Life Sciences 239 (2019) 117043

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Amentoflavone suppresses amyloid β1–42 neurotoxicity in Alzheimer's disease through the inhibition of pyroptosis

Ningning Zhao[a], Chengxin Sun[b], Mei Zheng[a], Shen Liu[a], Ran Shi[a,*]

[a] Department of Rehabilitation Medicine, The First Affiliated Hospital of Shandong First Medical University, Jinan, China
[b] Department of Digestive System, Yucheng People's Hospital, Dezhou, China

ARTICLE INFO

Keywords:
Alzheimer's disease
Amyloid β_{1-42}
Amentoflavone
Pyroptosis
AMPK

ABSTRACT

Background: The accumulation of Amyloid β (Aβ) plays key roles in Alzheimer's disease (AD) by inducing intracellular reactive oxygen species (ROS) and neuronal cell death. In this study, we aimed to identify the neuroprotective mechanisms of amentoflavone (AF) in Aβ-induce neuronal cell injury.

Materials and methods: The animal model was established by injecting $A\beta_{1-42}$ into the bilateral hippocampus. The effect of AF on $A\beta_{1-42}$-induced neurological dysfunction was examined using the Y-maze and radical maze tests. The hippocampal neuron viability was examined using Nissl staining and TUNEL assay. On the other hand, *in vitro* studies were conducted using SH-SY5Y cells. The expression level of marker proteins was measured using western blot. The activity of caspase-1 and the levels of pro-inflammatory cytokines were determined using ELISA assay. AMPKα knock down was carried out by transfecting SH-SY5Y cells with siRNA against AMPK transcript.

Results: Neurological tests showed that AF significantly attenuated $A\beta_{1-42}$-induced neurological dysfunction. AF suppressed $A\beta_{1-42}$-induced pyroptosis in the hippocampal region of the rat model, which was associated with the modulation of AMPK/GSK3β signaling. Similar results were obtained *in vitro* in SH-SY5Y cells exposed to $A\beta_{1-42}$, showing that the neuroprotective activity of AF is mediated by suppressing pyroptosis through AMPK/GSK3β signaling.

Conclusion: AF inhibits $A\beta_{1-42}$-induced neurotoxicity in animal and cellular models through AMPK/GSK3β-mediated pyroptosis suppression. Our results highlight AF as a clinical compound for the prevention and treatment of AD.

1. Introduction

Alzheimer's Disease (AD) is a neurodegenerative disorder that is considered the major cause of dementia in elderly people. A typical feature of AD is the accumulation of amyloid β (Aβ) deposits and neurofibrillary tangles [1]. The accumulation of Aβ greatly contributes to the pathology of AD by inducing neuronal cell death and intracellular reactive oxygen species (ROS) production [2–5]. Amyloid β_{1-42} ($A\beta_{1-42}$), a peptide consisting of 42 amino acids, represents a major neurotoxic form of Aβ during the development and progression of AD. Therefore, ameliorating $A\beta_{1-42}$–induced neurotoxicity has been shown to be an effective therapeutic approach for the treatment of AD [6,7].

Pyroptosis is an inflammatory form of programmed cell death that is associated with inflammasome activation [8]. The inflammasomes play a role as sensors of invading pathogens and cellular stress by promoting the production of potent pro-inflammatory cytokines that augment inflammatory cell injury [9]. Of interest, NOD-like receptor protein 3 (NLRP3) has been reported to initiate neuronal pyroptosis [10,11]. Indeed, NLRP3 inhibition has been shown to exhibit neuroprotective effects through the suppression of pyroptosis [12]. In this regard, the suppression of pyroptosis has been recently reported as a neuroprotective mechanism against cerebral ischemia reperfusion injury [12]. In addition, NLRP3 inhibition was shown to ameliorate isoflurane-induced neuronal loss and neurological dysfunction in aged mice [13]. Therefore, pyroptosis inhibition could be a neuroprotective strategy in the treatment of neurodegenerative diseases.

The beneficial effects of flavonoid compounds have been documented in several clinical trials on neurodegenerative disorders, including AD [14,15]. The citrus flavonoid nobiletin, for example, was recently reported to exhibit potential beneficial effects against AD and Parkinson's Disease [15]. Amentoflavone (AF), a major active ingredient of Selaginella tamariscina, has been reported to possess a

* Corresponding author. 16766 Jingshi Road, Jinan, 250014, China.
E-mail addresses: shiranjn@163.com, 2131@sdhospital.com.cn (R. Shi).

https://doi.org/10.1016/j.lfs.2019.117043
Received 19 September 2019; Received in revised form 22 October 2019; Accepted 4 November 2019
Available online 10 November 2019
0024-3205/

variety of biological activities such as anti-inflammatory [16], antimicrobial [17], anti-oxidative [42] , anti-radiation [18] and anti-tumor activities [19]. In addition, we recently showed that AF ameliorates $A\beta_{1\text{-}42}$-induced memory deficit and oxidative stress in an animal model of AD [20]. However, the underlying mechanisms are not fully understood. In this study, we aimed to identify the molecular mechanism by which AF inhibits $A\beta_{1\text{-}42}$-induced neurotoxicity.

2. Materials and Methods

2.1. Animal grouping and treatment

The ethical approval for this study was obtained from the institutional ethics committee (Approval No. 20180213). Male Wistar rats aged 3 months and weighing between 200 g and 230 g were used in this study. All rats were kept in a room with constant temperature (25 °C) and were given ad libitum access to food and water. Rats were acclimatized for 7 days and randomized into 5 treatment groups, including 10 rats per group: control (sham vehicle), Aβ, Aβ + AF (Aβ and AF injection at 40 mg/kg/day), Aβ and AF (Aβ and AF injection at 80 mg/kg/day), and Aβ + donepezil (positive control). $A\beta_{1\text{–}42}$ peptide (Sigma Aldrich, St. Louis, MO, USA) was dissolved in saline to a final concentration of 2.5 μg/μL and incubated at 37 °C for 3 days to form fibrils. The rats were anesthetized with ketamine (80 mg/kg) and xylazine (15 mg/kg) through intraperitoneal (i.p.) injection, their head was fixed into a stereotaxic instrument (Narishige, Japan), and Aβ (4 μg/side) was injected into the bilateral hippocampus as described in our previous study [20]. The used AF dose (purity ≥ 98%; Shanghai Research Center for Standardization of Traditional Chinese Medicine, Shanghai, China) was chosen based on our previous study (Fig. 1A) [20]. Donepezil (National Institutes for Food and Drug Control, Beijing, China) was administered at 1 mg/kg/day through i.p. injection. The experimental design is shown in Fig. 1B.

2.2. The Y-maze test

The Y-maze test was conducted, as previously described [21], to evaluate the cognitive and exploratory activities of rats. The test was conducted on last day (14th) of the experimental protocol. A Plexiglas Y-maze was built with an equilateral central area, and each arm with 25 cm height, 35 cm length, and 10 cm width. Briefly, each rat was put in the central area and allowed to move freely in the maze for 6 min and the arm entries were noted. Arms were wiped to remove retained odor. Alternation was defined as successive entry into the three arms on overlapping triplet sets. Alternation rate (%) was defined as the ratio of the number of actual alternations performed to the number of possible alternations [22].

2.3. The radical-arm maze test

The radical-arm maze test was conducted as previously described [23]. Briefly, each rat was put in the apparatus on day 15 and left there for 10 min to be familiar with the apparatus. The rats were then returned to their housing box with free access to water and restricted access to food. On day 16, each rat was put in the apparatus, with chocolate cereal placed in four of the eight arms of the maze. After completing the task, rats were returned to the housing box. The same protocol was employed to evaluate the spatial memory of the rats on days 17, 18 and 19. Each test session was conducted for 10 min. The total errors to find food was reported by the number of entries into each arm, and the latency to find food was measured by the time spent to locate the 4 cereal rewards. The number of working memory errors and the number of reference memory errors were also recorded [24]. After the last test, and 24 h after the last treatment, the rats were euthanatized, and samples were collected for biomedical assays.

2.4. Measurement of Aβ

The hippocampus from each rat was homogenized with PBS and centrifuged at 4 °C for 10 min at 10,000 g. The supernatant was retrieved, and equal volume of PBS was added. The level of Aβ was determined using the Mouse/Rat beta-Amyloid (1–42) ELISA Kit (Arigo Biolaboratories, Taiwan) according to the manufacturer's protocol.

2.5. Nissl staining and TUNEL assay

Following anesthesia, the brain was isolated from each rat and postfixed overnight in 50 mmol/L PBS containing 4% paraformaldehyde, then immersed in a 50 mmol/L PBS solution containing 30% sucrose, and stored at 4 °C until sectioning. The frozen brains were coronally sectioned on paraffin into 3 μm slices, which were stored at 4 °C in a storage solution. Nissl staining and TUNEL assay were conducted as previously described [20]. Briefly, the brain tissue was processed with paraffin for examination, and a TUNEL kit (Beyotime, Shanghai, China) was used to detect TUNEL-positive cells in the CA1 region of hippocampus.

2.6. Measurement of caspase-1 activity

The caspase-1 activity was measured using an ELISA kit (BioVision, Milpitas, CA) as per the manufacturer's protocol.

2.7. Measurement of IL-18 and IL-1β

The levels of IL-18 and IL-1β in hippocampal tissues and cell lysates were detected using ELISA kits (Blue Gene, Shanghai, China) as per the manufacturer's protocols.

2.8. Cell culture

Human neuroblastoma SH-SY5Y cells (American Type Culture Collection, ATCC, Shanghai, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin and streptomycin, at 37 °C in a humid incubator with 5% CO2. Cells in the exponential growth phase were used for all experiments.

2.9. Measurement of cell viability

Viable cells were detected using the Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai). Briefly, astrocytes cultured in 96-well plates were incubated in CCK-8 solution at 37 °C for 2 h, and the absorbance was measured at 450 nm.

2.10. LDH release assay

Cell death was determined using a lactate dehydrogenase (LDH) cytotoxicity detection kit (TaKaRa Bio, Kusatsu, Japan) according to the manufacturer's instructions.

2.11. AMPKα siRNA transfection

Transfection with AMPKα-targeting siRNA was performed using Lipfectamine 3000 (Invitrogen, Grand Island, NY) as previously described [25]. The protein expression level of AMPK was measured by western blot 48 h post transfection.

2.12. Western blot

Western blot was performed as per standard procedures using the following primary antibodies: Aβ antibody (1:1000, Abcam, UK), NLRP3 antibody (1:1000, Abcam, UK), ASC (1:500, Santa Cruz, CA),

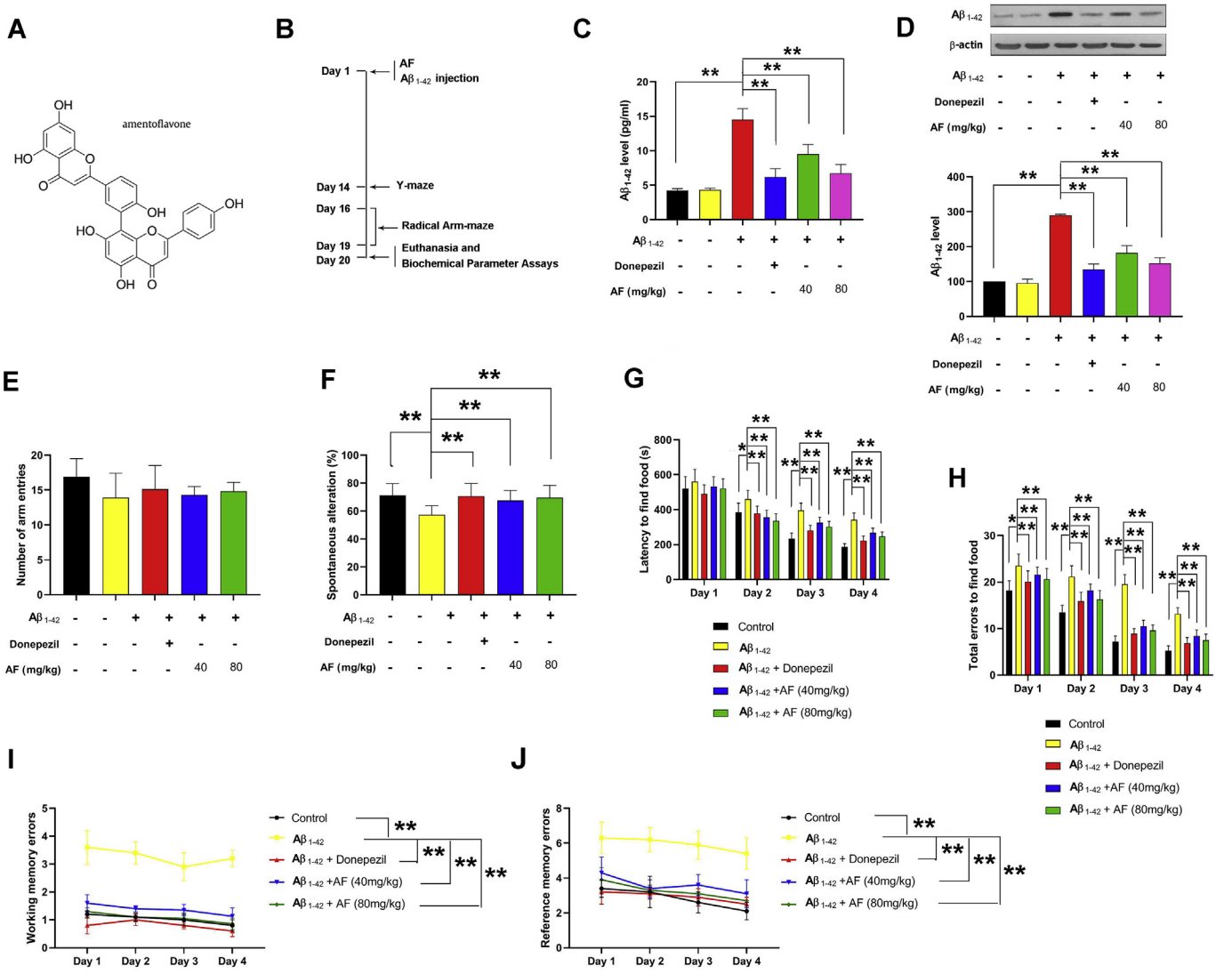

Fig. 1. Amentoflavone (AF) protects against Amyloid β ($Aβ)_{1-42}$-induced neurological dysfunction in the rat model. A. The chemical structure of AF. B. The experimental design of the animal study. C-D. The Accumulation of $Aβ_{1-42}$ in the hippocampus measured by ELISA assay and western blot, respectively. E-F. The results of the Y-maze test. G-J. The results of the radical maze test. **p < 0.01.

caspase-1 (1:500, Santa Cruz, CA), GSDMD (1:500, Abcam, UK), IL-18 antibody (1:1000, Abcam, UK), IL-1β antibody (1:1000, Abcam, UK), AMPK (1:1000, Abcam, UK) and GSK-3β (1:1000, Abcam, UK). β-actin was used as the internal control for equal loading. Goat anti-rabbit IgG-HRP (Beyotime, Shanghai, China) was used as the secondary antibody, and protein bands were visualized using ECL solution(Thermo Fisher, Shanghai, China).

2.13. Statistical analysis

The Shapiro-Wilk test was conducted to test the assumption of normality. Experimental results were displayed as mean ± SD for each of the biological triplicates *in vitro*, and for each of the 10-rat groups from *in vivo* experiments. One-way ANOVA followed by Dunnett's *t*-test was used for statistical comparison between groups. All statistical analysis was conducted using Graphpad 8.0 software, and a p value < 0.05 was used as a cutoff for statistical significance.

3. Results

3.1. AF attenuates $Aβ_{1-42}$-induced neurological dysfunction in vivo

The establishment of successful rat models was validated by examining the level of $Aβ_{1-42}$ accumulation in the hippocampus. $Aβ_{1-42}$ measurement by ELISA showed that hippocampal $Aβ_{1-42}$ levels significantly increased in Aβ injected rats, compared with control rats (Fig. 1C). In contrast, treatment with Donepezil and AF, at both 40 and 80 mg/kg/day, was able to effectively attenuate the elevated hippocampal $Aβ_{1-42}$ levels. Similar results were obtained when the hippocampal $Aβ_{1-42}$ levels were measured by western blot (Fig. 1D).

On the other hand, the protective activity of $Aβ_{1-42}$-induced neurological dysfunction was examined by conducting behavioral tests. The Y-maze test was conducted on day 14 after treatment, and the results showed that $Aβ_{1-42}$ injection did not cause a significant change in the number of total entries compared with the control group, which indicates that $Aβ_{1-42}$ did not induce a significant change in the locomotor activities of rats (Fig. 1E). In contrast, when the spontaneous alteration was compared, $Aβ_{1-42}$ injection resulted in a significant reduction in the alteration rate, compared with the control group, indicating that $Aβ_{1-42}$ compromised the short memory of rats (Fig. 1F). However, treatment

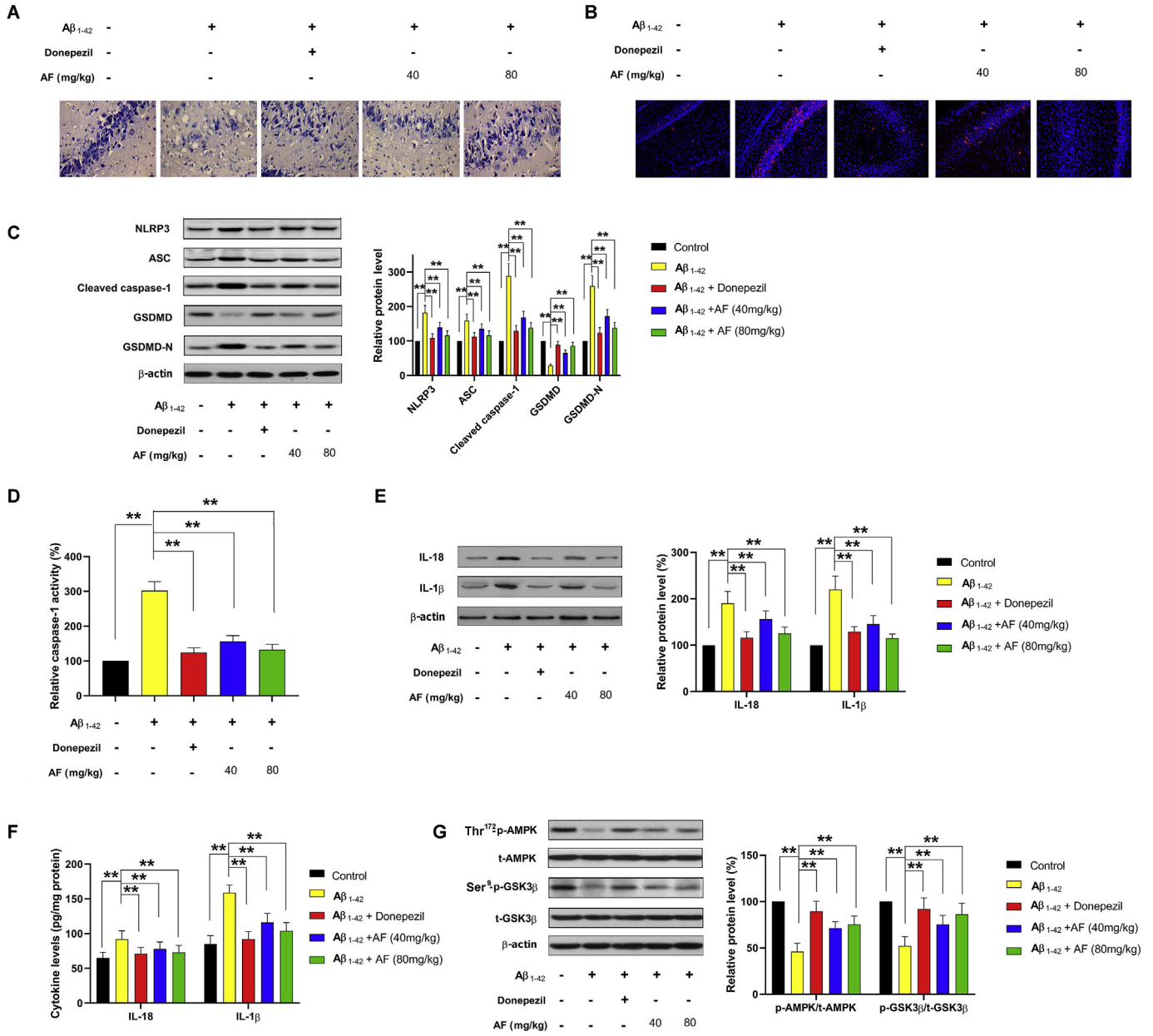

Fig. 2. AF suppresses Aβ$_{1-42}$-induced pyroptosis in the hippocampus. A. Nissling staining in the CA1 region of rat hippocampus. B. TUNEL assay in the CA1 region of rat hippocampus. C. The protein expression of pyroptosis markers in the hippocampus. D. measuring caspase-1 activity by ELISA assay. E-F. The expression of the pro-inflammatory cytokines IL-18 and IL-1β measured by western blot and ELISA assay, respectively. G. The level of phosphorylated AMPK and GSK3β measured by western blot. **p < 0.01.

with Donepezil or AF was able to improve the short memory loss in Aβ$_{1-42}$-treated rats (Fig. 1F).

The radical-arm maze was also performed to further explore the neuroprotective activity of AF. Aβ$_{1-42}$ injection led to a significant impairment in the spatial memory as shown by the increased latency to find food at days 2, 3 and 4, compared with the control group (Fig. 1G). The number of total errors to find food was also increased by Aβ$_{1-42}$ injection (Fig. 1H). Interestingly, treatment with Donepezil or AF was able to decrease the latency and total errors to find food (Fig. 1G and H). Besides, reference memory comparison showed that Aβ$_{1-42}$ injection led to impaired spatial memory at the last day of the test, which was partially restored by treatment with Donepezil or AF. Similarly, working memory errors evaluation showed that treatment with Donepezil or AF can also ameliorate Aβ$_{1-42}$ injection-induced working memory impairment (Fig. 1J). Altogether, these results indicate that AF can effectively attenuate Aβ$_{1-42}$-induced neurological impairment in our rat model.

3.2. AF rescues neuronal cells from Aβ$_{1-42}$–induced pyroptotic cell death

Neuronal cell death was the hallmark of Aβ$_{1-42}$-induced neurotoxicity. Indeed, Aβ$_{1-42}$ injection promoted cell death in rats' hippocampus, as detected by both Nissling staining and TUNEL assay (Fig. 2A and B). On the other hand, AF treatment was able to rescue hippocampal neurons from Aβ$_{1-42}$-induced cell death (Fig. 2A and B).

Hippocampal neuronal pyroptosis was recently proposed to play an important role in Aβ$_{1-42}$-induced neurological dysfunction. Therefore, we hypothesized that the modulation of pyroptotic cell death could be involved in the neuroprotective effects of AF. As shown in Fig. 2C, the protein expression of NLRP3 and ASC, and caspase-1 cleavage, were significantly elevated in the hippocampal region of Aβ$_{1-42}$-treated rats. In contrast, AF treatment at both dosages repressed Aβ$_{1-42}$-induced

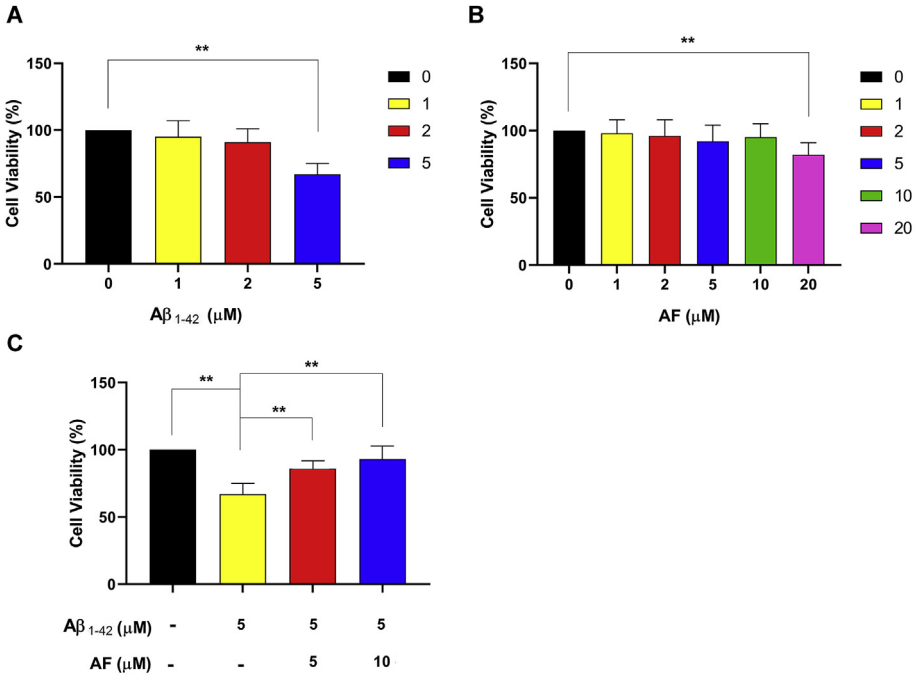

Fig. 3. AF rescues $A\beta_{1\text{-}42}$-induced cell death in SH-SY5Y cells. A. The effect of $A\beta_{1\text{-}42}$ on cell death *in vitro* at different dosages. B. The effect of AF on cell death *in vitro*. C. The protective effect of AF pretreatment against $A\beta_{1\text{-}42}$-induced cell death *in vitro*. **p < 0.01.

NLRP3 and ASC overexpression, as well as it reduced caspase-1 cleavage in the hippocampal region of rat models (Fig. 2C). In addition, ELISA assay was used to examine the effects of $A\beta_{1\text{-}42}$ injection, with or without AF treatment, on caspase-1 activity. $A\beta_{1\text{-}42}$ injection markedly increased caspase-1 activity, while AF treatment almost completely abolished $A\beta_{1\text{-}42}$–induced casapase-1 activation (Fig. 2D).

Pyroptosis is characterized by the overexpression of the pro-inflammatory cytokines IL-1β and IL-18. As shown in Figs. 2F and 3E, $A\beta_{1\text{-}42}$ injection significantly increased the levels of IL-1β and IL-18 in the hippocampus, as detected by both western blot and ELISA, which was inhibited by AF treatment (Fig. 2E). Similarly, $A\beta_{1\text{-}42}$ injection increased the cleavage of the N-terminal region of Gasdermin-D (GSDMD-N) in the hippocampus of rats, which is considered a hallmark of pyroptosis. This was reversed by treatment with AF (Fig. 2C), which indicate that AF can attenuate $A\beta_{1\text{-}42}$-induced pyroptosis in hippocampal neuronal cells.

AMPK/GSK3β signaling has been proposed to regulate pyroptosis during neuronal stress [12]. In line with our previous findings [20], $A\beta_{1\text{-}42}$ injection reduced the levels of phosphorylated AMPK (Thr^{172} p-AMPK) and phosphorylated GSK3β (Ser^{9} p-GSK3β), which were significantly increased by AF treatment, suggesting that AF can rescue neuronal cells from pyroptosis by inducing AMPK/GSK3β phosphorylation (Fig. 2G).

3.3. AF inhibits $A\beta_{1\text{-}42}$-induced neurotoxicity in vitro

The human neuroblastoma SH-SY5Y cells were used to study the neuroprotective effects of AF *in vitro*. Treatment with $A\beta_{1\text{-}42}$ at 5 μM for 24 h reduced the percentage of viable cells (Fig. 3A). As shown in Fig. 3B, AF treatment at 1, 2, 5 or 10 μM did not influence cell death, while it reduced the percentage of viable cells at 20 μM. To evaluate the neuroprotective effects of AF *in vitro*, SH-SY5Y cells were pretreated with AF for 6 h before $A\beta_{1\text{-}42}$ treatment. As shown in Fig. 3C, AF at 5 or 10 μM significantly reduced $A\beta_{1\text{-}42}$-induced cell death of SH-SY5Y cells.

3.4. The neuroprotective effects of AF are mediated through the inhibition of pyroptosis in vitro

The TUNEL assay was first used to examine the effect of AF on pyroptosis *in vitro*. Interestingly, the $A\beta_{1\text{-}}42$-induced increase in TUNEL positive cells was significantly abrogated by pretreatment with AF (Fig. 4A and B). Similarly, pretreatment with AF at 5 μM or 10 μM prevented $A\beta_{1\text{-}42}$-induced LDH release by SH-SY5Y cells (Fig. 4B). LDH is a soluble cytoplasmic enzyme that is present in almost all cells and is released into the extracellular space in damaged cells. The level of expression of pyroptosis marker proteins was also examined following $A\beta_{1\text{-}42}$ treatment, with or without AF pretreatment. As shown in Fig. 4C, the protein levels of NLRP3, ASC and cleaved caspase-1 were significantly increased in SH-SY5Y cells after $A\beta_{1\text{-}42}$ treatment, which were repressed by pretreatment with AF. ELISA assay also demonstrated that $A\beta_{1\text{-}42}$ treatment activated caspase-1, while pretreatment with AF suppressed caspase-1 activation (Fig. 4D). Similarly, pre-treatment with AF attenuated $A\beta_{1\text{-}42}$-induced cleavage of GSDMD-N (Fig. 4E). In addition, both western blot and ELISA measurements showed that pretreatment with AF suppressed $A\beta_{1\text{-}42}$-induced IL-18 and IL-1β expression in SH-SY5Y cells (Fig. 4F and G). Furthermore, AF also induced the phosphorylation of AMPK and GSK3β *in vitro* in SH-SY5Y cells, which was in line with the *in vivo* results from rat models (Fig. 4H).

3.5. AF protects against $A\beta_{1\text{-}42}$-induced pyroptosis by inducing AMPK/GSK3β signaling

In order to examine the role of AMPK/GSK3β signaling in AF-mediated suppression of $A\beta_{1\text{-}42}$-induced pyroptosis, AMPKα was repressed in SH-SY5Y cells by siRNA transfection (Supplementary Fig. 1A). As shown in Fig. 5A, the rescuing activity of AF against $A\beta_{1\text{-}42}$-induced cell death was inhibited by AMPKα knockdown. However, LDH release increased from less than 20% to more than 40% after AMPKα knockdown in SH-SY5Y cells treated with $A\beta_{1\text{-}42}$ and AF(Fig. 5B). AMPKα repression also restored the levels of NLRP3, ASC and cleaved caspase-1 in SH-SY5Y cells treated with $A\beta_{1\text{-}42}$ and AF (Fig. 5C). Similarly, ELISA assay showed that AMPKα knockdown abrogated AF-mediated suppression of $A\beta_{1\text{-}42}$-induced caspase-1 activity (Fig. 5D).

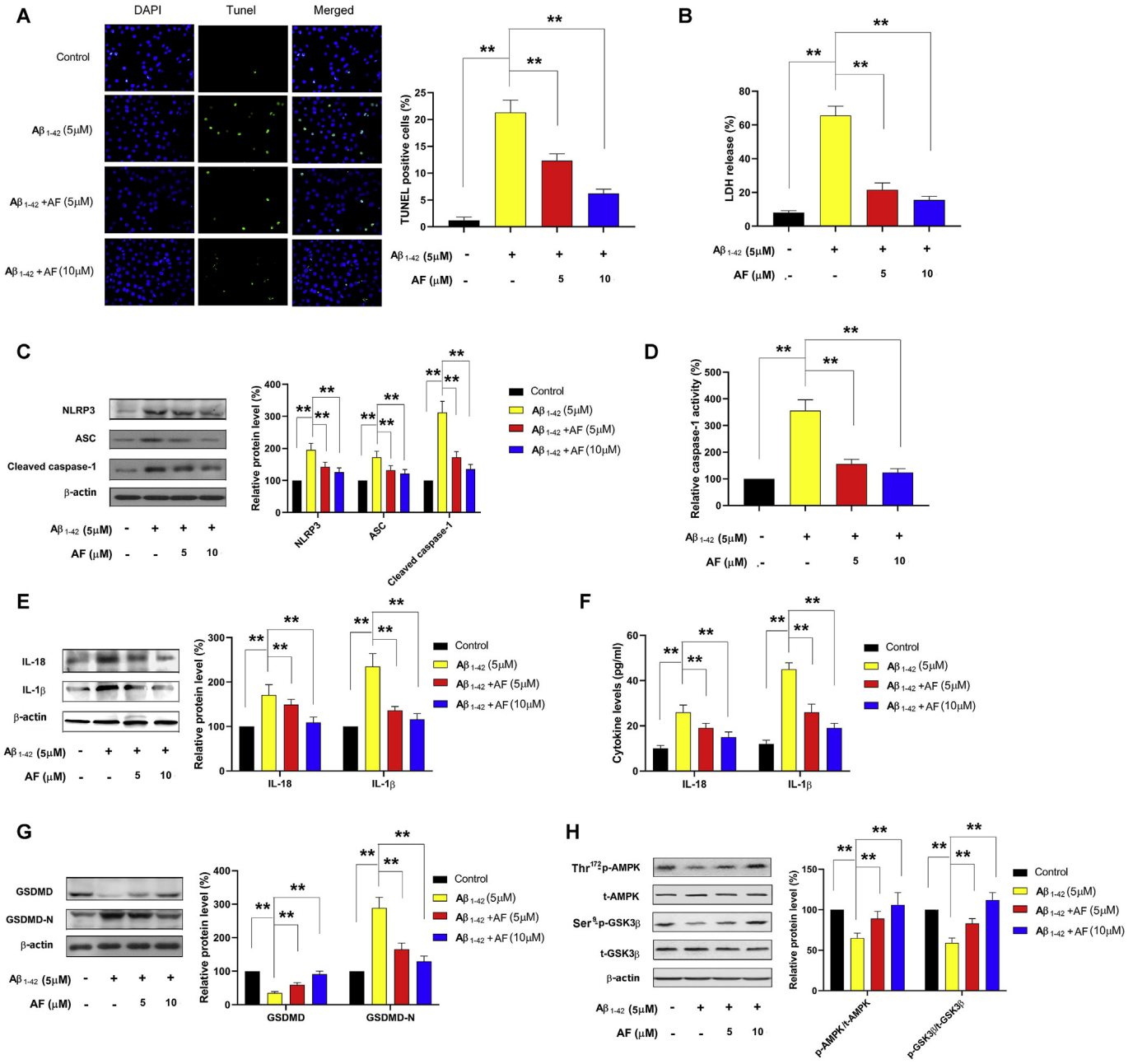

Fig. 4. AF suppresses $A\beta_{1-42}$-induced pyroptosis in SH-SY5Y cells. A. Cell pyroptosis examined by TUNEL assay. B. LDH release measured by ELISA assay. C. The protein level of NLRP3, ASC and cleaved casapase-1 determined by western blot. D. Caspase-1 activity measured by ELISA assay. E. Western blot of the N-terminal region of GSDMD. F. The protein levels of pro-inflammatory cytokines measured by western blot. G. The levels of pro-inflammatory cytokines measured by ELISA assay. H. The levels of phosphorylate of AMPK and GSK3β measured by western blot. **p < 0.01.

AMPKα knockdown also restored the level of GSDMD-N in SH-SY5Y cells treated with AF (Fig. 5E). Besides, AF-mediated repression of IL-18 and IL-1β was also restored by AMPKα knockdown in SH-SY5Y cells (Fig. 5F and G). Furthermore, a specific AMPK inhibitor, compound C, was used to further examine the role of AMPK activation in the anti-pyroptotic effects of AF (Supplementary Fig. 1B). Interestingly, AMPK inhibition was able to significantly compromise the protective effects of AF against $A\beta_{1-42}$-induced cell death (Fig. 6A). Compound C was also able to restore LDH release by SH-SY5Y cells (Fig. 6B) and restore the protein levels of NLRP3, ASC and cleaved caspase-1 in SH-SY5Y cells treated with AF (Fig. 6C). In addition, AMPKα inhibition by compound C abrogated the suppressing effect of AF on $A\beta_{1-42}$-induced caspase-1 activation (Fig. 5D) and restored the levels of GSDMD-N in SH-SY5Y cells treated with AF (Fig. 5E). Moreover, the expression of the pro-inflammatory cytokines IL-18 and IL-1β was also restored by compound C in SH-SY5Y cells treated with AF (Fig. 5F and G). Taken together, these results provide a strong evidence that AMPK signaling mediate the anti-pyroptotic effects of AF.

4. Discussions

The beneficial effects of dietary flavonoids on a variety of neurological degenerative disorders has been reported in animal models and in several clinical trials [26,27]. It has been demonstrated that the anti-inflammatory activity is involved in the neuroprotective effects of flavonoid compounds against neurological degenerative disorders, such as AD [28,29]. AF is one of the major flavonoids produced by the medicinal plant *Selaginella tamariscina*. The neuroprotective activity of AF

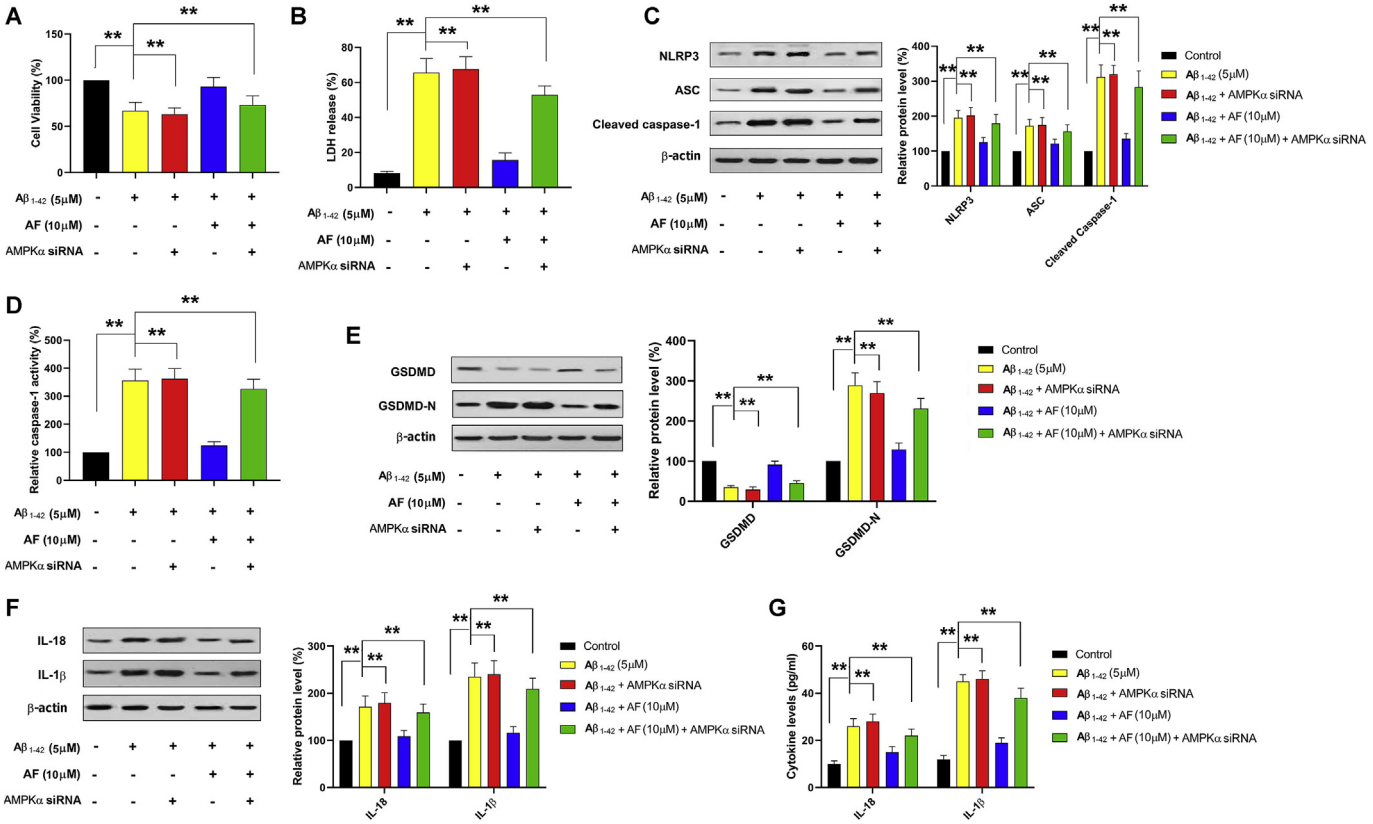

Fig. 5. AMPKα knockdown abolishes the cytoprotective effects of AF *in vitro*. A. AMPKα knockdown suppressed AF-mediated rescue of $A\beta_{1\text{-}42}$-induced cell death. B. AMPKα knockdown inhibited AF-mediated suppression of $A\beta_{1\text{-}42}$-induced LDH release. C. AMPKα knockdown inhibited AF-mediated suppression of $A\beta_{1\text{-}42}$-induced protein expression of NLRP3, ASC and cleaved caspase-1. D. AMPKα knockdown attenuated the ability of AF to suppress caspase-1 activation. E. AMPKα knockdown inhibited AF-mediated suppression of $A\beta_{1\text{-}42}$-induced GSDMD cleavage. F-G. AF was not able to suppress $A\beta_{1\text{-}42}$-induced expression of pro-inflammatory cytokines in cells with AMPKα knockdown. **p < 0.01.

was first described in hypoxia/ischemia-induced neuronal injury both *in vitro* and *in vivo* [30]. We have previously showed that AF can protect against $A\beta_{1\text{-}42}$-induced neurological dysfunction in a rat model [20], which underscores the potential use of AF in the prevention and treatment of AD. However, the mechanisms of AF neuroprotective effects remained unclear. In this study, we showed that AF can effectively ameliorate $A\beta_{1\text{-}42}$-induced cognitive impairment in an animal model through its anti-pyroptotic activity that is mediated by AMPK/GSK3β phosphorylation.

Pyroptosis is a form of inflammatory programmed cell death that has been reported in neurological pathogenesis [31]. Pyroptotic neuronal cell death has been proposed to be a contributor to the progression of neurological degenerative disorders, including Parkinson's disease and AD [32]. It has been shown that pyroptosis suppression can ameliorate the progression of Parkinson's disease in animal models [33]. In addition, pyroptosis suppression was also shown to alleviate neuronal cell death in a cellular model of Parkinson's disease *in vitro* [34]. The role of pyroptotic cell death in disease progression has also been reported in AD, where inhibitors of pyroptosis were shown to alleviate cognitive impairment in animal models [10]. These findings suggest that pyroptotic cell death inhibition could be an effective neuroprotective strategy for the treatment of neurodegenerative diseases.

Pyroptotic cell death is initiated by inflammasome-mediated caspase-1 activation and the subsequent release of pro-inflammatory cytokines, which eventually lead to the formation of pores in the cell membrane by GSDMD-N [35]. The NLRP3 inflammasome inhibitor has been shown to promote non-phlogistic clearance of Aβ and to improve neurological functions in a transgenic mouse model of AD [10]. In this study, we showed that NLRP3 expression was significantly elevated following $A\beta_{1\text{-}42}$ treatment in both cellular and animal models. In addition, we showed that $A\beta_{1\text{-}42}$ treatment also promoted caspase-1 activation, increased the expression of the pro-inflammatory cytokines IL-18 and IL-1β, and induced the exposure of GSDMD-N domain. These results highlight the role of neuronal cell pyroptosis in the progression of AD. Of importance, our results showed that AF repressed $A\beta_{1\text{-}42}$-induced NLRP3 expression and subsequent caspase-1 activation and IL-18 and IL-1β expression, as well as inhibited the exposure of GSDMD-N domain, which suggests that the neuroprotective effects of AF against $A\beta_{1\text{-}42}$-induced neurotoxicity are mediated by suppressing NLRP3-mediated pyroptosis.

AMP-activated protein kinase (AMPK), a heterotrimeric serine/threonine kinase [25,36,37], has been shown to play an important role in the development and progression of neurodegenerative conditions such as AD [38]. Interestingly, AMPK can act an upstream regulator of NLRP3 inflammasome [39]. Indeed, *in vivo* studies on animal models of depression showed that inhibition of GSK3β can block NLRP3 activation in the central nervous system [40,41]. On the other hand, we previously showed that AF can modulate AMPK/GSK3β signaling in an animal model of AD [20]. Therefore, we proposed that AF suppresses NLRP3-mediated pyroptosis by modulating AMPK/GSK3β signaling. Indeed, this study showed that AMPKα knockdown and pharmacological inhibition abrogated AF-mediated suppression, which suggest that $A\beta_{1\text{-}42}$-induced pyroptosis is inhibited by AF, at least partly, by inducing AMPK/GSK3β signaling.

In conclusion, AF exhibits a neuroprotective activity against $A\beta_{1\text{-}42}$-induced neurotoxicity in animal and cellular models. Moreover, our results suggest that the suppression of NLRP3-mediated pyroptosis plays a key role in the neuroprotective effects of AF.

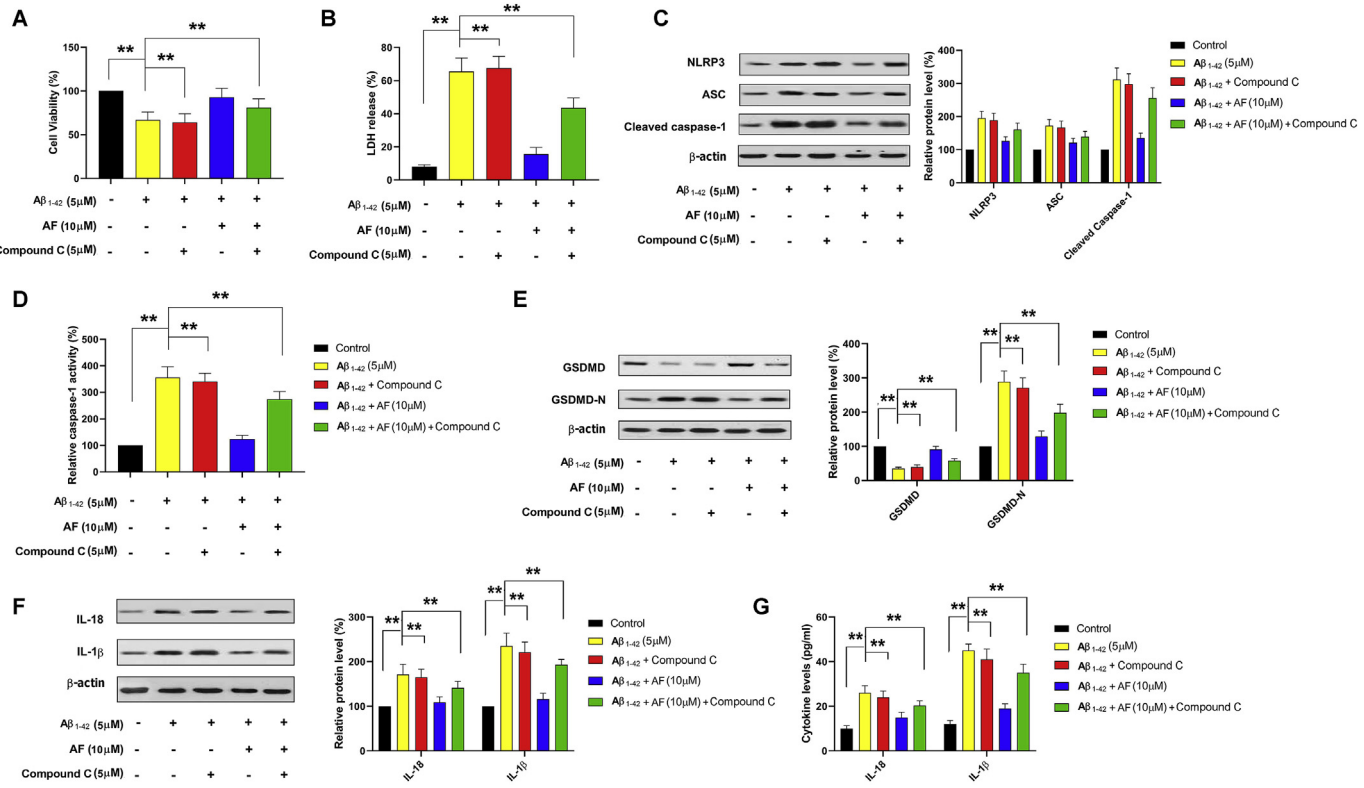

Fig. 6. AMPK inhibition by compound C abrogates the cytoprotective effects of AF against $A\beta_{1\text{-}42}$-induced cytotoxicity *in vitro*. A. Compound C compromised the ability of AF to rescue $A\beta_{1\text{-}42}$-induced cell death. B. Compound C suppressed AF's activity in suppressing $A\beta_{1\text{-}42}$-induced LDH release. C. Compound C inhibited the ability of AF to suppress $A\beta_{1\text{-}42}$-induced elevation in the protein levels of NLRP3, ASC and cleaved caspase-1. D. Compound C attenuated the ability of AF to suppress caspase-1 activation. E. Compound C attenuated AF-mediated suppression of $A\beta_{1\text{-}42}$-induced GSDMD cleavage. F-G. Compound C attenuated AF-mediated suppression of pro-inflammatory cytokines. **p < 0.01.

Authors contribution

Ran Shi designed the study and wrote the paper.

Ningning Zhao, Chengxin Sun and Mei Zheng performed the experiments.

Shen Liu analyzed the data.

Declaration of competing interest

We declare that we have no conflict of interest.

Acknowledgement

The study is supported by Jinan science and Technology Bureau clinical medicine science and technology innovation plan project (201805077).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.117043.

References

[1] Z. Zhu, J. Yan, W. Jiang, X.G. Yao, J. Chen, L. Chen, et al., Arctigenin effectively ameliorates memory impairment in Alzheimer's disease model mice targeting both beta-amyloid production and clearance, J. Neurosci. : the official journal of the Society for Neuroscience 33 (2013) 13138–13149.

[2] C. Behl, F. Holsboer, [Oxidative stress in the pathogenesis of Alzheimer's disease and antioxidant neuroprotection], Fortschr. Neurol. Psychiatr. 66 (1998) 113–121.

[3] D.L. Marcus, C. Thomas, C. Rodriguez, K. Simberkoff, J.S. Tsai, J.A. Strafaci, et al., Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease, Exp. Neurol. 150 (1998) 40–44.

[4] Q.L. Peng, A.R. Buz'Zard, B.H. Lau, Pycnogenol protects neurons from amyloid-beta peptide-induced apoptosis, Brain research Molecular brain research 104 (2002) 55–65.

[5] L. Zheng, K. Kagedal, N. Dehvari, E. Benedikz, R. Cowburn, J. Marcusson, et al., Oxidative stress induces macroautophagy of amyloid beta-protein and ensuing apoptosis, Free Radic. Biol. Med. 46 (2009) 422–429.

[6] G. Aliev, M.E. Obrenovich, V.P. Reddy, J.C. Shenk, P.I. Moreira, A. Nunomura, et al., Antioxidant therapy in Alzheimer's disease: theory and practice, Mini Rev. Med. Chem. 8 (2008) 1395–1406.

[7] Y.H. Lin, A.H. Liu, H.L. Wu, C. Westenbroek, Q.L. Song, H.M. Yu, et al., Salvianolic acid B, an antioxidant from Salvia miltiorrhiza, prevents Abeta(25-35)-induced reduction in BPRP in PC12 cells, Biochem. Biophys. Res. Commun. 348 (2006) 593–599.

[8] E.A. Miao, I.A. Leaf, P.M. Treuting, D.P. Mao, M. Dors, A. Sarkar, et al., Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria, Nat. Immunol. 11 (2010) 1136–1142.

[9] H. Guo, J.B. Callaway, J.P. Ting, Inflammasomes: mechanism of action, role in disease, and therapeutics, Nat. Med. 21 (2015) 677–687.

[10] C. Dempsey, A. Rubio Araiz, K.J. Bryson, O. Finucane, C. Larkin, E.L. Mills, et al., Inhibiting the NLRP3 inflammasome with MCC950 promotes non-phlogistic clearance of amyloid-beta and cognitive function in APP/PS1 mice, Brain Behav. Immun. 61 (2017) 306–316.

[11] Y. Lu, G. Xiao, W. Luo, Minocycline suppresses NLRP3 inflammasome activation in experimental ischemic stroke, Neuroimmunomodulation 23 (2016) 230–238.

[12] P. An, J. Xie, S. Qiu, Y. Liu, J. Wang, X. Xiu, et al., Hispidulin exhibits neuroprotective activities against cerebral ischemia reperfusion injury through suppressing NLRP3-mediated pyroptosis, Life Sci. 232 (2019 Sep 1) 116599.

[13] Y. Fan, L. Du, Q. Fu, Z. Zhou, J. Zhang, G. Li, et al., Inhibiting the NLRP3 inflammasome with MCC950 ameliorates isoflurane-induced pyroptosis and cognitive impairment in aged mice, Front. Cell. Neurosci. 12 (2018) 426.

[14] M. Ayaz, A. Sadiq, M. Junaid, F. Ullah, M. Ovais, I. Ullah, et al., Flavonoids as prospective neuroprotectants and their therapeutic propensity in aging associated neurological disorders, Front. Aging Neurosci. 11 (2019) 155.

[15] A. Nakajima, Y. Ohizumi, Potential benefits of nobiletin, A citrus flavonoid, against alzheimer's disease and Parkinson's disease, Int. J. Mol. Sci. 20 (2019).

[16] H.Y. Tian, Z.X. Li, H.Y. Li, H.J. Wang, X.W. Zhu, Z.H. Dou, Effects of 14 single herbs on the induction of caspase-3 in tumor cells: a brief review, Chin. J. Integr. Med. 19 (2013) 636–640.

[17] P. Coulerie, M. Nour, A. Maciuk, C. Eydoux, J.C. Guillemot, N. Lebouvier, et al., Structure-activity relationship study of biflavonoids on the Dengue virus polymerase DENV-NS5 RdRp, Planta Med. 79 (2013) 1313–1318.

[18] C.W. Lee, Y. Na, N.H. Park, H.S. Kim, S.M. Ahn, J.W. Kim, et al., Amentoflavone inhibits UVB-induced matrix metalloproteinase-1 expression through the modulation of AP-1 components in normal human fibroblasts, Appl. Biochem. Biotechnol. 166 (2012) 1137–1147.
[19] V. Tarallo, L. Lepore, M. Marcellini, F. Dal Piaz, L. Tudisco, S. Ponticelli, et al., The biflavonoid amentoflavone inhibits neovascularization preventing the activity of proangiogenic vascular endothelial growth factors, J. Biol. Chem. 286 (2011) 19641–19651.
[20] C. Chen, B. Li, G. Cheng, X. Yang, N. Zhao, R. Shi, Amentoflavone ameliorates abeta1-42-induced memory deficits and oxidative stress in cellular and rat model, Neurochem. Res. 43 (2018) 857–868.
[21] B. He, F. Xu, F. Xiao, T. Yan, B. Wu, K. Bi, et al., Neuroprotective effects of nootkatone from Alpiniae oxyphyllae Fructus against amyloid-beta-induced cognitive impairment, Metab. Brain Dis. 33 (2018) 251–259.
[22] H. Yuan, C. Jiang, J. Zhao, Y. Zhao, Y. Zhang, Y. Xu, et al., Euxanthone attenuates abeta1-42-induced oxidative stress and apoptosis by triggering autophagy, J. Mol. Neurosci. : MN 66 (2018) 512–523.
[23] J. Budni, D.P. Feijo, H. Batista-Silva, M.L. Garcez, F. Mina, T. Belletini-Santos, et al., Lithium and memantine improve spatial memory impairment and neuroinflammation induced by beta-amyloid 1-42 oligomers in rats, Neurobiol. Learn. Mem. 141 (2017) 84–92.
[24] L. Hritcu, O. Cioanca, M. Hancianu, Effects of lavender oil inhalation on improving scopolamine-induced spatial memory impairment in laboratory rats, Phytomedicine : Int. J. Phytotherapy. Phytopharmacol. 19 (2012) 529–534.
[25] M. Han, H. Gao, J. Xie, Y.P. Yuan, Q. Yuan, M.Q. Gao, et al., Hispidulin induces ER stress-mediated apoptosis in human hepatocellular carcinoma cells in vitro and in vivo by activating AMPK signaling pathway, Acta Pharmacol. Sin. 40 (2019) 666–676.
[26] I. Carrera, R. Cacabelos, Current drugs and potential future neuroprotective compounds for Parkinson's disease, Curr. Neuropharmacol. 17 (2019) 295–306.
[27] D. Vauzour, K. Vafeiadou, A. Rodriguez-Mateos, C. Rendeiro, J.P. Spencer, The neuroprotective potential of flavonoids: a multiplicity of effects, Genes & nutrition 3 (2008) 115–126.
[28] K. Szczechowiak, B.S. Diniz, J. Leszek, Diet and Alzheimer's dementia - nutritional approach to modulate inflammation, Pharmacol. Biochem. Behav. 184 (2019) 172743.
[29] A. Thapa, E.Y. Chi, Biflavonoids as potential small molecule therapeutics for alzheimer's disease, Adv. Exp. Med. Biol. 863 (2015) 55–77.
[30] D.H. Shin, Y.C. Bae, J.S. Kim-Han, J.H. Lee, I.Y. Choi, K.H. Son, et al., Polyphenol amentoflavone affords neuroprotection against neonatal hypoxic-ischemic brain damage via multiple mechanisms, J. Neurochem. 96 (2006) 561–572.
[31] A. Zychlinsky, M.C. Prevost, P.J. Sansonetti, Shigella flexneri induces apoptosis in infected macrophages, Nature 358 (1992) 167–169.
[32] S. Wang, Y.H. Yuan, N.H. Chen, H.B. Wang, The mechanisms of NLRP3 inflammasome/pyroptosis activation and their role in Parkinson's disease, Int. Immunopharmacol. 67 (2019) 458–464.
[33] R. Gordon, E.A. Albornoz, D.C. Christie, M.R. Langley, V. Kumar, S. Mantovani, et al., Inflammasome inhibition prevents alpha-synuclein pathology and dopaminergic neurodegeneration in mice, Sci. Transl. Med. 10 (2018).
[34] E.F. Pitorak, J.C. Kraus, Pain control with sublingual morphine. The advantages for hospice care, Am. J. Hospice Care 4 (1987) 39–41.
[35] Z. Dong, K. Pan, J. Pan, Q. Peng, Y. Wang, The possibility and molecular mechanisms of cell pyroptosis after cerebral ischemia, Neurosci Bull 34 (2018) 1131–1136.
[36] M. Han, H. Gao, P. Ju, M.Q. Gao, Y.P. Yuan, X.H. Chen, et al., Hispidulin inhibits hepatocellular carcinoma growth and metastasis through AMPK and ERK signaling mediated activation of PPARgamma, Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 103 (2018) 272–283.
[37] D.G. Hardie, AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy, Nat. Rev. Mol. Cell Biol. 8 (2007) 774–785.
[38] S.J. Byun, Y. Son, H.O. Pae, Cytoprotective effect of beta-lapachone by inducing heme oxygenase-1 expression and AMP-activated protein kinase activation in human endothelial cells, Eur. Rev. Med. Pharmacol. Sci. 18 (2014) 949–958.
[39] L. Qing, J. Fu, P. Wu, Z. Zhou, F. Yu, J. Tang, Metformin induces the M2 macrophage polarization to accelerate the wound healing via regulating AMPK/mTOR/NLRP3 inflammasome singling pathway, American journal of translational research 11 (2019) 655–668.
[40] M.T. Song, J. Ruan, R.Y. Zhang, J. Deng, Z.Q. Ma, S.P. Ma, Astragaloside IV ameliorates neuroinflammation-induced depressive-like behaviors in mice via the PPARgamma/NF-kappaB/NLRP3 inflammasome axis, Acta Pharmacol. Sin. 39 (2018) 1559–1570.
[41] C.Y. Zhang, M.J. Zeng, L.P. Zhou, Y.Q. Li, F. Zhao, Z.Y. Shang, et al., Baicalin exerts neuroprotective effects via inhibiting activation of GSK3beta/NF-kappaB/NLRP3 signal pathway in a rat model of depression, Int. Immunopharmacol. 64 (2018) 175–182.
[42] I. Erdogan-Orhan, M.L. Altun, B. Sever-Yilmaz, G. Saltan, Anti-acetylcholinesterase and antioxidant assets of the major components (salicin, amentoflavone, and chlorogenic acid) and the extracts of Viburnum opulus and Viburnum lantana and their total phenol and flavonoid contents, J. Med. Food 14 (2011) 434–440.