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*Original Article*

# Protective effects of wogonoside against β-amyloid-induced neurotoxicity and neuroinflammation

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**Abstract:** Accumulation of beta-amyloid (Aβ) is one of the most important features of Alzheimer's disease (AD), which plays a critical role in AD-related neurotoxicity, neuroinflammation, oxidative stress and neuronal cell death. In the current study, we were aiming to investigate the effects of wogonoside on $A\beta_{1-42}$-induced neurotoxicity and neuroinflammation. We found in HT22 cells, wogonoside treatment significantly ameliorated $A\beta_{1-42}$-induced cell death, cell apoptosis and suppressed the release of lactate dehydrogenase (LDH). Moreover, we observed that in BV-2 cells, wogonoside treatment inhibited pro-inflammatory cytokines production and oxidative stress induced by $A\beta_{1-42}$. Furthermore, we found that wogonoside treatment significantly increased Bcl-2/Bax ratio, suppressed Akt/NF-κB pathway but activated Nrf2/HO-1 pathway. In conclusion, our study demonstrated that wogonoside may exert anti-apoptotic, anti-inflammatory and anti-oxidative effects against Aβ-induced neurotoxicity and neuroinflammation, and suggested wogonoside as potential drug candidate for AD.

**Keywords:** Beta-amyloid, Alzheimer's disease, wogonoside, Akt/NF-κB, Nrf2/HO-1

## Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease in the elderly [1, 2]. The main pathological features of AD are the extracellular formation of beta-amyloid (Aβ) plaques and the intracellular formation of neurofibrillary tangles [3]. Although the exact cause and related mechanisms of AD still remain largely unknown, many evidences show that neuronal apoptosis, mitochondria dysfunction and oxidative stress played critical roles in the pathogenesis of AD [4-7].

Microglia-mediated neuroinflammation, characterized by excessive microglia activation and overproduction of pro-inflammatory cytokines and chemokines, is also an important component of AD, which starts as a defense mechanism against the Aβ deposition in the brain, but can also lead to neurodegeneration [8]. Excessive activation of microglia will not only release inflammatory cytokines, but will also synthesize and release some cytotoxic factors, such as nitric oxide and reactive oxygen species, leading to significant neuronal cell damage [9]. This indicates that inhibition of microglia-mediated inflammatory responses can play a potential therapeutic role in the treatment of AD.

More than 40 flavonoids derivatives have been extracted and identified from the roots of *Scutellaria baicalensis* Georgi [10]. Among them, the most abundant content is baicalin and wogonoside [11]. Both baicalin and wogonoside were found to exhibit anti-tumor, anti-oxidative and anti-inflammatory activities [12-19]. Recently, it has been reported that baicalin has protective effects on Aβ-induced cognitive impairment, oxidative stress and neuronal apoptosis in rat [20, 21], as well as the inhibitory effects on Aβ-induced microglial cell activation [22], which suggested the protective role of baicalin in the pathogenesis of AD. However, the effect of wogonoside on AD, especially on $A\beta_{1-42}$-induced neurotoxicity and neuroinflammation, has not been reported.

In this study, we were aiming to investigate the role of wogonoside in Aβ-induced neurotoxicity and neuroinflammation in cultured HT22 hippocampal cells and BV-2 microglial cells. We found that wogonoside treatment significantly in-

creased HT22 cell viability, inhibited the release of LDH and suppressed cell apoptosis after $A\beta_{1-42}$ challenge. Furthermore, we observed that treatment of wogonoside increased the expression of Bcl-2 and decreased Bax protein level, leading to the increased level of Bcl-2/Bax ratio. Moreover, we found wogonoside treatment decreased the expression of pro-inflammatory cytokines, ameliorated $A\beta_{1-42}$-in - duced oxidative stress in $A\beta_{1-42}$-treated BV-2 cells, which may relate to the suppressed Akt/NF-κB pathway and activated Nrf2/HO-1 pathway modulated by wogonoside.

## Material and methods

### *Cells and materials*

HT22 hippocampal cells and BV-2 microglial cells were obtained from American Type Culture Collection (Manassas, VA). $A\beta_{1-42}$ was purchased from American Peptide Company (Sunnyvale, CA, USA). Wogonoside was purchased from Langze Pharmaceutical Company (Nanjing, China). Antibodies used for β-actin (#3700), Nrf2 (#12721), HO-1(#70081), Bax (#2772), Bcl-2 (#2764), p-Akt (#4060), Akt (#4685), p-P65 (#3033), P65 (#8242) were all obtained from Cell Signaling Technology.

### *Flow cytometry and caspase assay*

After pre-incubation with wogonoside followed by $A\beta_{1-42}$ treatment, HT22 cells were collected and suspended in 100 μL binding buffer. 5 μL of Annexin V-PE and 5 μL of 7-amino-actinomycin D (7-AAD) were added and the mixture was incubated in the dark for 15 min. The caspase-3 activity was examined by commercial detection kit (ab39401, Abcam) according to the manufacturer's protocol.

### *Cell viability assay and Lactate dehydrogenase (LDH) activity assay*

A commercial LDH diagnostic kit (STANBIO Laboratory, Boerne, TX, USA) was used to measure LDH activity according to the manufacturer's instructions. The procedure of cell viability assay was as follow: After pre-incubation with wogonoside followed by $A\beta_{1-42}$ treatment, 20 μL MTT was added to each well and incubated at 37°C. Four hours later, the supernatants were aspirated off and 130 μL of DMSO was added to. SpectraMax Plus384 Microplate Reader (Molecular Devices, USA) was used to detect the value of optical density.

### *ELISA and qRT-PCR*

TNF-α and IL-6 ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to measure the protein levels of TNF-α and IL-6 in cell-free supernatants. Total RNA was extracted by TRIzol reagent (Invitrogen). Synthesis of cDNA was performed with M-MLV First Strand Kit (Taraka, China). Real-time PCR was implemented with GoTaq qRT-PCR Master Mix (Promega, USA). GAPDH was used as the internal control, and the $2^{-\Delta\Delta CT}$ method was used to analyze the results. Primer sequences: TNF-α: 5'-AGG GCC ATT CCT ACT CCC AT-3' (F), 5'-TGT AGC CCC GGA TAC ACA GA-3' (R); IL-6: 5'-CAC TTC ACA AGT CGG AGG CT-3' (F), 5'-TCT GAC AGT GCA TCA TCG CT-3' (R); GAPDH: 5'-GGA GAG TGT TTC CTC GTC CC-3' (F), 5'-ACT GTG CCG TTG AAT TTG CC-3' (R).

### *Measurement of intracellular MDA and activities of SOD and CAT*

MDA level was measured with a commercial kit (Nanjing Jiancheng Biochemistry Co., Nanjing, China) according to the manufacturer's instructions. The activities of SOD and CAT were measured using colorimetric assay kits (Abcam, USA) according to the protocols provided by the manufacturer.

### *Western blot analysis*

Cell were collected and lysed with RIPA Lysis (Thermo Fisher scientific) with a protease inhibitor cocktail tablet (Roche diagnostic, Basel, Switzerland). Western blot analysis was performed as described [23].

### *Statistical analysis*

All the data were reported as mean ± S.D. and statistical differences were analyzed by one-way ANOVA followed by Turkey test. $P<0.05$ was considered statistically significant.

## Results

### *Wogonoside attenuated $A\beta_{1-42}$-induced cytotoxicity*

To investigate the effect of wogonoside on $A\beta_{1-42}$-induced neurotoxicity, we firstly exam-

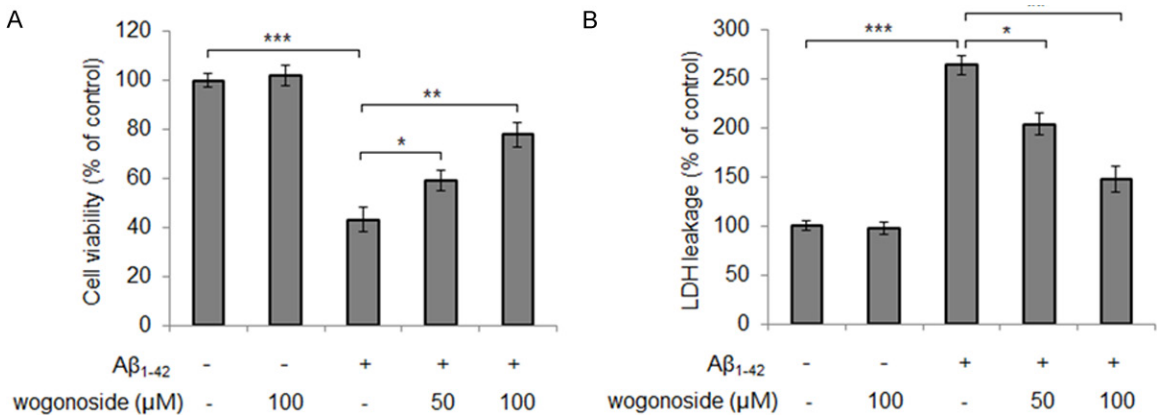


**Figure 1.** Wogonoside attenuated $A\beta_{1-42}$-induced cytotoxicity. HT22 cells were incubated without or with wogonoside (50, 100 μM) for 4.0 h, followed by incubation with $A\beta_{1-42}$ for another 24 h. A. Cell viability was determined with the MTT assay. B. The release of LDH into extracellular surrounding was measured with LDH assay. Results are shown as the mean ± S.D. and represent three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

ined the role of wogonoside in cell viability in $A\beta_{1-42}$-treated HT22 cells. As shown in **Figure 1A**, treatment of HT22 cells with $A\beta_{1-42}$ significantly decreased the level of cell viability, but when the cells were pretreated with wogonoside followed by exposure to $A\beta_{1-42}$, the level of cell viability was increased in a wogonoside dose-dependent manner. Next, we determined the level of LDH release, which is also an indicator of cell injury, from $A\beta_{1-42}$-treated HT22 cells. As shown in **Figure 1B**, the level of LDH release was significantly improved by $A\beta_{1-42}$ treatment. Conversely, in the presence of wogonoside, LDH activity was obviously reduced compared to the control group.

*Wogonoside suppressed $A\beta_{1-42}$-induced cell apoptosis*

Apoptotic cell death plays a vital role in the pathogenesis of AD. Therefore we used flow cytometry analysis to detect the effect of wogonoside on $A\beta_{1-42}$-induced cell apoptosis. Consistently with previous studies, we found $A\beta_{1-42}$ treatment indeed promoted cell apoptosis in HT22 cells. However, pre-incubation with wogonoside resulted in a significant decrease in the apoptotic rate (**Figure 2A** and **2B**). Furthermore, we examined the activity of caspase-3 and we found treatment of wogonoside decreased caspase-3 activity in a dose-dependent manner (**Figure 2C**). As shown in **Figure 2D**, we observed that wogonoside treatment significantly increased the protein level of Bcl-2 and inhibited the expression of Bax, leading to the increased Bcl-2/Bax ratio (**Figure 2E**) in $A\beta_{1-42}$-treated HT22 cells, which indicated that wogonoside could regulate $A\beta_{1-42}$-induced HT22 cell apoptosis by modulate the expression level of Bax and Bcl-2.

*Wogonoside inhibited $A\beta_{1-42}$-induced pro-inflammatory cytokines production in microglial cells*

Wogonoside has been reported to play anti-inflammatory roles in diverse disease models [12, 15]. Therefore, we examined the effect of wogonoside on $A\beta_{1-42}$-induced inflammation in BV-2 microglial cells. We found the mRNA levels of pro-inflammatory cytokines such as TNF-α and IL-6 were both up-regulated by $A\beta_{1-42}$ challenge, but pre-treatment with wogonoside significantly decreased the expression of these cytokines in a dose-dependent manner (**Figure 3A**). Consistently, the secreted protein levels of TNF-α and IL-6 in wogonoside-treated groups were also decreased compared to the control group (**Figure 3B**).

*Wogonoside attenuated $A\beta_{1-42}$-induced oxidative stress in microglial cells*

Furthermore, we examined the effect of wogonoside on $A\beta_{1-42}$-induced oxidative stress. As shown in **Figure 4**, oxidative stress was assessed by measuring the level of malondialdehyde (MDA) (**Figure 4A**), and the activities of antioxidant enzymes including superoxide dismutase (SOD) (**Figure 4B**) and catalase (CAT) (**Figure 4C**). We found pre-treatment of wogonoside significantly reduced the produc-

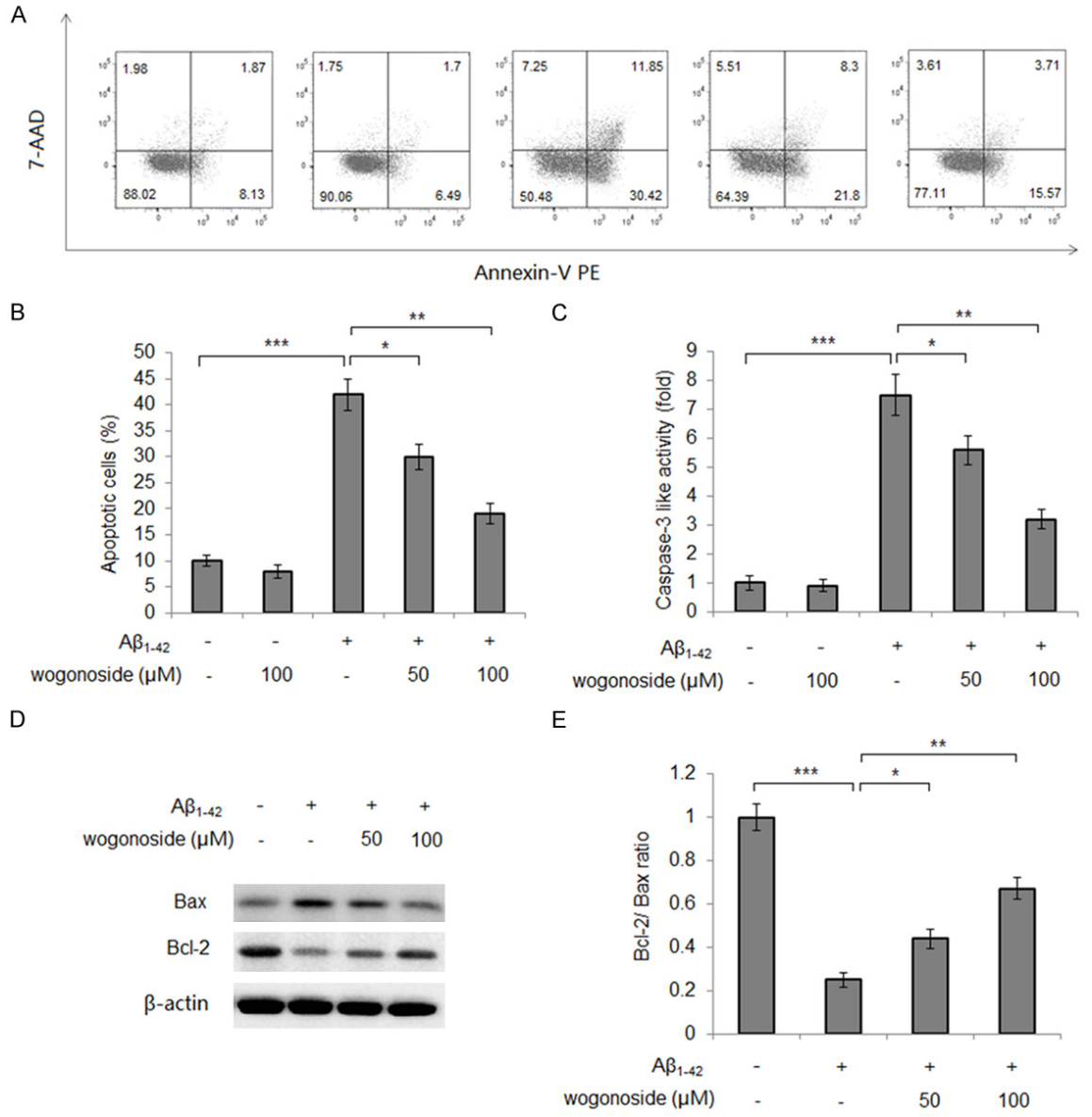


**Figure 2.** Wogonoside suppressed $A\beta_{1-42}$-induced cell apoptosis. HT22 cells were incubated without or with wogonoside (50, 100 μM) for 4.0 h, followed by incubation with $A\beta_{1-42}$ for another 24 h. (A and B) The percentage of apoptotic cells was measured by flow cytometry analysis. (C) Caspase-3 activity was detected with a fluorometric assay. (D) Protein levels of Bax and Bcl-2 were examined by western blot. (E) Quantification of protein levels of Bax and Bcl-2 in (D). Results are shown as the mean ± S.D. and represent three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

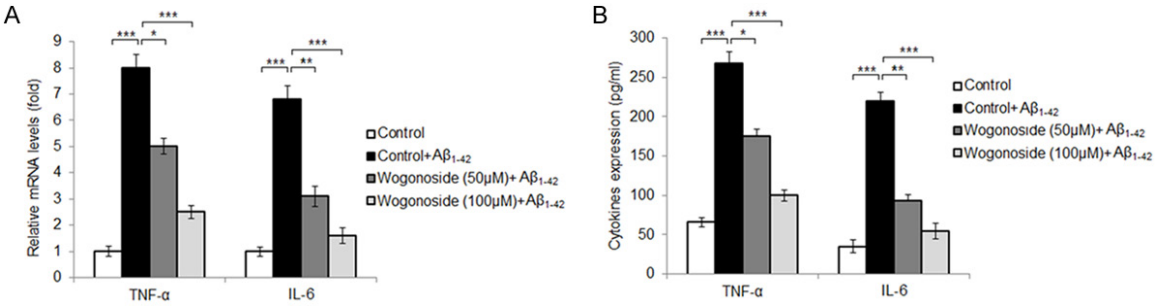

**Figure 3.** Wogonoside inhibited $A\beta_{1-42}$-induced pro-inflammatory cytokines production in microglial cells. BV-2 cells were incubated without or with wogonoside (50, 100 μM) for 4.0 h, followed by incubation with $A\beta_{1-42}$ for another 24 h. A. mRNA levels of TNF-α and IL-6 were examined by qRT-PCR. B. Protein levels of TNF-α and IL-6 were measured by ELISA. Results are shown as the mean ± S.D. and represent three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

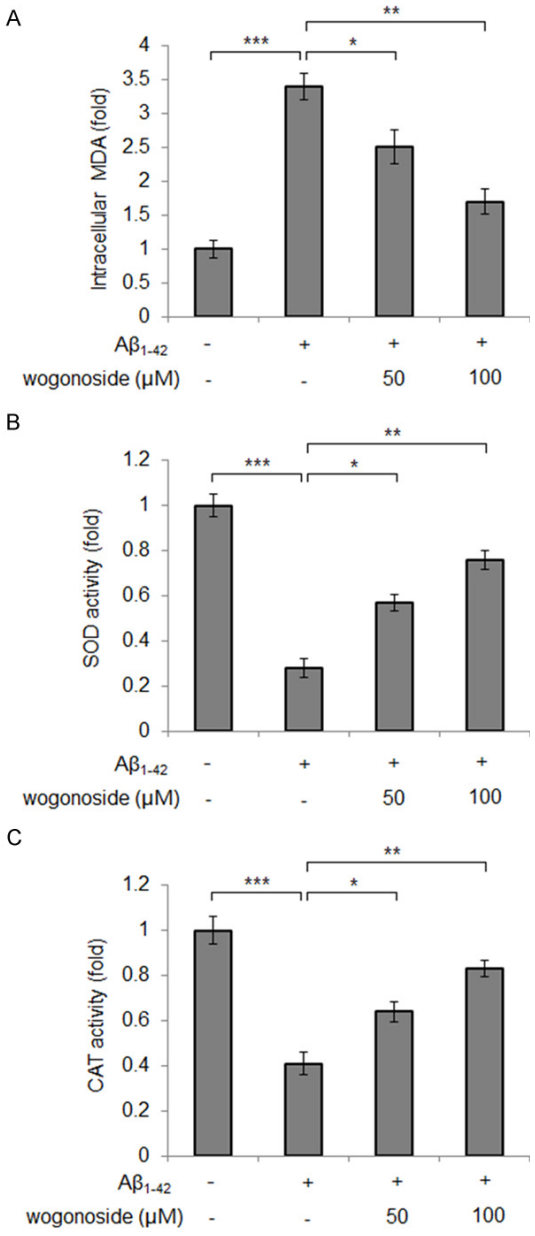


**Figure 4.** Wogonoside attenuated $A\beta_{1-42}$-induced oxidative stress in microglial cells. BV-2 cells were incubated without or with wogonoside (50, 100 μM) for 4.0 h, followed by incubation with $A\beta_{1-42}$ for another 24 h. Intracellular MDA level (A), SOD activity (B) and CAT activity (C) were detected. *, P<0.05; **, P<0.01; ***, P<0.001.

tion of the oxidative marker MDA in $A\beta_{1-42}$-treated BV-2 cells, and increased the activities of antioxidant enzymes such as SOD and CAT in a dose-dependent manner.

*Wogonoside suppressed Akt/NF-κB pathway and activated Nrf2/HO-1 pathway in $A\beta_{1-42}$-treated microglial cells*

Previous studies revealed that wogonoside plays an inhibitory role in Akt/NF-κB pathway [12, 15]. In order to investigate how wogonoside regulates the pro-inflammatory cytokines production and oxidative stress, we examined the protein levels of p-Akt, Akt, p-P65 and P65 in $A\beta_{1-42}$-treated microglial cells (**Figure 5A**). We found that the expression of p-Akt and p-P65 was significantly reduced in wogonoside-administrated BV-2 cells in a dose-dependent manner (**Figure 5B** and **5C**). It has been reported that Nrf2/HO-1 pathway plays critical roles in controlling antioxidant responses and inhibition of NF-κB signaling in microglia cells [24, 25], therefore we also examined the effect of wogonoside on Nrf2 and HO-1 expression. We found the protein levels of Nrf2 and HO-1 were both decreased in BV-2 cells after $A\beta_{1-42}$ treatment (**Figure 5A**). Most importantly, we found wogonoside could up-regulate the expression of Nrf2 and HO-1 in a dose-dependent manner (**Figure 5D** and **5E**) in $A\beta_{1-42}$-treated BV-2 microglial cells.

## Discussion

In the current study, in order to investigate the function of wogonoside in AD, we examined the effect of wogonoside on $A\beta_{1-42}$-induced neurotoxicity and neuroinflammation in cultured HT22 hippocampal cells and BV-2 microglial cells. To the best of our knowledge, this is the first article to demonstrate the protective role of wogonoside against $A\beta_{1-42}$-induced nervous system disease.

Alzheimer's disease is the most common neurodegenerative disorder in the world [26]. This disease is characterized by synaptic impairment [27], neurotrophin and neurotransmitter imbalance, mitochondrial dysfunction, oxidative stress, intracellular calcium increase and

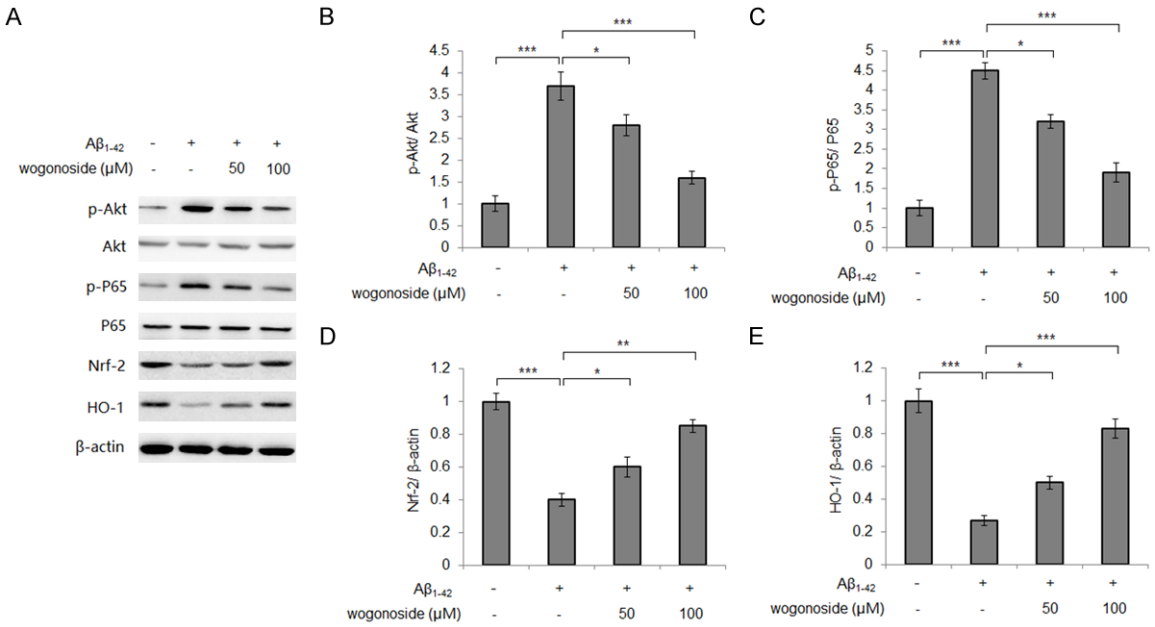


**Figure 5.** Wogonoside suppressed Akt/NF-κB pathway and activated Nrf2/HO-1 pathway in $Aβ_{1-42}$-treated microglial cells. BV-2 cells were incubated without or with wogonoside (50, 100 μM) for 4.0 h, followed by incubation with $Aβ_{1-42}$ for another 24 h. A. Protein levels of p-Akt, Akt, p-P65, P65, Nrf-2 and HO-1 were examined by western blot. B-E. Quantification of protein levels of p-Akt, p-P65, Nrf-2 and HO-1 in (A). *, P<0.05; **, P<0.01; ***, P<0.001.

cell cycle failure [28]. The most severe changes are in hippocampus, as well as in cortical and subcortical regions [29], which are associated with the AD memory deficits [30]. Abnormal cleavage of amyloid precursor protein results in the accumulation of Aβ in the brain, and leads to the pathological manifestations of AD [31]. It has been well studied that treatment with or intracerebral injection of $Aβ_{1-42}$ significantly induced neurotoxicity and AD related cognitive and cerebral changes *in vivo* and *in vitro* [32, 33]. Similar with their findings, in the current study, we found stimulated with $Aβ_{1-42}$ indeed decreased the cell viability of HT22 cells, increased the release of LDH and promoted cell apoptosis. However, we found pre-treatment with wogonoside significantly reversed these phenomenon induced by $Aβ_{1-42}$, and wogonoside treatment increased Bcl-2/Bax ratio which indicated that wogonoside could protect HT22 cells from $Aβ_{1-42}$-induced cell apoptosis and neurotoxicity.

Oxidative stress has been found to be implicated in the neurotoxicity of Aβ and in the pathogenesis of AD, and there is growing evidence that oxidative stress is closely related to the inflammatory response of microglia [34, 35]. Consistently, we found $Aβ_{1-42}$ treatment significantly increased the level of MDA and suppressed the activities of SOD and CAT in BV-2 cells. Whereas after pre-incubation with wogonoside, MDA level in BV-2 cells was greatly decreased, as well as the increased activities of SOD and CAT. Similar with oxidative stress, neuroinflammation, characterized by excessive glial activation and overproduction of pro-inflammatory cytokines and chemokines, was also found to play critical roles in the pathogenesis of neurodegeneration in AD and related neurodegenerative disorders [36]. In the current study, we observed that both mRNA and protein levels of pro-inflammatory cytokines such as TNF-α and IL-6 induced by $Aβ_{1-42}$ were significantly decreased in the presence of wogonoside. Our findings suggested that wogonoside played anti-apoptotic, anti-oxidative and anti-inflammatory roles in the protection against $Aβ_{1-42}$ stimulation.

Akt/NF-κB signaling has represented a paradigm for signal transduction and pro-inflammatory cytokines production implicated in numerous diseases including AD [37-39]. Previous studies also reported that wogonoside could inhibit Akt/NF-κB signaling activation in diverse diseases [12, 15]. Consistently, in the present research, we found wogonoside treatment greatly inhibited Akt/NF-κB pathway activation through suppressing the phosphorylation of Akt

and P65, and this inhibition may lead to the decreased production of pro-inflammatory cytokines. Nrf2 is crucial for the regulation of antioxidant genes, including HO-1 and NQO1, and it has been reported that the Nrf2/HO-1 signaling pathway plays an important role in the action of neuroprotectant [40, 41]. In this study, for the first time, we found wogonoside induced HO-1 expression and increased Nrf2 levels in a dose-dependent manner in $A\beta_{1-42}$-stimulated BV-2 cells.

In conclusion, these results indicated that wogonoside could increase Bcl-2/Bax ratio, inhibit Akt/NF-κB signaling and activate Nrf2/HO-1 pathway, therefore played anti-apoptotic, anti-oxidative and anti-inflammatory roles to protect HT22 hippocampal cells and BV-2 microglial cells against $A\beta_{1-42}$-induced neurotoxicity and neuroinflammation.

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## Disclosure of conflict of interest

None.

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