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Lychee Seed Fraction (LSF) Suppressed Aβ_{1-42}-induced Neuroinflammation, Apoptosis and Facilitated Viabilities of PC12 Cells via NF-κB Signalling Pathway

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KEYWORDS Alzheimer's Disease. Beta-Amyloid$_{1-42}$. Inflammation. Lychee Seed Fraction. Nuclear Factor Kappa-B

ABSTRACT Traditional Chinese medicines have been widely used for Alzheimer's disease (AD) treatment. This study examined impacts of lychee seed fraction (LSF) on modulating PC12 cell apoptosis, viability and inflammatory responses after Aβ_{1-42} induction. CCK-8 was applied to measure PC12 cell viabilities, showing that Aβ_{1-42}-induced low PC12 cell viabilities were promoted by LSF treatment dose-dependently. Moreover, using JC-1 staining, mitochondrial membrane potential (MMP) was suppressed with Aβ_{1-42} induction while LSF treatment reversed impacts of Aβ_{1-42}. LSF also suppressed Aβ_{1-42}-induced elevated TNF-α, IL-1β and iNOS mRNA and protein expressions. Additionally, LSF treatment restrained Aβ_{1-42}-induced apoptosis through downregulating cleaved-PARP and Bax protein expressions and upregulating Bcl-2 protein expressions. Furthermore, LSF also downregulated IκBα and phosphorylated p65 protein expressions in PC12 cells after Aβ_{1-42} induction. Hence, LSF restrained Aβ_{1-42}-induced low PC12 cell viability and high apoptosis through suppressing Bax and cleaved-PARP and elevating Bcl-2 via NF-κB signalling pathway.

INTRODUCTION

Alzheimer's disease (AD) is one progressive and chronic neurodegenerative disorder that is associated with memory loss, behavioural, and cognitive dysfunction (Chen et al. 2014). At present, about 50 million people are suffering from AD, and this amount is increasing with time (Breijyeh and Karaman 2020). It was also observed that most of the patients suffering from AD are over 75 years old (Leira et al. 2017). Cholinergic and amyloid hypotheses are two main hypotheses that were proposed as reasons for AD (Breijyeh and Karaman 2020). The two leading causes of AD are Aβ peptide accumulation in SPs and hyperphosphorylation of tau in NTFs (Sevigny et al. 2016). Tau is a protein associated with microtubules that can accumulate in the neurons leading to the death of neuron cells (Khan and Bloom 2016). Accumulation of amyloid β has also been verified to act as a promoter in the occurrence of AD (Scheltens et al. 2021). Many shreds of evidence showed that Aβ plays a crucial role in AD progression such as phosphorylation of tau proteins, apoptotic induction, DNA fragmentation, and mitochondrial dysfunction (Ye et al. 2015). Hence, suppressing amyloid β accumulation might be a promising way for treating AD.

There is a long history of Traditional Chinese Medicines (TCMs) in treating diseases, which have been demonstrated to act critically on neurodegenerative disorders (Wu et al. 2017; Yuan et al. 2017). Lychee seed extracts have been reported to facilitate prostate cancer cell apoptosis and suppress cell viability through inactivating AKT/mTOR and NF-κB signalling pathway (Chang et al. 2021). Furthermore, lychee seed saponins, a fraction of lychee, have been detected to reduce neuronal damages in AD rats through suppressing nerve cell apoptosis (Wang et al. 2017; Zhao et al. 2018). However, how LSF exerts its neuroprotective effects on AD remains unknown.

In this study, mechanisms about LSF in PC12 cells after Aβ_{1-42} induction were investigated. Through examining PC12 cell viabilities, mitochon-

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drial membrane potential, apoptosis, pro-inflammatory expressions, and activation of NF-κB signalling pathway after $A\beta_{1-42}$ induction, impacts of LSF were examined in vitro.

Objectives

This study aimed at exploring impacts of LSF on modulating cell viability and MMP in PC12 cells after $A\beta_{1-42}$ induction. Effects of LSF on modulating proinflammatory cytokines expressions and biomarkers in apoptosis and factors in NF-κB signalling pathway were examined as well.

Experimental

Main Reagents

The reagents used in this study included RPMI 1640 medium (Gibco, USA), FBS (LuBioScience, Zurich, Switzerland), streptomycin (LuBioScience), penicillin (LuBioScience), $A\beta_{1-42}$ (Abcam, UK), CCK-8 kit (Dojindo EU GmbH, Munich, Germany), JC-1 Dye (Mitochondrial Membrane Potential Probe, Invitrogen), Prime Script RT reagent kit (Takara Bio Inc., Japan), RIPA lysis buffer (Thermo Scientific, USA), Tween and Tris-buffered saline (BioLegend, CA, USA), and ECL Detection Reagent (Abcam).

MATERIAL AND METHODS

Cell Culture and Treatment

PC12 cells, obtained from ECM Biosciences (Kentucky, USA), were cultivated in an incubator with 10 percent. FBS, 50 µg/mL streptomycin, and 1 percent penicillin (LuBioScience GmbH, Zurich, Switzerland) in RPMI 1640 medium (Gibco,) having 5 percent CO_2 at 37^0C . Moreover, $A\beta_{1-42}$ (5 µM, Abcam, UK) was used to treat PC12 cells for 12 hours to prepare for the following experiments.

Preparation of Drug

Lychee seed was purchased from Frieda's, 90720 CA, USA, and verified by Professor Can Tang (Department of Pharmacy, Southwest Medical University, China). The purification and isolation of LSF components were done by using the method used in the previous studies (Tang et al. 2018). 1 kg of lychee seed (air-dried) was crushed and saturated with 1 L 80 percent methanol for 12 hours followed by the extraction with 10 L of 80 percent methanol by using filter paper. This filtered solution was collected and evaporated under a vacuum. This dried lychee seed was then dissolved in water and loaded onto the D101 macroporous resin column and adsorbed with 80 percent methanol. After adsorption, a brown powder obtained is ready to use for further experiments.

Cell Viability

The CCK-8 kit (Dojindo EU GmbH, Munich, Germany) was used according to the manufacturer's protocol to measure the cell viability of PC12 cells. A 96-well plate with density (1×10^5 cells/well) was used to culture PC12 cells for 24 hours. Thereafter, cells were exposed into 5µM of $A\beta_{1-42}$ to establish AD cell models. Then, cells were treated by LSF (0.12 and 0.48 $mg.L^{-1}$). Later, 10µL of CCK-8 was added followed by cultivation with cells for 1 hour at 37^0C. A standard plate-reader was applied for examining the absorbance of the solute mixture at 450 nm.

JC-1 Fluorescence Analysis

JC-1, a cationic fluorescent dye that exists as a monomer or dimer in living cells, was used to determine mitochondrial membrane potential. A 96-well plate was used to seed cells and treated with different concentrations of LSF (0.12 and 0.48 $mg.L^{-1}$). Cells were washed by 10 percent warm PBS and cultured with 2µM JC-1 (Invitrogen) for 15 minutes at 37^0C. A fluorescent microscope (Biocompare, San Francisco, USA) was used to observe staining of cells. A percentage of red stained cells to total cells were analysed using Image Pro Plus Software (Media Cybernetics, Inc., MD, USA).

Real-Time PCR

Trizol reagent (Invitrogen) was applied for total RNA isolation, and cDNA was synthesised using Prime Script RT reagent kit (Takara Bio Inc., Japan). A $2^{-\Delta\Delta Ct}$ method was used to find the results of RT-qPCR to qualified expressions of RNAs. Primer sequences used in this experiment were listed in Table 1.

Table 1: Primer sequences for RT-qPCR

Primers		*Sequence*
iNOS	Forward	ACCAACTGACGGGAGATGAG
iNOS	Backward	GTTGCCATTGTTGGTGGAGT
TNF-α	Forward	AGGACACCATGAGCACTGAA
TNF-α	Backward	CCGATCACTCCAAAGTGCAG
IL-1β	Forward	CTAGGGACTTAGGTGCTGTC
IL-1β	Backward	CTCTGCCTTTGCTTCCAAGC
β-actin	Forward	ACCCAGAAGACTGTGGATGG
β-actin	Backward	TCAGCTCAGGGATGACCTTG

Western Blot

PC12 cells were lysed by RIPA lysis buffer (Thermo Scientific, USA) to segregate total protein followed by isolation of protein using SDS-PAGE. Then, proteins were migrated onto PVDF membranes (Biocompare, CA, USA) after electrophoresis followed by the blocking with 5 percent dried milk having no fat in Tween and Tris-buffered saline (BioLegend, CA, USA) for 60 minutes. After washing these membranes three times, the blot was incubated with primary antibodies including TNF-β (210-TA; 1:1000; Novus Biologicals, CO, USA), IL-1β (201-LB; 1:1000), iNOS (NB300-605; 1:1000), IκBα, p-IκBα, PARP (NB100-56708; 1: 1000), cleaved-PARP (NB100-56599; 1:1000), β-actin (NB600-501; 1: 1000), Bax (NBP1-28566; 1: 1000) and Bcl-2 (NB500-249; 1:1000) for 12 hours at 4^0C. Then these blots were cultured with horseradish peroxide labelled anti-rabbit IgG (1: 2000; Jackson Immunoresearch Laboratories Inc., CB7 4EX, UK) for 1 hour. The bands were observed using ECL Detection Reagent (Abcam). The protein band intensity was analysed by using ChemDoc Image J software (BioRad, California, USA).

Statistical Analysis

Data were displayed as mean ± standard deviation (SD). Student's t-test and one-way univariate analysis of variance (ANOVA) were applied for detecting differences of the data among two or more groups. $P < 0.05$ was meaningful statistically. Graphpad Prism 9 (GraphPad, USA) was used to present data in graphical form.

RESULTS

LSF Suppressed $A\beta_{1-42}$-induced PC12 Cell Viabilities

To analyse impacts of lychee seed fraction on PC12 cell viabilities, cells were first treated by $A\beta_{1-42}$. Results of CCK-8 showed that cell viability was inhibited in PC12 cells treated with $A\beta_{1-42}$ (5 μM) (Fig. 1, $^{****}P<0.0001$). Conversely, LSF (0.12 and 0.48 $mg.L^{-1}$) treatment reversed the inhibitory impacts of $A\beta_{1-42}$ and increased PC12 cell viabilities dose-dependently (Fig. 1, $^{**}P=0.029$ and $^{****}P<0.0001$). These results suggested that LSF could restrain low PC12 cell viabilities caused by $A\beta_{1-42}$.

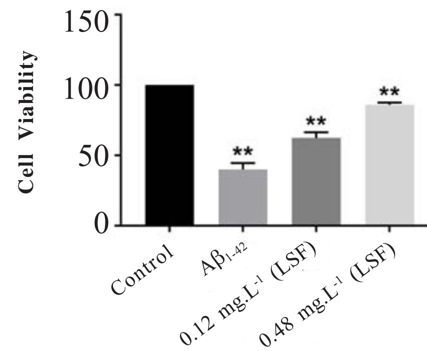

Fig.1. LSF treatment facilitated PC12 cell viabilities after $A\beta_{1-42}$ induction. CCK-8 was applied for measuring PC12 cell viabilities with $A\beta_{1-42}$ induction and LSF treatment, $^{**}P<0.05$
Source: Authors

LSF Promoted Mitochondrial Membrane Potential after $A\beta_{1-42}$ Induction

After PC12 cell viabilities were investigated, the mitochondrial membrane potential was examined to measure cell apoptosis by using JC-1 fluorescence analysis. As seen from the results fluorescent intensity was reduced by the $A\beta_{1-42}$ treatment (Fig. 2, $^{****}P<0.0001$). Reversely, LSF increased the inhibited fluorescent intensity in PC12 cells after $A\beta_{1-42}$ induction (Fig. 2, $^{***}P= 0.0003$ and $^{****}P<0.0001$). The findings revealed that mitochondrial membrane potential was decreased in PC12 cells induced by $A\beta_{1-42}$ but restored by LSF treatment.

LSF Inhibited Pro-inflammatory Cytokines Expressions in $A\beta_{1-42}$-induced PC12 Cells

To analyse impacts of LSF on modulating inflammatory responses, pro-inflammatory cytokines expressions were examined. Results showed that $A\beta_{1-42}$

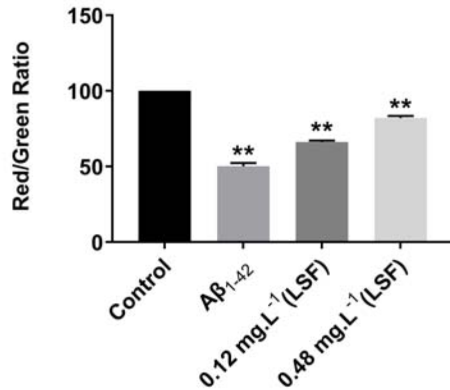

Fig.2. LSF increased mitochondrial membrane potential in $A\beta_{1-42}$-induced PC12 cells. JC-1 was applied for evaluating MMP in PC12 cells after $A\beta_{1-42}$ induction and LSF treatment, $^{**}P<0.05$
Source: Authors

induction upregulated TNF-α, IL-1β and iNOS mRNA and protein expressions while expressions of these three were all downregulated by LSF treatment dose dependently (Fig. 3A, B, C, $^{**}P<0.05$). Hence, LSF treatment suppressed $A\beta_{1-42}$-induced PC12 cell inflammatory responses via downregulating proinflammatory cytokines expressions.

LSF Inhibited Aβ1-42-induced PC12 Cell Apoptosis

To figure out impacts of LSF on regulating $A\beta_{1-42}$-induced PC12 cell apoptosis, Bcl-2, Bax and cleaved-PARP protein expressions were evaluated. In Figure 4A and B, results of flow cytometry indicated that $A\beta_{1-42}$ downregulated Bcl-2 protein expressions but upregulated Bax and cleaved-PARP protein expressions ($^{**}P<0.05$). In contrast, LSF treatment elevated Bcl-2 protein expressions but decreased Bax and cleaved-PARP protein expressions (Fig. 4A. B, $^{**}P<0.05$). Besides that, the

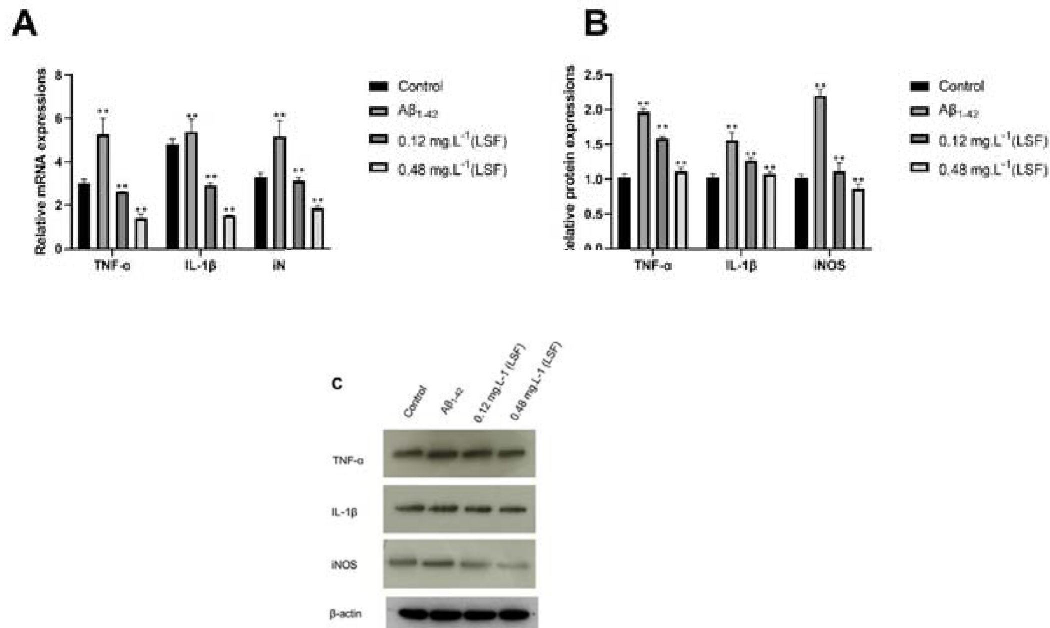

Fig. 3. LSF suppressed proinflammatory cytokines expressions after $A\beta_{1-42}$ induction. A: TNF-α, IL-1β and iNOS mRNA expressions were assessed using RT-qPCR, $^{**}P<0.05$. B, C: TNF-α, IL-1β and iNOS protein expressions were detected by western blot, $^{**}P<0.05$
Source: Authors

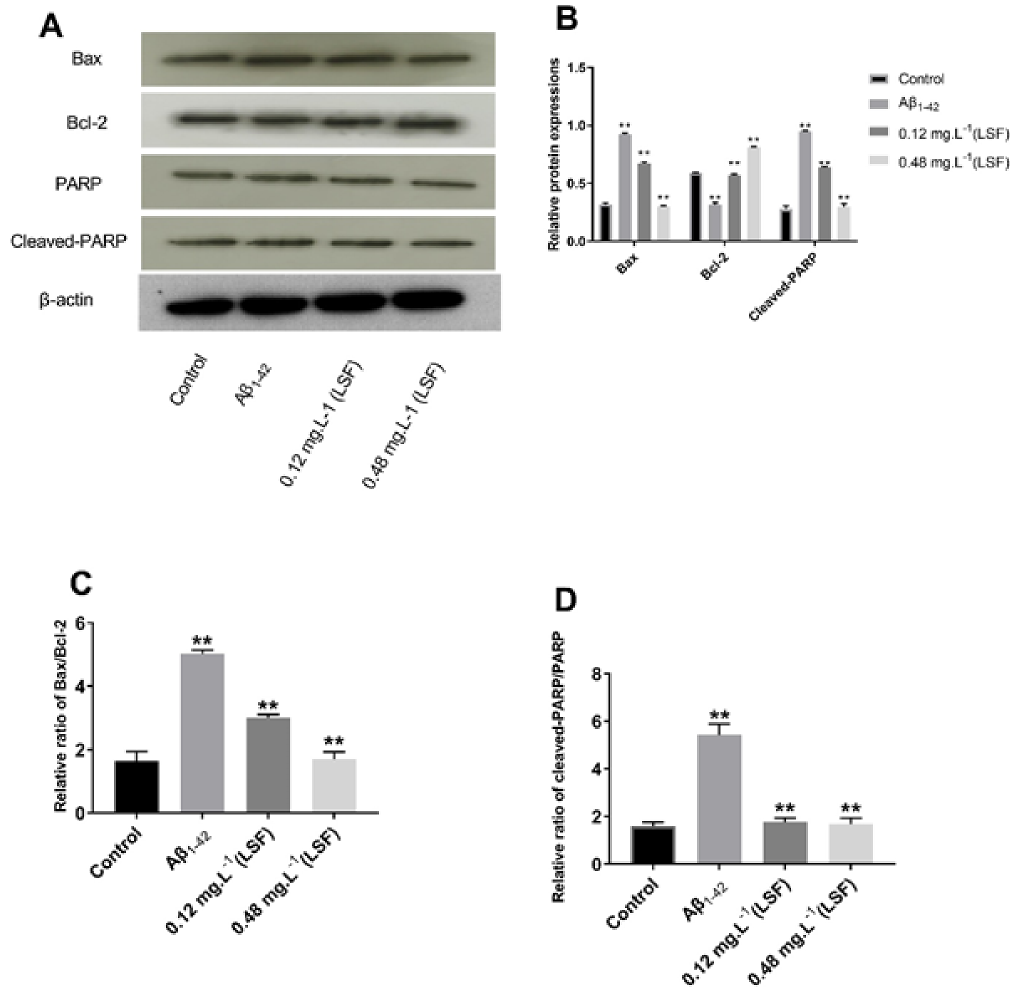

Fig. 4. LSF inhibited $A\beta_{1-42}$-induced apoptosis in PC12 cells. A, B: Western blot was applied for validating Bax, Bcl-2, cleaved-PARP and PARP protein expressions. C: The ratio of Bax/Bcl-2. D: The ratio of cleaved-PARP/PARP
Source: Authors

ratio of Bax/Bcl-2 was decreased with LSF treatment, so was the ratio of cleaved-PARP/PARP (Fig. 4C, D, $^{**}P<0.05$). Therefore, LSF modulated apoptosis related biomarkers to suppress PC12 cell apoptosis after $A\beta_{1-42}$ induction.

LSF Inhibited $A\beta_{1-42}$-induced PC12 Cell Progression via NF-κB Signaling Pathway

Expressions of pro-inflammatory cytokines are regulated by the NF-κB signalling pathways, which are associated with the inhibitory factor IκBα. Aβ has been reported to regulate pro-inflammatory cytokines via NF-κB activation. Therefore, factors in the NF-κB signalling pathway were examined in this study to figure out further roles about LSF with $A\beta_{1-42}$ induction. Results showed that p-IκBα, IκBα, p-p65, p65 protein expressions were up regulated in $A\beta_{1-42}$-induced PC12 cells (Fig. 5A). However, treatment of LSF downregulated p-IκBα, IκBα, p-p65, p65 protein expressions to hamper $A\beta_{1-42}$ induction in PC12 cells (Fig. 5A).

Additionally, LSF suppressed the ratio of p-IκBα/IκBα and p-p65/p65 in $A\beta_{1-42}$-induced PC12 cells, indicating that LSF modulated PC12 cell progressions via NF-κB signalling pathway (Fig. 5B, C).

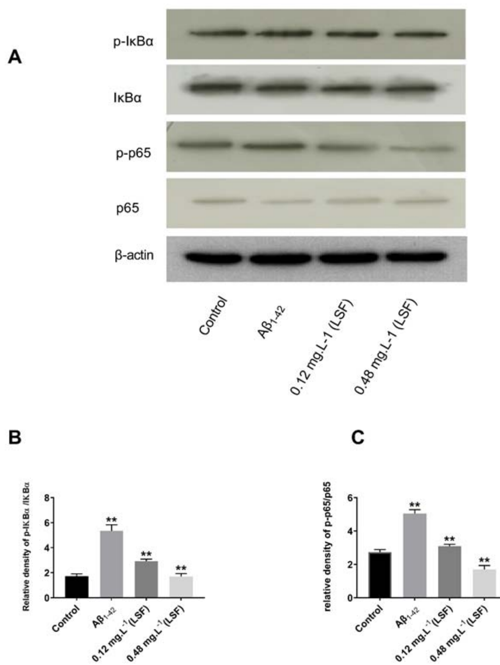

Fig. 5. LSF regulated $A\beta_{1-42}$-induced PC12 cell progression via NF-κB signaling pathway. A: Phosphorylated IκBα, IκBα, phosphorylated p65 and p65 protein expressions in PC12 cells with $A\beta_{1-42}$ induction and LSF treatment were examined using western blot. B: The ratio of p- IκBα/ IκBα. C: The ratio of p-p65/p65.
Source: Authors

AD is a pathological neurodegenerative disorder causing memory deficits and cognition. From previous studies, it can be elucidated that the activation of microglia can significantly improve the Aβ clearance, which in return can protect the central nervous system from pathogens and also reduce the inflammation (Alarcon et al. 2005). Lychee seed, a traditional herbal medicine, has been demonstrated to provide many biological functions such as enhancing insulin resistance (IR), anti-virus, anti-oxidation, and anti-inflammation (Xu et al. 2010). Results from the previous research showed the impact of LSF on the protection against injury induced by Aβ, improved cognition in a mouse model and inhibited neuroinflammation in PC12 cells induced by $A\beta_{1-42}$ (Wang et al. 2017; Wang et al. 2017). However, the exact molecular mechanism of LSF exerting protective roles in AD remains unclear. Therefore, the present study involved the validation of the neuroprotective effects of LSF on Aβ-induced PC12 cells in AD.

Results from previous studies revealed that there was a degeneration of neurons in AD due to Aβ-induced inflammation (Kitamura 2011; Wullaert et al. 2011). Other research demonstrated that in AD patient's brains, the level of pro-inflammatory cytokines increased significantly, leading to the pathogenesis of AD (Ghosh and Karin 2002). Upregulated pro-inflammatory cytokines such as TNF-α and IL-6 have been verified to induce neuron damage in AD patients (Hayden and Ghosh 2008). Therefore, in this study, a cellular model of AD was established to investigate impacts of LSF on modulating proinflammatory cytokines expressions. The findings revealed that LSF reduced TNF-α, iNOS and IL-1β mRNA and protein expressions in $A\beta_{1-42}$-induced PC12 cells, suggesting a protective role of LSF in AD development via regulating inflammatory responses.

The Bcl-2 family is also critical in modulating apoptosis with pro-apoptotic proteins and anti-apoptotic ones (Tang et al. 2018). Furthermore, PARP is another protein associated with apoptosis, which might involve genomic stability, repairing of DNS, and programmed cell death. For the activation of caspase 3 and apoptosis, cleaved-PARP was considered as a crucial indicator (Zeng et al. 2016). Based on these results, the researchers examined cleaved-PARP, Bax and Bcl-2 protein expressions to verify LSF effects on modulating PC12 cells apoptosis induced by $A\beta_{1-42}$. Results revealed that LSF reduced the ratio of Bax/Bcl-2 and cleaved-PARR/PARP in PC12 cells after $A\beta_{1-42}$ induction. Hence, LSF inhibited the apoptosis in $A\beta_{1-42}$-induced PC12 cells.

NF-κB plays a vital role in the regulation of cell survival, the release of pro-inflammatory cytokines, and DNA transcription (Seo et al. 2018). NF-κB activation is associated with the release of pro-inflammatory cytokines such as IL-6, COX-2, IL-1β, and TNF-α (Park et al. 2015). IκBα is an inhibitory factor that might be produced by NF-κB in the cytoplasm (Shi et al. 2016). Activation of the NF-κB signalling pathway also leads to the

phosphorylation of IκBα by IκB kinase. The previous study indicated that LSF could inhibit apoptosis in BV-2 cells and neurons, which were directly connected with the suppression of NF-κB signalling pathways (Wang et al. 2017). The results revealed that LSF inhibited the NF-κB signalling pathways by inhibiting the p-IκBα/IκBα and p-p65/p65 expressions in $A\beta_{1-42}$-induced PC12 cells, Therefore, LSF might modulate PC12 cell progression via inactivating NF-κB signalling pathway.

CONCLUSION

LSF facilitated $A\beta_{1-42}$-induced PC12 cells viabilities but decreased cell apoptosis and inflammatory reactions via downregulating NF-κB signalling pathway, suggesting that LSF might be used as a potential drug against neuroinflammation in AD.

RECOMMENDATIONS

LSF treatment promoted PC12 cell viabilities and MMP dose-dependently after $A\beta_{1-42}$ induction. Moreover, LSF decreased TNF-α, IL-1β and iNOS mRNA and protein expressions inhibited Bax and cleaved-PARP protein expressions but upregulated Bcl-2 protein expressions. Furthermore, LSF treatment suppressed NF-κB signalling pathway activation to modulate PC12 cells progression after $A\beta_{1-42}$ induction. Herein, LSF was verified to prevent $A\beta_{1-42}$-caused damages in PC12 cells, showing a protective impact of this TCM. Further studies should focus on impacts of LSF in vivo and clinical stages to gain more knowledge.

ABBREVIATION LIST

- AD: Alzheimer's Disease
- MMP: Mitochondrial Membrane Potential
- $A\beta_{1-42}$: Beta-Amyloid$_{1-42}$
- TCM: Traditional Chinese Medicine
- PARP: Poly-ADP-ribose Polymerase
- CNS: Central Nervous System
- LSF: Lychee Seed Fraction
- NF-κB: Nuclear Factor Kappa-B
- RIPA: Radio Immunoprecipitation Assay

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