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The Protective Effects of Flavonoids from *Scutellaria Baicalensis* Georgi Stems and Leaves on Oligodendrocyte Damage Induced by A$\beta_{1\text{-}42}$

Tangtang Song[1], Yinhui Yao[1], Yazhen Shang[1,2*]

[1]Institute of Traditional Chinese Medicine, Chengde Medical College, Chengde, China
[2]Hebei Province Key Research Office of Traditional Chinese Medicine against Dementia, Hebei Province Key Laboratory of Traditional Chinese Medicine Research and Development, Hebei Key Laboratory of Nerve Injury and Repair, Chengde, China
Email: *973358769@qq.com

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Abstract

Aim: This study aimed to investigate the protective effects of flavonoids from the stem and leaves of Scutellaria baicalensis Georgi (SSFs) against A$\beta_{1\text{-}42}$-induced oligodendrocytes (OL) damage. **Methods:** Immunofluorescence was used for the detection of myelin-associated glycoprotein (MAG), a characteristic protein of rat oligodendrocytes (OLN-93 cells). To evaluate the potential protective effects of SSFs on OLN-93 cells injured by A$\beta_{1\text{-}42}$, an injury model was established by subjecting OLN-93 cells to A$\beta_{1\text{-}42}$ exposed. Cell morphology was examined using an inverted microscope, while cell viability was assessed using the colorimetric method of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Additionally, lactate dehydrogenase (LDH) was measured using the pyruvic acid reduction assay. The Ginkgo biloba leaf extract (GBE) injection was used as a positive control. **Results:** A total of >95% of the MAG immunofluorescence-positive cells were identified as oligodendrocytes. Gradually increasing concentrations of SSFs impaired the cells, and the maximum nondetrimental dose for OLN-93 cells was 75 mg/L. This study assessed the effects of SSFs on OLN-93 cells damaged by A$\beta_{1\text{-}42}$. The results indicated that SSFs significantly improved OLN-93 cell morphological abnormal changes, increased the OLN-93 cell survival rate, and reduced LDH release. **Conclusion:** SSFs can alleviate A$\beta_{1\text{-}42}$-induced damage of OL.

Keywords

Flavonoids form the Stem and Leaves of *Scutellaria baicalensis* Georgi, A$\beta_{1\text{-}42}$, Oligodendrocytes, Damage

*Corresponding author.

1. Introduction

In Alzheimer's disease (AD), memory and cognitive function deteriorate with age are characterized by a progressive nature [1]. It is a neurodegenerative disease strongly linked to aging. The formation of senile plaques via extracellular β-amyloid (Aβ), along with the formation of neurofibrillary tangles (NFTs) caused by hyperphosphorylation of intracellular tau proteins in neuronal cells, are defining pathologies of AD [2] [3] [4]. The amyloid cascade hypothesis proposes that the amyloid precursor protein (APP) is a transmembrane glycoprotein that produces Aβ in response to β- and γ-secretase, and as the amount of Aβ increases, Aβ forms oligomers that eventually aggregate to form extracellular plaques [5] [6]. Aβ_{42} is thought to have an important role in AD pathology and is the predominant form deposited in senile plaques, which is associated with its higher hydrophobicity and propensity to self-aggregation [7]. The tau protein hypothesis suggests that excessive phosphorylation of tau proteins leads to the depolymerization of normal microtubules in the brain and the formation of NFTs, which causes widespread damage such as synaptic dysfunction and neuronal death [8]. In addition to Aβ and tau protein factors, recent studies have shown that the degeneration of oligodendrocytes (OL) and myelin are closely related to AD development [9] [10] [11] [12].

Myelin comprises Schwann cells from the peripheral nervous system (PNS) and OL from the central nervous system (CNS) [13] [14]. It is a lipid-rich structure that surrounds axons, sustains the jumping transmission of information and provides nourishment to nerves [15]. The toxic effects of Aβ play a critical role in the abnormal changes in myelin and OL, with OL being more susceptible to the toxic effects of Aβ than neurons [16] [17]. Pathological alterations in myelin thickness, percentage of nuclear heterochromatin in OL, and reduced expression of myelin basic protein were observed in the medial prefrontal cortex (mPFC) in 5- and 8-month-old APP/PS1 mice [18]. Studies have shown that damage to the cortical myelin sheath occurs during the preclinical stages of AD and is associated with Aβ [19] [20]. Interestingly, recent studies have found that damage to myelin is likely to further disrupt the homeostasis of the brain's environment, accelerating the production of protein plaques, including accelerated Aβ production triggered by axonal swelling and slower Aβ clearance by microglia [9]. Over time, myelin damage can create a vicious cycle in the AD environment. Therefore, attenuating the toxic effects of Aβ on myelin and OL may be a crucial research topic for treating AD [21].

It is reported that the primary constituent of flavonoids from the stem and leave of *Scutellaria baicalensis* Georgi (SSFs) is baicalin. It has anti-inflammatory, antioxidant and neuroprotective effects. Preliminary results from our laboratory showed that SSFs significantly improved learning-related memory deficits in AD models prepared with composite Aβ [22] [23]. Additionally, SSFs inhibited the hyperphosphorylation of multiple tau protein sites and promoted nerve regeneration [24]. However, there is no report that demonstrates whether SSFs can

ameliorate memory deficits by protecting OL, and advanced to ameliorate AD. Ginkgo biloba extract (GBE) is composed primarily of ginkgo flavonoids, and studies have shown that GBE has a protective effect on OL [25]. In the present study, the damage of $A\beta_{1\text{-}42}$-induced OLN-93 cells was used to establish a mimic AD cell model to study the protective effects of SSFs, which may lay the foundation for elucidating the role of SSFs in preventing AD through affecting the OL.

2. Materials and Methods

2.1. Cells and Main Reagents

Rats' OL cell line OLN-93 (Hunan Fenghui Bio, No. CL0251); SSFs (98%, prepared in the laboratory of the Institute of Traditional Chinese Medicine, Chengde Medical College, Chengde, China) [26]; $A\beta_{1\text{-}42}$ (Dalian Meilun, No. MB10425); DMEM (High Sugar Medium, Cytiva, Cat.No. SH30022.01); Fetal bovine serum (Shanghai Xiaopeng, Cat. No. C04001-050X10); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide (MTT) (Report, Cat. No. D0801); Lactate dehydrogenase kit (LDH) (Nanjing Jiancheng, Cat. No. A020-2-2); MAG rabbit polyclonal antibodies (ABclonal, Cat. No. A16914); DAPI staining (Beijing Solarbio, Cat. No. C0065); Dylight 488 goat anti-rabbit IgG (H+L) (Boster, Cat. No. BA1127) were used the studies.

2.2. Instrumentation

CO_2 Incubator (Thermo Fisher, USA); Biological Safety Cabinets (Shanghai Lishen Scientific Equipment Co., Ltd.); Microplate Reader (Thermo Fisher, USA); Inverted Microscope (Olympus Corporation, Japan); Fluorescence Orthostatic Microscope (Olympus Corporation, Japan) were supplied for the experiment.

2.3. Experimental Method

2.3.1. Experimental Design

Figure 1 displays the experimental design of the entire study. OLN-93 cells were cultured *in vitro* and the oligodendrocyte-specific protein MAG was identified with immunofluorescence method. OLN-93 cells were exposed to different concentrations of SSFs (25, 50, 75, 100, 125 and 150 mg/L) for 24 hours. Morphological observation, cell viability and LDH release were conducted to evaluate the cytotoxic effects of SSFs on OLN-93 cells. The protective effect of SSFs on $A\beta_{1\text{-}42}$ induced OLN-93 cells was evaluated by morphological observation, cell viability, and LDH release. Ginkgo biloba leaf extract (GBE) injection was used as the positive control.

2.3.2. OLN-93 Cell Culture and Identification

The recovered OLN-93 cells were cultured in high-sugar complete DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin. One to two days for media change. The cells were cultured at 37°C under 5% CO_2, and when the cell density reached 80% - 90%, the cells were either passaged

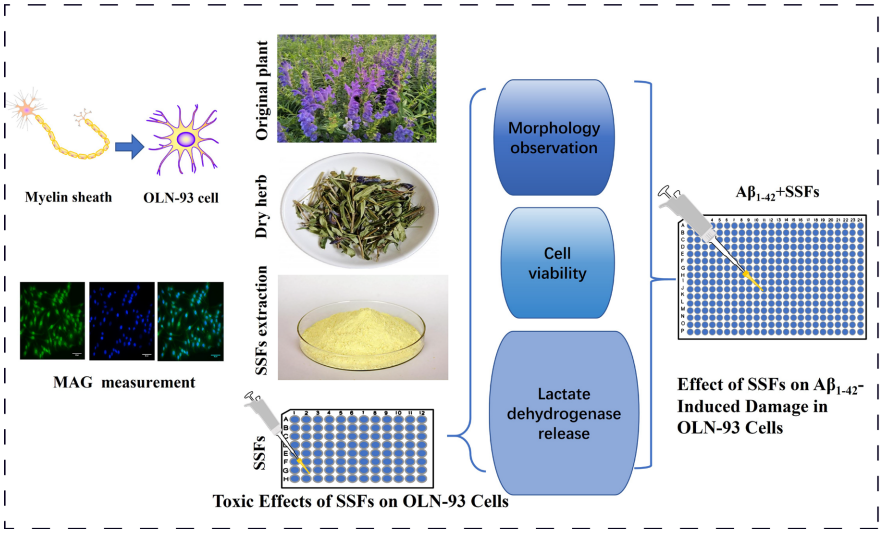

Figure 1. Experimental design.

1:3 or cryopreserved. The third generation of OLN-93 cells was inoculated at a density of 3×10^4 cells/ml into 24-well plates (500 μL per well) and cultured for 24 hours. Cell climbing slides were fixed with 4% paraformaldehyde, incubated with 0.25% Triton X-100 dropwise for 15 min, then 5% BSA dropwise for 30 minutes, and then added primary antibody MAG (dilution 1:300) overnight. On the next day, fluorescent secondary antibody Dylight488 (dilution 1:250) was added, incubated at 37˚C for 1 hour (protected from light), stained with DAPI dropwise, and finally with anti-fluorescence quencher dropwise, and photographed using a fluorescence microscope.

2.3.3. Toxicity of SSFs to OLN-93 Cells

OLN-93 cells in the logarithmic growth phase were inoculated into 96-well plates at a density of 6×10^4 cells/mL, with 100 μL per well. Concentrations of the SSFs (25, 50, 75, 100, 125, 150 mg/L) were set up, using complete culture medium as the control group. The cells were incubated for 24 hours in a cell culture incubator based on the pre-experimental screening results. The concentration at which the SSFs produced cytotoxic effects was determined by basis on the cell morphology, cell viability, and LDH release.

1) Cytomorphological Observations

After exposing OLN-93 cells to increasing concentrations (25, 50, 75, 100, 125 and 150 mg/L) of SSFs for 24 hours, the cells were observed for morphological changes and photographed using an inverted microscope.

2) Cell Viability Determination

After exposing OLN-93 cells to various concentrations of SSFs (25, 50, 75, 100, 125, 150 mg/L) for 24 hours, the culture medium was removed, and the medium was replaced with a mixture of high-glucose DMEM and 10% MTT. The cells were then incubated at 37˚C for 4 hours before being observed and photographed under an inverted microscope. The culture solution was discarded and DMSO was added for complete dissolving the purple crystals. The absorbance

(OD) of purple solution was assayed at 490 nm.

The formula is as follows:

$$\text{The cellviability rate}(\%) = \frac{(OD_e - OD_b)}{(OD_c - OD_b)} \times 100\%$$

OD_e: OD of the experimental group OD_c: OD of the control group OD_b: OD of the blank group

3) LDH Release Assay

After exposing OLN-93 cells to a range of SSFs (25, 50, 75, 100, 125, 150 mg/L) for 24 hours, the culture was aspirated and mixed. The assayed procedure of LDH was performed in accordance with the instruction provided by the reagent kit.

The formula is as follows:

$$\text{LDH activity}(U/L) = \frac{(OD_e - OD_c)}{(OD_s - OD_b)} \times 0.2 \times 1000$$

OD_e: OD of the experimental group OD_c: OD of the control group OD_s: OD of the standard group OD_b: OD of the blank group

2.3.4. A$\beta_{1\text{-}42}$-Induced OLN-93 Cell Injury and SSFs Intervention

OLN-93 cells in the logarithmic growth phase were cultured in 96-well plates at a density of 6×10^4 cells/ml (100 μl per well) for 24 hours. There are numerous reports on the damaging effects of Aβ on oligodendrocytes, and it is practical to create an AD cell injury model in which A$\beta_{1\text{-}42}$ acts on OLN-93 [27] [28]. The cells were randomly allocated into the following groups: control, model (7.5 μmol/L A$\beta_{1\text{-}42}$), 15 mg/L SSFs (7.5 μmol/L A$\beta_{1\text{-}42}$ + 15 mg/L SSFs), 30 mg/L SSFs (7.5 μmol/L A$\beta_{1\text{-}42}$ + 30 mg/L SSFs), 60 mg/L SSFs (7.5 μmol/L A$\beta_{1\text{-}42}$ + 60 mg/L SSFs), and 60 mg/L GBE (7.5 μmol/L A$\beta_{1\text{-}42}$ + 60 mg/L GBE). After continuing the culture for 24 hours, the cell morphology was observed by an inverted microscope and photographed. The measurements of cell viability and LDH release were performed as the same as above methods.

2.4. Statistical Analysis

The data was used to analyze with GraphPad Prism 8.0.1 software and expressed as the mean ± standard deviation (mean ± SD). One-way analysis of variance (ANOVA) was used to analyze the groups with homogeneous variance, whereas the nonparametric test (Kruskal-Wallis test) was applied for the groups with heterogeneous variance. $P < 0.05$ was considered as statistical significance.

3. Results

3.1. OLN-93 Morphology and Identification

Figure 2(a) displays the morphology of OLN-93 cells. Freshly resuscitated cells are small, spherical, and refractive. Upon adherence, the cell protrusions exhibited a short, pike-like, or triangular morphology that was characterized by a full

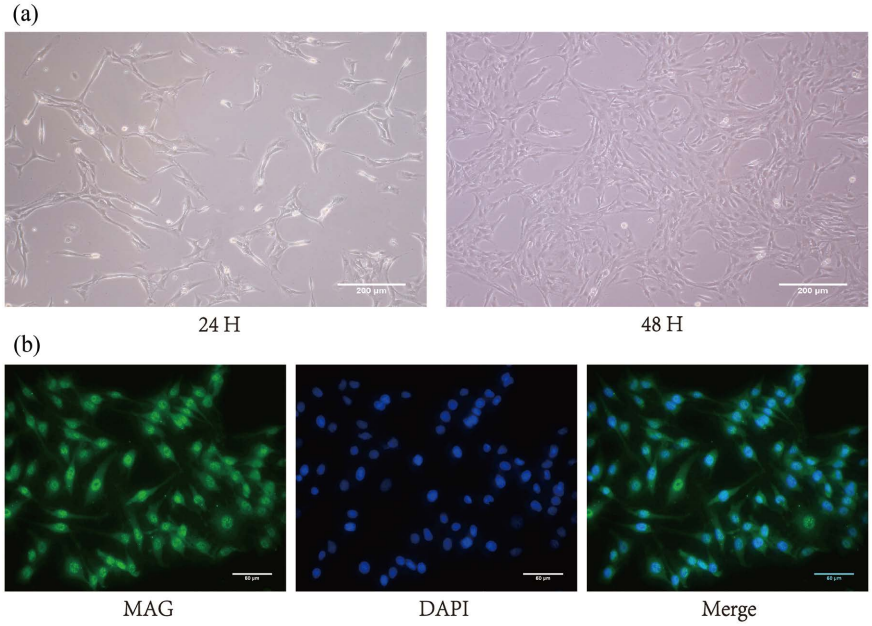

Figure 2. (a) Morphology of OLN-93 (100×, Scale bar = 200 μm). (b) Identification of OL by MAG immunofluorescence (400×, Scale bar = 50 μm).

and bright appearance. Further culture of these cells produced narrow and long protrusions, eventually connecting to form web-like structures. **Figure 2(b)** shows the results of MAG immunofluorescence staining of OLN-93 cells. The results showed that the bodies of the MAG-positive cells were long and spindle-shaped with green fluorescence, and the nuclei were large, elliptical, or rounded with blue fluorescence. The calculation showed that OLN-93 cells can be identified as OL with confidence because the number of MAG-positive cells exceeded 95%.

3.2. Toxic Effects of SSFs on OLN-93 Cells

3.2.1. Effect of SSFs on the Morphology of OLN-93 Cells

As shown in **Figure 3(a)**, OLN-93 cells exposed to 25 - 75 mg/L SSFs exhibited normal growth and intact membranes along with a bright, pike-shaped body. Additionally, reticular junctions were observed between the protruding cells. Treatment with 100 - 150 mg/L SSFs decreased proliferation, cell membrane fragmentation, shorter processes, spherical cytosol crumpling, intercellular reticular junction disruption, and death to varying degrees as the dose of SSFs increased. It is suggested that 100 - 150 mg/L SSFs can disrupt the cell structure and produce the dramatic cytotoxicity.

3.2.2. Effect of SSFs on OLN-93 Cell Viability

In **Figure 3(b)** and **Figure 3(c)**, the MTT assay results are shown for each group of cells. A negative correlation was observed between the MTT-stained area of the cells in each group of SSFs. Compared with the control group, the cell viability increased by 12% (25 mg/L SSFs, $P < 0.01$) and 10.7% (50 mg/L SSFs, $P <$

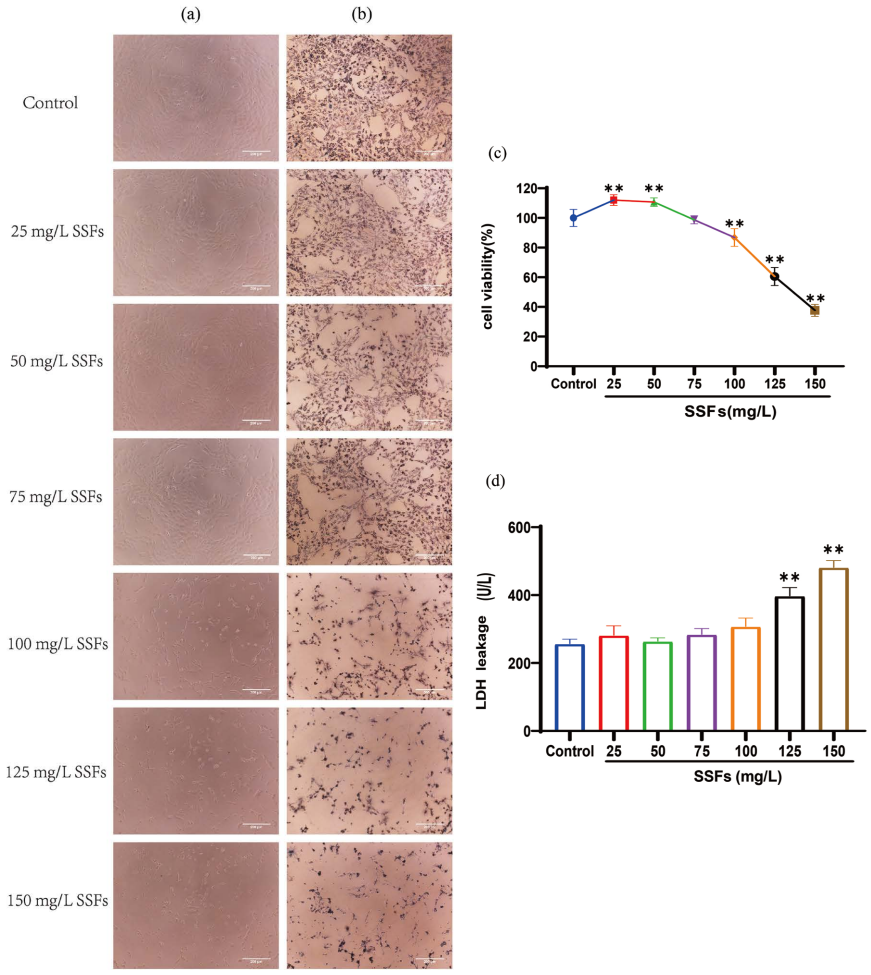

Figure 3. Effect of SSFs on OLN-93 cytotoxicity. (a) Effect of SSFs on OLN-93 cell morphology (100×, Scale bar = 200 μm). (b), (c) Effect of SSFs on OLN-93 cell viability (100×, Scale bar = 200 μm) Mean ± SD, n = 7; **P < 0.01, vs. the control group. (d) Effect of SSFs on LDH release from OLN-93 cells (mean ± SD, n = 7). **P < 0.01, vs. the control group.

0.01) at concentrations of 25 - 50 mg/L SSFs. The cell survival was reduced by 1.23% (75 mg/L SSFs, P > 0.05), 13.19% (100 mg/L SSFs, P < 0.01), 39.49% (125 mg/L SSFs, P < 0.01), and 62.29% (150 mg/L SSFs, P < 0.01) in 75 - 150 mg/L SSFs concentration range, respectively, as compared with the control group. These results suggest that SSFs appeared a significant cytotoxic effect on OLN-93 cells at a concentration of 100 - 150 mg/L.

3.2.3. Effect of SSFs on LDH Release from OLN-93 Cells

In **Figure 3(d)**, Compared with the control group, the LDH release of OLN-93 cells was not significantly change exposing to SSFs at concentrations of 25 mg/L, 50 mg/L, 75 mg/L and 100 mg/L SSFs. However, LDH release from OLN-93 cells increased 54.98% and 87.54% (all P < 0.01), respectively at 125 mg/L and 150 mg/L SSFs. These results suggest that SSFs show cytotoxic effect above 125 mg/L concentration and result in cell membrane damaged and the cell membrane permeability increased.

3.3. Effect of SSFs on A$\beta_{1\text{-}42}$-Induced Damage in OLN-93 Cells

3.3.1. Effect of SSFs on A$\beta_{1\text{-}42}$-Induced Morphological Damage to OLN-93 Cells

Figure 4(a) shows the effect of SSFs on A$\beta_{1\text{-}42}$-induced morphological damage in OLN-93 cells. The cells in the control group had a normal proliferation rate, with pike-shape, intact cell membranes, and clearly intercellular connection. In the model group, the cell outlines were unclear, swollen or vacuolization. The some cells' intercellular junctions or membranes were broken. The number of cells was significantly reduced. However, all three doses of SSFs ameliorated the A$\beta_{1\text{-}42}$-induced aberrant changes of OLN-93 cell in morphology, with hold the cells long spindle morphology, decrease the cell membrane integrity and the cell fragmentation.

3.3.2. Effect of SSFs on A$\beta_{1\text{-}42}$-Induced OLN-93 Cell Viability

The MTT cell viability results for the all groups are shown in **Figure 4(b)**. The results indicate that the MTT staining area was significantly lower in the model group as compared with the control group. However, this decreased stained area by MTT can be reversed by three doses of SSFs treated. **Figure 4(c)** shows the cell viability statistics for each group, with 34.24% reduction in the model group, as compared to the control group ($P < 0.01$). Three-dose of SSFs and GBE increased the cells survival by 7.07% (15 mg/L SSFs, $P < 0.01$), 15.12% (30 mg/L SSFs, $P < 0.01$), 23.56% (60 mg/L SSFs, $P < 0.01$) and 7.19% (60 mg/L GBE, $P < 0.01$). These results indicated that SSFs dose-dependently reversed the reduction in OLN-93 cell viability induced by A$\beta_{1\text{-}42}$.

3.3.3. Effect of SSFs on A$\beta_{1\text{-}42}$-Induced LDH Release from OLN-93 Cells

Figure 4(d) shows the effect of SSFs on A$\beta_{1\text{-}42}$-induced LDH release in OLN-93 cells. LDH release was increased 21.90% ($P < 0.01$) in the model group as compared with the control group. In contrast, the LDH release in the three-doses of SSFs and GBE groups was reduced by 11.93% (15 mg/L SSFs, $P < 0.01$), 17.21% (30 mg/L SSFs, $P < 0.01$), 14.47% (60 mg/L SSFs, $P < 0.01$), and 15.84% (60 mg/L GBE, $P < 0.01$).

4. Discussion

AD is a neurodegenerative disease characterized by the cognitive decline, which mainly be occurred in the elderly population. The contemporary researches have indicated that AD is typified by the abnormal aggregations of Aβ and tau protein hyperphosphorylation. In additional, the myelin sheaths impairment, synaptic loss, and oxidative stress also contribute to the development of AD [11] [29]. The pathogenesis and treatment of AD encompass a highly intricate process involving the multifactorial pathology, and the effective pharmaceutical intervention remains elusive [30]. As a result, AD prevention and treatment are increasingly focused on finding effective drugs [31]. SSFs are flavonoids extracted from the dried stem and leaves of Scutellaria baicalensis Georgi; these flavonoids have

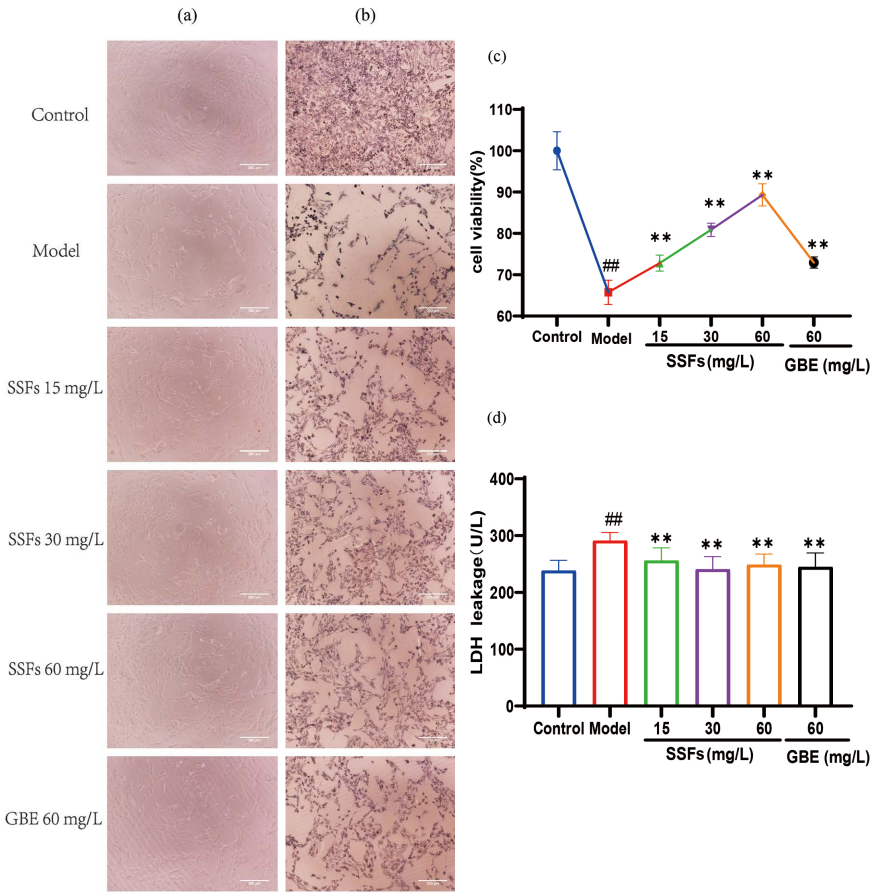

Figure 4. Effect of SSFs on A$\beta_{1\text{-}42}$-induced OLN-93 cell damage. (a) Effect of SSFs on A$\beta_{1\text{-}42}$-induced morphological damage in OLN-93 cells (100×, Scale bar = 200 μm). (b), (c) Effect of SSFs on A$\beta_{1\text{-}42}$-induced OLN-93 cell viability (100×, Scale bar= 200 μm) Mean ± SD, n = 7; $^{\#\#}$P < 0.01, vs. the control group; **P < 0.01, vs. the model group. (d) Effect of SSFs on A$\beta_{1\text{-}42}$-induced LDH release from OLN-93 cells (mean ± SD, n = 7); $^{\#\#}$P < 0.01, vs. the control group; **P < 0.01, vs. the model group.

multiple pharmacological activities, including anti-inflammatory, antioxidant, and neuroprotective activities. Additionally, they have unique therapeutic advantages and potential for treating AD through complex pathological mechanisms.

OL form myelin sheaths and wrap around axons, and normal differentiation and migration of OL maintain myelin integrity and repair [29] [32]. Neuronal networks require the integrity of the myelin sheath to maintain rapid information transmission and neuronal nutrition [33]. Aβ-mediated oxidative stress is a major cause of OL damage, which in turn leads to abnormal myelin function [34]. A$\beta_{1\text{-}42}$ is more likely to form Aβ oligomers and more neurotoxic than other Aβ peptides. Then, A$\beta_{1\text{-}42}$ is often used as a clue to build the AD model both *in vivo* and *in vitro* [35].

To prevent the cytotoxic effects of SSFs on normal OLN-93 cells, we designed an assessment of SSFs at safety dosage through a serial concentration SSFs exposing to OLN-93 cells. The results found that after 24 hours of exposure to

SSFs, OLN-93 cells exhibited cytotoxicity at concentrations exceeding 75 mg/L. The maximum non-injurious dose was determined to be 75 mg/L. The study results indicate that SSFs is not un-cytotoxicity for OLN-93 cells and shows cytotoxicity over concentrations 75 mg/L.

This study investigates the potential ameliorative effects of SSFs on OLN-93-related injury using a well-established A$\beta_{1\text{-}42}$-induced OLN-93 cell model. Morphological observation is employed as a reliable method for assessing the cellular condition. The experimental results demonstrate that A$\beta_{1\text{-}42}$ disrupts the integrity of the cell membrane, resulting in cell swelling, vacuolization, and ultimately cell rupture and death. Importantly, the administration of three different doses of SSFs effectively mitigates the abnormal morphological alterations induced by A$\beta_{1\text{-}42}$ in OLN-93 cells. MTT is a routine method for determining cell viability and cytotoxicity [36] [37]. The principle of the MTT assay is to enzymatically reduce MTT to purple-blue formazan using succinate dehydrogenase within living cell mitochondria, dissolve the formazan, and determine cell survival rates through a colorimetric assay. The results indicated that A$\beta_{1\text{-}42}$ at a concentration of 7.5 μmol/L caused significant cytotoxicity in OLN-93 cells, which was consistent with the findings of previous experiments. SSFs at three doses exhibited a significant protective effect on A$\beta_{1\text{-}42}$-induced OLN-93 cells damage, leading to a dose-dependent increase in the cell survival rate. Interestingly, the groups receiving SSFs at 30 mg/L and 60 mg/L exhibited superior results in terms of promoting cell survival, as compared to the 60 mg/L GBE. LDH release is a crucial measure for determining the severity of tissue and cell injury [38]. During cellular damage, the permeability of the cell membrane increases, and LDH is released. The present experimental results demonstrated that A$\beta_{1\text{-}42}$ augmented the permeability of the cell membrane to OLN-93, which resulted in increased LDH levels. Conversely, three different doses of SSFs resulted in decreased LDH levels and increased cell membrane integrity, possibly related to the inhibition of cell membrane lipid peroxidation from SSFs anti-oxidative activity.

5. Conclusion

Aβ-induced oxidative stress is a significant contributor to OL damage. SSFs are flavonoids obtained from the dried stems and leaves of Scutellaria baicalensis. These compounds demonstrate strong inhibition of oxidative damage. The polyhydroxy moiety of flavonoids has potent reducing effects and serves as the structural foundation for their antioxidant, free radical scavenging, reactive oxygen species, and neuroprotective effects. This present showed that SSFs can effectively counteract A$\beta_{1\text{-}42}$-induced OLN-93 cells damage, and advance to improve myelin degeneration, which provided an experimental basis for the clinical application of SSFs in the prevention and treatment of AD.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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