J = 1.2 Hz, H-26), 0.71 (3H, s, H-18); ^{13}C-NMR(100 MHz, $CDCl_3$) δ_C (ppm): 37.2 (C-1), 31.7 (C-2), 71.7 (C-3), 42.4 (C-4), 140.6 (C-5), 121.7 (C-6), 31.9 (C-7), 31.9 (C-8), 50.4 (C-9), 36.5 (C-10), 21.1 (C-11), 39.7 (C-12), 42.2 (C-13), 56.7 (C-14), 24.4 (C-15), 28.3 (C-16), 55.8 (C-17), 11.8 (C-18), 19.4 (C-19), 36.4 (C-20), 18.7 (C-21), 35.2 (C-22), 25.7 (C-23), 146.7 (C-24), 34.8 (C-25), 22.1 (C-26), 22.2 (C-27), 115.8 (C-28), 13.2 (C-29)

fucosterol at various concentrations, and 20 μL of AChE or BuChE (each with a stock concentration of 0.3125 U mL^{-1}). The mixture was incubated for 15 min at room temperature. Thereafter, 10 μL of 0.5 mM DNTB was added and the reaction was initiated with the addition of 10 μL of 0.5 mM acetylthiocholine iodide or butyrylthiocholine iodide. The reaction was monitored by following the formation of yellow 5-thio-2-nitrobenzoate anion at 405 nm using a microplate reader (Infinite F200, Tecan). Galanthamine served as positive control. The percentage of AChE or BuChE inhibition was calculated using the formula $\frac{(E-S)}{E} \times 100$, where E is the absorbance of control (without fucosterol) while S is the absorbance of sample with fucosterol at different concentrations.

Effects of fucosterol from *P. australis* on the viability of C8-B4 cells

The C8-B4 microglia cells were cultured in DMEM supplemented with 10% FBS and 1% (*v*/*v*) penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO_2. Microglia cells at a cell density of 2×10^4 cells well^{-1} were treated with various concentrations of fucosterol or LPS (0.1 μg mL^{-1}) for 24 h prior to the addition of 20 μL of 0.5 mg mL^{-1} MTT. After incubation for 3 h at 37 °C and 5% CO_2, the supernatant was removed and DMSO (100 μL) was added to dissolve the purple crystal (formazan). Absorbance was determined at a test wavelength of 570 nm and a reference wavelength of 630 nm using a microplate reader.

Effects of fucosterol from *P. australis* on the production of pro-inflammatory mediators in LPS-induced C8-B4 cells

C8-B4 microglial cells (2×10^4 per well) were pretreated with various concentrations of fucosterol for 0.5 h prior to the addition of LPS (0.1 μg mL^{-1}). After 24 h of incubation at 37 °C, the ELISA (R&D systems) were conducted to determine the levels of TNF-α, IL-1β, IL-6, and PGE_2 in the cell supernatants. In addition, accumulated nitrite in the cell supernatants was measured using the Griess reagent assay kit.

Effects of fucosterol on the production of NO in Aβ-induced BV-2 cells

Fucosterol (CAS number 17605-67-3; Sigma-Aldrich, USA) was used to assess its effects on Aβ-induced murine microglia BV-2 cells. It was used as a commercially available alternative source of fucosterol to assess the potential neuroprotective effects of fucosterol. BV-2 cells were cultured in DMEM supplemented with 10% FBS and 1% (*v*/*v*) penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO_2. In order to study the cytotoxicity effects of fucosterol on BV-2 cells, the cells were seeded overnight at a density of 5×10^3 cells well^{-1} in a 96-well plate. After 24 h, the cells were treated with different concentrations of fucosterol for 24 h. The cell viability was determined using the MTT assay.

To study the effects of fucosterol on the production of NO in Aβ-induced BV-2 cells, $A\beta_{1\text{-}42}$ was dissolved in DMSO and diluted with PBS into 20 μM. The Aβ stock solution was incubated at 4 °C for 24 h and stored at − 20 °C until use. BV-2 cells were seeded overnight at a density of 4×10^5 cells well^{-1} in a six-well plate. After 24 h, the cells were pretreated with various concentrations of fucosterol for 4 h at 37 °C prior to the addition of 2 μM Aβ. After 24 h of incubation at 37 °C, the amount of NO was determined using the Griess reagent assay. Based on the observation, 0.2 μM fucosterol was used for the subsequent experiments.

Effects of fucosterol on the production of IL-6 and TNF-α in Aβ-induced BV-2 cells

BV-2 cells were seeded overnight at a density of 4×10^5 cells well^{-1} in a six-well plate. The cells were pretreated with 0.2 μM fucosterol for 4 h followed by treatment with 2 μM Aβ for 24 h. Total RNA was extracted from BV-2 cells using RNeasy plus mini kit and reverse-transcribed into cDNA using QuantiNova Reverse Transcription Kit. Real-time PCR assay was conducted in iQ5 cycler system (Bio-Rad, USA) using QuantiNova SYBR green PCR Kit and Quantitect primer assays for the detection of IL-6 (Mm_Il6_1_SG, catalogue no. QT00098875), TNF-α (Mm_Tnf_1_SG, catalogue no. QT00104006), and GAPDH

(Mm_Gapdh_3_SG, catalogue no. QT01658692). The PCR protocol was 95 °C for 2 min, 40 cycles at 95 °C for 5 s and 60 °C for 10 s, followed by 91 cycles at 50 °C for 10 s and 16 °C at 30 s. Data obtained for IL-6 and TNF-α were normalized with GAPDH as an endogenous control. Data were presented as fold change relative to the control group (untreated cells). In addition, the levels of TNF-α and IL-6 in cell supernatants were determined using ELISA (Qiagen).

Statistical analysis

All data were analyzed by using either the Statistical Package for Social Science (SPSS version 18) or the GraphPad Prism software. The data were analyzed using one-way ANOVA, followed by Tukey's post hoc test. Data are considered significant difference versus the respective control group if $p < 0.05$.

Results

Inhibitory effects of fucosterol from *P. australis* on cholinesterase enzymes

Fucosterol exhibited significant inhibitory activities against both AChE and BuChE in a dose-dependent manner (Fig. 2, $p < 0.05$). It was observed that at concentrations ≤ 56 μM, fucosterol exhibited greater inhibition on AChE (10.99–20.71%) when compared with its inhibitory effect on BuChE (4.53–17.53%). At the highest tested concentration (112 μM), the inhibitory effects of fucosterol on AChE and BuChE were recorded as 27.42 and 28.75%, respectively.

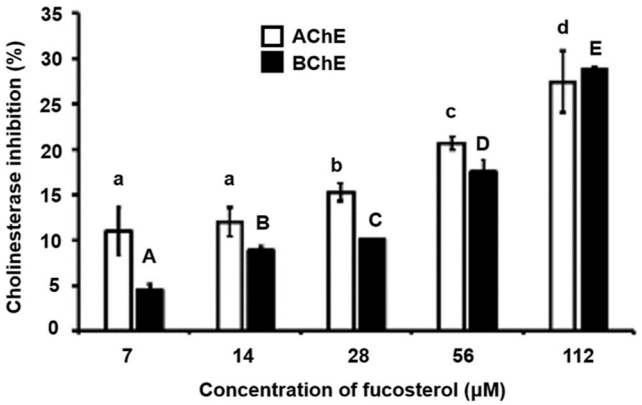

Fig. 2 Inhibitory activities of fucosterol against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Cholinesterase inhibitory assays were determined using the Ellman's colorimetric method with modifications (Ellman et al. 1961). The percentage of inhibition was calculated as $\frac{E-S}{E} \times 100$, where E is the absorbance of control (without fucosterol) while S is the absorbance of sample with fucosterol at different concentration. All values presented correspond to mean ± SD ($n = 6$). Treatment groups marked with the same letter are not significantly different ($p > 0.05$)

Effects of fucosterol from *P. australis* on the viability of C8-B4 cells

At concentrations ranging from 12 to 192 μM, fucosterol did not cause any significant cytotoxicity effect in C8-B4 cells when compared to control (untreated cells) (approximately 100% cell viability, $p > 0.05$; Fig. 3). Similarly, the treatment with 0.1 μg mL^{-1} LPS did not show any significant difference in percentage of cell viability when compared to control ($p > 0.05$).

Effects of fucosterol from *P. australis* on the production of pro-inflammatory mediators in LPS-induced C8-B4 cells

As shown in Fig. 4, 0.1 μg mL^{-1} LPS significantly induced the production of TNF-α (821.11 ± 21.99 pg mL^{-1}), IL-1β (964.85 ± 55.93 pg mL^{-1}), IL-6 (365.35 ± 33.20 pg mL^{-1}), PGE_2 (440.84 ± 32.58 pg mL^{-1}), and NO (55.39 ± 1.25 μM) when compared with untreated control ($p < 0.05$). The inflammatory effects of LPS were markedly suppressed when C8-B4 cells were pretreated with fucosterol for 0.5 h prior to the addition of LPS. The anti-inflammatory effects of fucosterol followed a dose-dependent manner. The levels of inflammatory mediators induced by LPS were reduced with increased concentrations of fucosterol. It was noted that fucosterol at concentrations ranging from 12 to 192 μM significantly reduced the production of IL-6 in LPS-induced C8-B4 cells while significant reduction of TNF-α, IL-1β, NO, and PGE_2 in LPS-induced C8-B4 cells was noted only when cells were pretreated with fucosterol at ≥ 48 μM ($p < 0.05$). The levels of pro-inflammatory mediators recorded for treatment groups subjected to pretreatment with fucosterol at the highest tested concentration (192 μM) prior to LPS induction were 329.7 ±

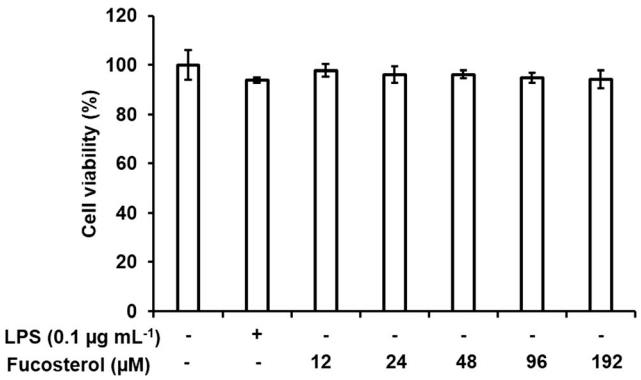

Fig. 3 Effects of fucosterol on the viability of C8-B4 microglial cells. Microglial cells were incubated with various concentrations of fucosterol or LPS (0.1 μg mL^{-1}) at 37 °C for 24 h. Control consisted of untreated cells (in the absence of LPS and fucosterol). Cell viability was evaluated using MTT assay. Data are presented as mean ± SD ($n = 6$). Percentage of viable cells in treated groups was compared with untreated control. Results were considered significant different if $^*p < 0.05$

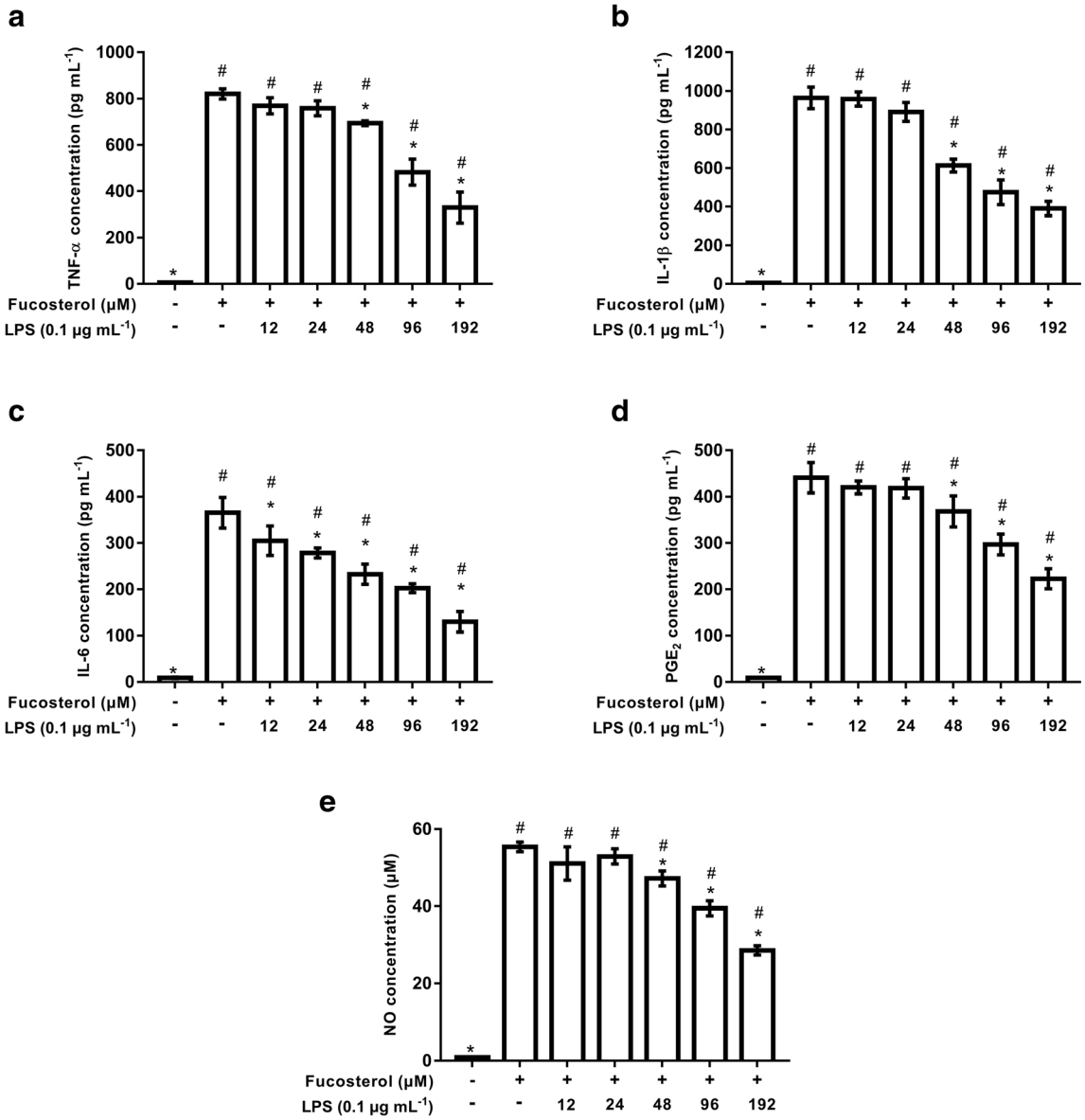

Fig. 4 Effects of fucosterol on the production of inflammatory mediators in LPS-induced microglial cells: **a** tumor necrosis factor-α, **b** interleukin-1β, **c** interleukin-6, **d** prostaglandins E_2, and **e** nitric oxide. C8-B4 cells were pretreated with various concentrations of fucosterol for 0.5 h, followed by treatment with 0.1 μg mL^{-1} of LPS for 24 h. Untreated cells and cells treated with LPS only were used as controls. Levels of TNF-α, IL-1β, IL-6, and PGE_2 were determined using the enzyme-linked immunosorbent assays (ELISA). Level of NO was determined using the Griess reagent assay. All values presented correspond to mean ± SD ($n = 6$). $^{\#}p < 0.05$ indicates a significant difference versus the untreated control group, while $^{*}p < 0.05$ indicates a significant difference versus LPS-induced group (without fucosterol)

66.55 pg mL^{-1} (TNF-α), 390.38 ± 36.96 pg mL^{-1} (IL-1β), 129.71 ± 22.12 pg mL^{-1} (IL-6), 222.77 ± 21.56 pg mL^{-1} (PGE_2), and 28.57 ± 1.22 μM (NO).

Effects of fucosterol on the production of NO in Aβ-induced BV-2 cells

Fucosterol at concentrations ranging from 0.0032 to 10 μM did not cause significant cytotoxicity to BV-2 cells (approximately 100% cell viability, $p > 0.05$; Fig. 5) when compared to untreated control. Based on this observation, three different concentrations (0.004, 0.2, and 10 μM) of fucosterol were used to assess its effects on NO production in Aβ-induced BV-2 cells. The NO level was significantly increased when BV-2 cells were treated with Aβ (14.7 μM, $p < 0.05$; Fig. 6) when compared to untreated control. However, the production of NO induced by Aβ significantly decreased when the cells were pretreated with fucosterol (approximately < 3 μM, $p < 0.05$; Fig. 6).

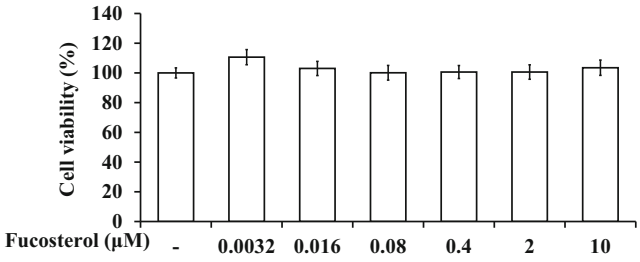

Fig. 5 Cytotoxic effects of fucosterol on BV-2 cells. BV-2 cells were exposed to different concentrations of fucosterol for 24 h. The cell viability was assessed by MTT assay. All values presented correspond to mean ± SD ($n = 12$). Percentage of viable cells in treated groups was compared with untreated control. Results were considered significant different if $^{*}p < 0.05$

Effects of fucosterol on the production of IL-6 and TNF-α in Aβ-induced BV-2 cells

Aβ significantly up-regulated the mRNA expression of IL-6 (ninefold, $p < 0.05$; Fig. 7a) and TNF-α (10-fold, $p < 0.05$; Fig. 7b) when compared to untreated cells. However, pretreatment with fucosterol ameliorated the effects of Aβ by down-regulating the expression of IL-6 and TNF-α (undetectable and twofold, respectively, $p < 0.05$ when compared with the Aβ-induced group, without fucosterol). Similarly, higher levels of IL-6 (142%, $p < 0.05$; Fig. 7c) and TNF-α (164%, $p < 0.05$; Fig. 7d) were observed in supernatants of Aβ-induced BV-2 cells when compared with untreated control. Pretreatment with 0.2 μM fucosterol reduced the Aβ-induced production of IL-6 (86%) and TNF-α (89%) when compared with Aβ-induced cells, without fucosterol ($p < 0.05$).

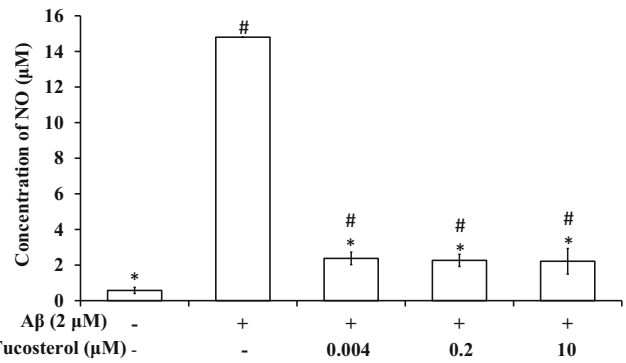

Fig. 6 Effects of fucosterol on the production of NO in Aβ-induced BV-2 cells. BV-2 cells were pretreated with various concentrations of fucosterol for 4 h, followed by 2 μM $A\beta_{1\text{-}42}$. After 24 h, the amount of NO was determined using the Griess reagent. All values presented correspond to mean ± SEM of three independent experiments ($n = 3$). $^{\#}p < 0.05$ indicates a significant difference versus the untreated control group, while $^{*}p < 0.05$ indicates a significant difference versus Aβ-induced group (without fucosterol)

Discussion

Initially, cholinergic therapy for AD focused on the inhibition of AChE, the main enzyme involved in the breakdown of ACh in the normal brain. Recently, many studies were conducted to define the roles of BuChE and its importance as a diagnostic and therapeutic target for AD (Mesulam et al. 2002; Nordberg et al. 2013; Darvesh 2016). Selective BuChE inhibition was shown to elevate brain acetylcholine, improve cognitive performance, and reduce β-amyloid peptide in rats (Greig et al. 2005). In accordance with previous study conducted by Yoon et al. (2008), fucosterol (Fig. 1) acted as a dual inhibitor for AChE and BChE. It was observed that at lower concentrations, fucosterol exhibited higher inhibition on AChE when compared to BuChE (Fig. 2). However, its inhibitory effect against BuChE increased significantly with increased concentrations. Targeting BuChE is a promising strategy for treating AD because as the disease progresses, the activity of AChE decreases significantly while the activity of BuChE increases significantly (Perry et al. 1978; Ballard 2002). Although several cholinesterase inhibitors including rivastigmine (a dual inhibitor of AChE and BuChE) are presently available for the treatment of AD, they are associated with adverse effects (Hansen et al. 2008; Tan et al. 2014; Ali et al. 2015). It is of paramount importance to discover and develop new compounds derived from the natural product that demonstrate dual cholinesterase inhibitory properties yet with lesser side effects. Thus, the dual cholinesterase inhibitory effects are value-added properties of fucosterol.

On the other hand, increasing reports have supported the important role of immunological mechanisms in the pathogenesis of AD (Heneka et al. 2015). LPS contributes to neuroinflammation and neurodegeneration (Qin et al. 2007; Lukiw 2016). Neuroinflammation induced by LPS resulted in the pathogenesis of AD through increased amyloidogenic processing of APP into Aβ peptides (Lee et al. 2008). Both LPS and Aβ peptides induce microglia activation which leads to the release of pro-inflammatory mediators such as IL-6, TNF-α, and NO (Mandrekar and Landreth 2010; Krabbe et al. 2013; Cai et al. 2014; Fan et al. 2015). Consistently, in the present study, LPS and Aβ significantly induced the production of pro-inflammatory mediators in microglia cells when compared to untreated cells (Figs. 4, 6, and 7). Interestingly, fucosterol significantly inhibited the production of pro-inflammatory mediators in LPS-induced C8-B4 microglia cells (Fig. 4) and in Aβ-induced BV-2 microglia cells (Figs. 6 and 7) when compared to LPS- or Aβ-induced cells, respectively. It caused the down-regulation of pro-inflammatory gene expressions at transcriptional level (Fig. 7). Although mechanistic studies were not conducted in the present study, anti-inflammatory activities of fucosterol might be associated with the negative regulation of mitogen-activated protein kinase (p38 MAPK) and nuclear factor κ-

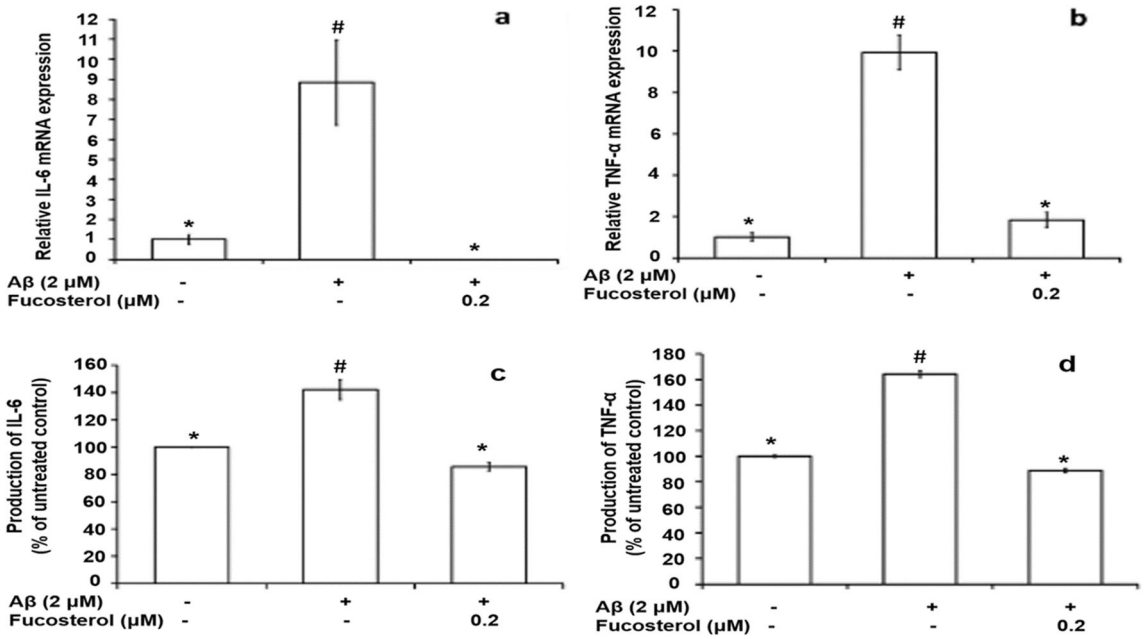

Fig. 7 Effects of fucosterol on mRNA expression and production of pro-inflammatory mediators in Aβ-induced BV-2 microglial cells: IL-6 (**a**, **c**) and TNF-α (**b**, **d**). BV-2 cells were pretreated with 0.2 μM fucosterol for 4 h, followed by 2 μM $A\beta_{1\text{-}42}$. After 24 h, supernatants were subjected to ELISA. Total RNA was extracted from BV-2 cells and subjected to RT-PCR assays. mRNA expression data were normalized with GAPDH. All values presented correspond to mean ± SEM of three independent experiments ($n = 3$). $^{\#}p < 0.05$ indicates a significant difference versus the untreated control group, while $^{*}p < 0.05$ indicates a significant difference versus Aβ-induced group (without fucosterol)

light-chain-enhancer of activated B cells (NF-κB) pathways, which are important pathways associated with neuroinflammation (Mahtani et al. 2001; Bhat et al. 2002; Bachstetter et al. 2011; Corrêa and Eales 2012; Yoo et al. 2012; Yang et al. 2014; Shih et al. 2015). It was indicated in Yoo et al. (2012) that fucosterol prevented the LPS-induced phosphorylation of p38 MAPK in RAW 264.7 macrophages by inhibiting the phosphorylations of mitogen-activated protein kinase kinases 3/6 (MKK3/6) and mitogen-activated protein kinase-activated protein kinase 2 (MK2) (Zhu et al. 2001; Zhou et al. 2014). Furthermore, phytosterols such as fucosterol were found to reduce the transcriptional activity of NF-κB (Chao et al. 2010; Yoo et al. 2012).

Many reports have shown correlations between the levels of PGE_2, TNF-α, IL-1β, and IL-6 and the progression of neurodegenerative diseases (Liang et al. 2005; Patel et al. 2005; Ramos et al. 2006; Belkhelfa et al. 2014; Rossi et al. 2014). Numerous reports have also shown that anti-inflammatory drug therapy could inhibit microglial activation and decrease the brain Aβ levels (Aisen 2002; Yan et al. 2003; Townsend and Praticò 2005). Hence, the ability of fucosterol to regulate the release of pro-inflammatory mediators by LPS- or Aβ-induced microglia suggested its potential application as an effective strategy targeting neuroinflammation (Bronzuoli et al. 2016). Although further investigations are needed to understand the neuroprotective mechanisms of fucosterol on neuronal cells against activated microglial-induced toxicity, the present study provides the basis for future research which might further confirm the neuroprotective properties of fucosterol.

Conclusion

Currently, the drugs available for treating AD are limited to three cholinesterase inhibitors (rivastigmine, donepezil, and galantamine) and memantine. In addition to associated side effects, these drugs could only delay the loss of cognitive function without offering a cure from AD. Hence, it is important to source and develop new drug candidates for the treatment of AD. Fucosterol demonstrated dual cholinesterase inhibitory properties and potential neuroprotective effects against LPS- and Aβ-induced neuroinflammation with its ability to regulate the production of pro-inflammatory mediators. This study indicated the possible application of fucosterol in the treatment of AD, a multifactorial neurodegenerative disorder.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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