International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2019.593.603

Research Article
Reverse of β-Amyloid Induced Apoptosis in PC12 Cells by Nattokinase: Role of SIRT1-ROCK1 Pathway

[1]Hongyuan Zhang, [2]Yanan Kang, [3]Youqun Han, [4]Xuemei Chen and [5]Runli Wang

[1]Department of Neurology, Jiyang Public Hospital, Jinan, 251400 Shandong, China
[2]Department of Internal Medicine, Laoling Municipal Hospital of Traditional Chinese Medicine, Dezhou, 253600 Shandong, China
[3]Department of Anesthesiology, Jinan Central Hospital Affiliated to Shandong University, Jinan, 250013 Shandong, P.R. China
[4]Innoscience Research SdnBhd, Jalan USJ 25/1,47650Subang Jaya, Selangor, Malaysia
[5]Department of Neurology, Pingyi County People's Hospital, No. 7, Jinhua Road, Linyi, 273300 Shandong, China

Abstract

Background and Objective: It is well-established fact that Alzheimer's disease (AD) is a neurodegenerative disease of CNS with progressive nature. The available treatment for AD unable to treat the pathology of disease. Due to this limitation urgent need the newer treatment of AD. The current investigation was aimed to scrutinize the neuro-protective effect of nattokinase against β-amyloid peptide$_{25-35}$ (Aβ_{25-35}) induced neurotoxicity and explored the underlying mechanism. **Materials and Methods:** The PC12 cells were damaged by Aβ_{25-35} and different concentration of nattokinase was added into the culture medium. Lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were performed for the estimation of cell viability and also estimation the hydrogen peroxide (H_2O_2) generation for estimation the intracellular signalling pathways. Furthermore, determination of mitochondrial membrane potential changes and caspase-3 effect of this compound occurred in this study. Additionally, ROS intracellular and expression of silent information regulator 1 (SIRT1) and Rho-associated kinase 1 (ROCK1) were also estimated. **Results:** The concentration-dependent treatment of nattokinase down-regulated the cell viability counts. The result exhibited that cell death induced via Aβ_{25-35} was mediated through an up-regulation of ROS production. It is estimated that there was enhanced caspase-3 and caspase-8 activity attributing to depolarization of mitochondrial membrane and reduced by the nattokinase. Nattokinase reduced the reactive oxygen species and down-regulated the overproduction of IL-1β, IL-6 and TNF-α, respectively. Nattokinase reduced the cell apoptosis and expression of ROCK1 and increased the expression of SIRT1. **Conclusion:** On the basis of the result, concluded that SIRT1-ROCK1 pathway plays a significant role in the AD pathology and nattokinase reduced the AD complication via SIRT1-ROCK1 pathway.

Key words: Alzheimer's disease, SIRT1, ROCK1, oxidative stress, nattokinase

Citation: Hongyuan Zhang, Yanan Kang, Youqun Han, Xuemei Chen and Runli Wang, 2019. Reverse of β-amyloid induced apoptosis in PC12 cells by nattokinase: Role of SIRT1-ROCK1 pathway. Int. J. Pharmacol., 15: 593-603.

Corresponding Author: Runli Wang, Department of Neurology, Pingyi County People's Hospital, No. 7, Jinhua Road, Linyi, 273300 Shandong, China
Tel: +86-10-65255378

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The first term Alzheimer's disease (AD) was coined by the Alois Alzheimer about a century age[1,2]. Studies suggested that AD is the most common neurodegenerative disease commonly found in the older patient, which ultimately damages the cognitive function of brain[3,4]. Research suggested that the AD is considered as the most common form of dementia and also considered as the progressive neurodegenerative disease of brain described by disordered cognitive function, modulated behaviour and progressive memory injury[5,6]. Previous studies suggested that the incidence of AD exponentially increases with the age[5,7]. Epidemiology studies suggested that nearly 1/8 older people (more than age 65 years) suffered from the AD and the incidence of AD is predictable to be close to the 30 million[8-10] by 2050.

Generally, Aβ protein unusually pledges in the brain, which is the significant hallmark of AD[11]. During the toxic effect into the brain, Aβ accumulation plays an important role in the progression of AD pathogenesis. Aβ is considered as the major module of senile plaques, resultant from the sequential proteolysis of amyloid precursor protein via sequential cleavages of β and γ-secretase and play a considerable role in the AD pathophysiology[12,13]. In spite of, studies have been designed to scrutinize the underlying mechanism Aβ neurotoxicity, the possible mechanism still remain unclear. Studies suggested that inflammation and oxidative stress play an important role in the Aβ-induced neurotoxicity[14,15]. The deposition of Aβ in the primary neurons could be persuaded by oxidative stress and Aβ boots extra inflammatory reaction via activation of cyclooxygenase-2 in the astrocytes through interaction with tumor necrosis factor-α (TNF-α), nuclear factor κ-B (NF-κ B) and interleukin-1β (IL-1β) mechanism in rat brain[15-17].

The ROCK is the serine/threonine protein kinase, which are two types of diastereomers (ROCK1 and ROCK2). The ROCK played an important role in the reduction of the non-amyloid ogenica secretase processing of amyloid precursor protein[18,19]. Previous research suggested that the over-expression of SIRT1 in the primary neurons decrease the expression of ROCK1 and Aβ secretion and increases the cell viability, signifying that the SIRT1 boosts a secretase arbitrated non-amyloidogenic APP processing through ROCK1 signaling pathway[18,20,21]. Based on the above fact, hypothesized that nattokinase sheltered the neurons against Aβ neurotoxicity and scrutinize the possible role of SIRT1 expression, probably relating the regulation of ROCK1. For confirmation of the hypothesis, there is need to examined the beneficial effect of nattokinase on the $A\beta_{25-35}$ injury using the PC12 cells. In the current experimental study was aimed to performed the cell viability test, MTT assay and LDH assay; intracellular calcium level. Furthermore, RT-PCR was used for the estimation of mRNA expression of ROCK1 and SIRT1.

MATERIALS AND METHODS

Nattokinase was received as a gift sample. The PC12 cell lines were procured from the Shanghai Institute for Biological Sciences, Chines Academy of Sciences, Shanghai, China. The $A\beta_{25-35}$, $A\beta_{1-42}$, 5,5,6,6-tetrachloro-1,1,3,3-tetraethyl-benzimidazolyl-carbocyanineiodide (JC-1), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Hoechst 33342 and nicotinamide were purchased from the Molecular Probes, USA and Sigma, USA. Dulbecco's modified Eagle's medium (DMEM) and Fetal Bovine Serum (FBS) purchased from the Sigma Aldrich, USA. Anti-phospho-ERK1/2, horseradish peroxidase-conjugated anti rabbi, anti-ERK1/2, anti-Akt-anti-GAPDH, anti Tublin and anti-phosphor Akt473 were procured from the Cell Signaling Technology, U.S.A. The current research performed in the month of December, 2018-January, 2019.

Protective effect on cultured cells: About 24 well plates were used for the cultured the PC12 cells at 37°C in the presence of CO_2. After that the various group divided of $A\beta_{25-35}$ treatment as: $A\beta_{25-35}$ control group, $A\beta_{25-35}$ was mixed into the culture medium at a concentration (20 mM); nattokinase treated, which contains 4 sub-groups with addition of 1.25, 2.5, 10 and 20 mL of 5 mM Nattokinase, respectively, into the culture medium prior to $A\beta_{25-35}$ injury; thus the final concentration of nattokinase was 12.5-100 mM, respectively. After the incubation (24 and 48 h), cells were collected from all groups and subjected to perform the various experimentation[22,23].

MTT assay: Briefly, 96 well plates were seeded in PC12 cells at a density of $4\text{-}8\times10^5$ cells/well in serum medium (1%) for 24 h as using the previously reported method with minor modification. After serum starvation, the cultures were incubated for 24 h with inhibitors or drugs and treated with Aβ; later performed the MTT assay (24 h later). After that, the cells were further incubated with 0.5 mg mL^{-1} MTT for more 3 h. After that, the medium removed from each well and 200 µL DMSO was added. After that, the optical density was estimated at a wavelength of 570 nm. Normal control group,

cultured without mixed of either Nattokinase or $A\beta_{25-35}$. An equal volume of serum-free DMEM and DMSO was added into the $A\beta_{25-35}$ injury group and normal control group[22,24].

Neurotoxic cell model: For establish the neurotoxic cell model with $A\beta_{25-35}$ PC12 cell lines was maintained in the FBS (10%), DMEM at 37°C in a humidified atmosphere supplied CO_2 (5%). The culture medium was changed every 3 days. Before the experimental study, the cells seeded in the culture plate at a density of 2×10^4 cells cm^{-2}. After the 24 h different concentration of $A\beta_{25-35}$ was added into the cell culture. Cells were exposed at a different time interval (24 and 48 h) after the exposure[25] to $A\beta_{25-35}$.

LDH assay: Lactate dehydrogenase assay was performed for the estimation of cell cytotoxicity. Briefly, LDH released were estimated into the incubation medium when the cellular membrane was demolished. The PC12 cells were seeded into the 96 well plates. Briefly, the released LDH activity was estimated according to CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, USA). M200 PRO Multimode Microplate has used the estimation of the fluorescent intensity at 560 nm and emission at 590 nm. All the values of LDH (%) released were normalized to the control group[22,24,25].

Estimation of intracellular ROS level: For the estimation of intracellular ROS, Cell ROXs Deep Red Reagent (Thermo Fisher Scientific, USA), 5 mM Cell ROXs Deep Red Reagent were incubated with the PC12 cells and incubated in DMEM for 1 h in dark room, washed with PBS solution and finally fluorescence was estimated via using the fluorescent microscope at a wavelength 640 nm and an emission wavelength (665 nm). Image J-software was used for the estimation of the ROS level. All the value of ROS (%) level were normalized to the control group[23,24].

Caspase 3/7 activity: The commercially available kits of caspase-Glos 3/7 were used for the estimation of caspase 3/7 activity via using the manufacturer's protocol (Invitrogen, USA). Briefly, the lysis buffer was used for lysate the PC12 cell and centrifuged at 12,500 g rpm for 5 min and incubated at room temperature[22-24].

Real-time quantitative PCR: For the estimation, the protective mechanism of Nattokinase, in order to the expression of ROCK1 and SIRT1 were evaluated by using the real-time quantitative PCR. Trizol reagent was extracted the total RNA from the PC12 cells and finally the RNA integrity was examined spectroscopically according to the A260/280 absorption. Consequently, reverse transcription used for the synthesized the cDNA. Mastercycler-ep-realplex (Eppendorf, Hamburg, Germany) was used for the performance of PCR. The following primers used:

SIRT1:

- Reverse primer: 59-CAGCAAGGCGAGCATAAATAC-39
- Forward primer: 59-CCAGAAACAATTCCTCCACCT-39

ROCK1:

- Reverse primer: 59-AGGCACATCGTAGTTGCTCAT-39
- Forward primer: 59-ATCCACCAGGAAGGTTTATGC-39

GAPDH:

- Reverse primer: 59-TGTCATATTTCTCGTGGTTCA-39
- Forward primer: 59-TGGAGTCTACTGGCGTCTT-39

Finally, the relative expression of the targeted gene at the mRNA level was estimated by using the $2^{-\Delta\Delta Ct}$ method.

Statistics analysis: Graph Pad Prism 7.0 statistical software (GraphPad Software, Inc., San Diego, CA, USA) was used for analysis of the statistics. All the experiments were done in the Triplicate. All the data are presented as mean±standard deviation (SD). One-way ANOVA followed by Tukey's multiple comparisons was used for the statistical analysis. The $p<0.05$ was considered as statistically significant.

RESULTS

$A\beta_{25-35}$ induced cytotoxicity in PC12 cells: In the current study, investigation of cytotoxicity of Aβ peptide (peptide $A\beta_{25-35}$) on the PC12 cells occurred by MTT assay. Figure 1a showed the notable reduction of the cell viability in a concentration-dependent manner, which suggested that the $A\beta_{25-35}$ could induce the toxicity in the PC12 cell lines. Figure 1b showed that the $A\beta_{25-35}$ (0.3 μM) lower dose and 2-10 μM (higher dose) able to induce cell death, but for the current study, 0.3 μM dose of $A\beta_{25-35}$ because it induced the 30-40% cell death. To estimate the potential effects of nattokinase on PC12 cells, the cells were treated with the nattokinase for 1 h before the treatment to $A\beta_{25-35}$ for the next 24 h. The MTT assay, the treatment of $A\beta_{25-35}$ (0.3 μM)

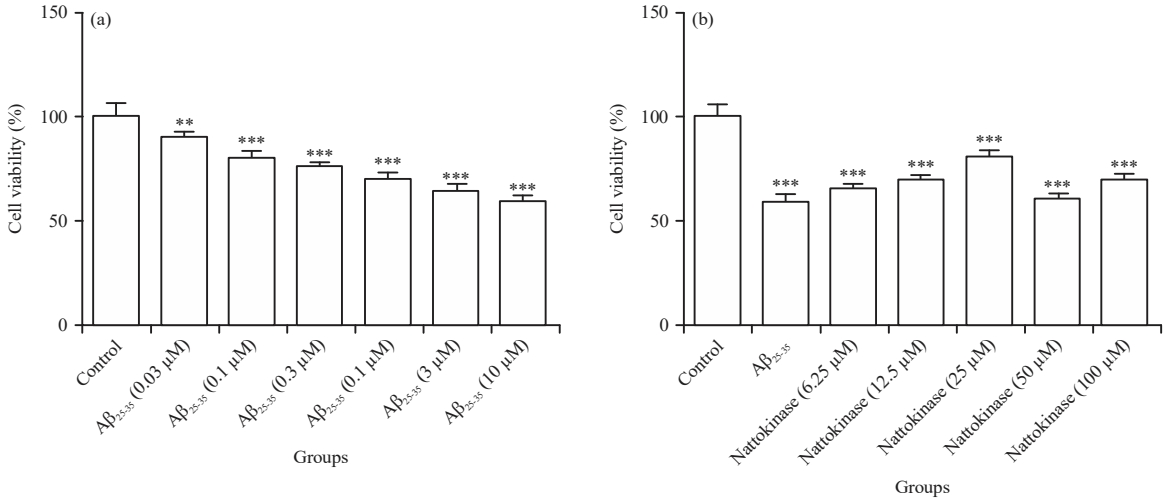

Fig. 1(a-b): Effect of nattokinase on the $A\beta_{25-35}$ induced cell viability loss in PC12 cells, (a) Cells were treated different concentration of $A\beta_{25-35}$ and (b) Nattokinase

***p<0.01, **p<0.01, compared with the $A\beta_{25-35}$ injury group compared by student's t-test (n = 3)

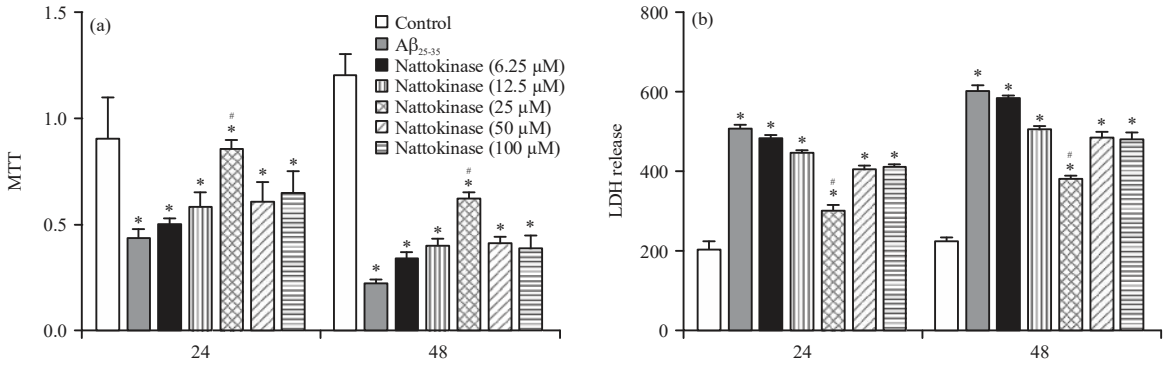

Fig. 2(a-b): Effect of nattokinase on PC12 cells against $A\beta_{25-35}$ on (a) MTT and (b) LDH

Cells treated with different concentration of nattokinase and compared against $A\beta_{25-35}$, *p<0.01, compared with the $A\beta_{25-35}$ injury group, #p<0.01, compared with values of different concentration of nattokinase by student's t-test (n = 3)

showed the dominant cell death and pre-treatment of nattokinase significantly decreased the $A\beta_{25-35}$ induced cell death.

Effect on the LDH, apoptotic rate and intracellular ROS: Lactate dehydrogenase (LDH) and MTT assay were used for the estimation of the protective effect of nattokinase. Figure 2a showed the effect of nattokinase on the MTT assay. Figure 2b showed that the pre-treatment of nattokinase significantly decreased the $A\beta_{25-35}$ induced LDH leakage.

Pre-treatment of nattokinase significantly reduced the cytotoxicity (Fig. 3a). A similar observation was found in the apoptotic rate. Nattokinase pre-treatment showed the downstream of apoptotic rate (Fig. 3b).

$A\beta_{25-35}$ showed the augmented level of intracellular ROS (191±7.6) and $A\beta_{25-35}$ induced group pre-treated with nattokinase showed the down-regulation (125.6±6.4) of intracellular ROS (Fig. 3c).

Effect of nattokinase on caspase 3/7 activity and mitochondrial membrane: The effect of nattokinase on the mitochondrial membrane potential ($\Delta\psi m$) loss (Fig. 4a), $A\beta_{25-35}$ showed the reduction of mitochondrial membrane potential ($\Delta\psi m$) loss and nattokinase significantly (p<0.001) increased the activity of mitochondrial membrane potential ($\Delta\psi m$) loss. Caspase 3/7 is the significant biomarker for activation of apoptosis in the neuronal cells. Figure 4b exhibited that the treatment of

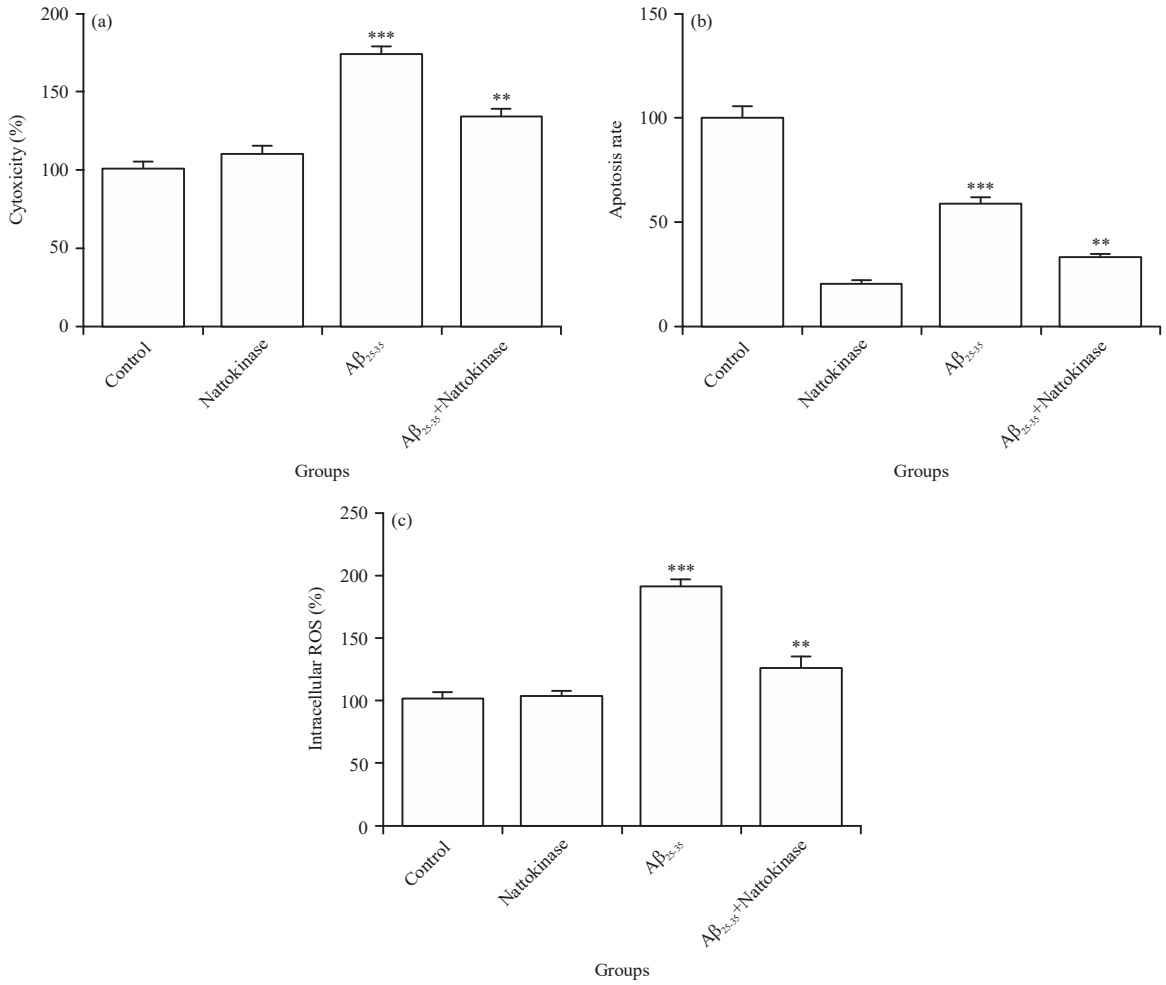

Fig. 3(a-c): Effect of nattokinase reduced the $A\beta_{25-35}$ induced apoptosis and LDH in the PC12 cells, (a) Cytotoxicity effect, (b) Apoptosis rate and (c) Intracellular ROS

Cells treated with different concentration of $A\beta_{25-35}$, ***p<0.01, **p<0.01, compared with the $A\beta_{25-35}$ injury group compared by student's t-test (n = 3)

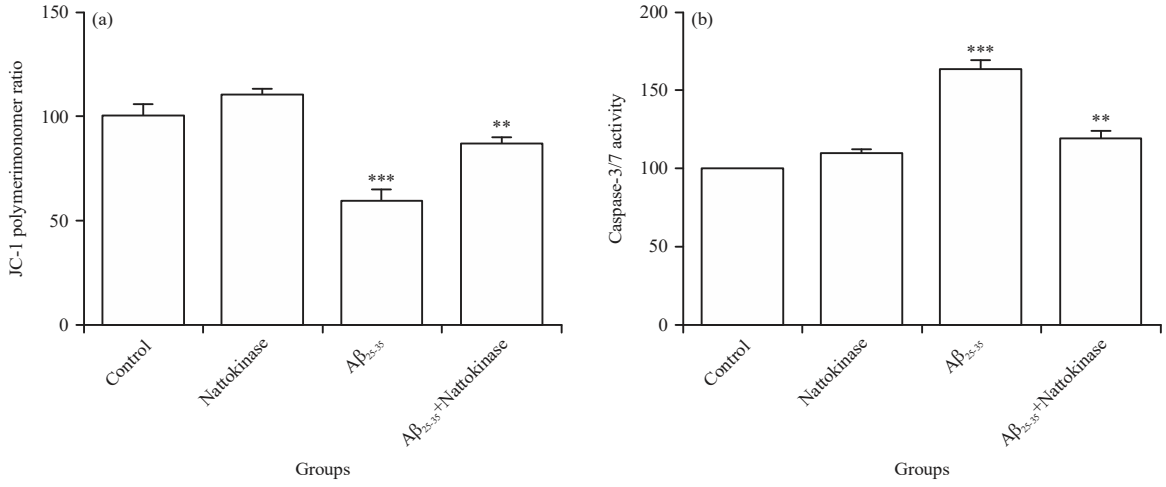

Fig. 4(a-b): Effect of nattokinase on Aβ-induced caspase 3/7 activity and mitochondrial membrane potential ($\Delta\psi m$) loss, (a) Mitochondrial membrane potential ($\Delta\psi m$) loss and (b) Caspase 3/7 activity

Cells treated with different concentration of $A\beta_{25-35}$, ***p<0.01, **p<0.01, compared with the $A\beta_{25-35}$ injury group compared by student's t-test (n = 3)

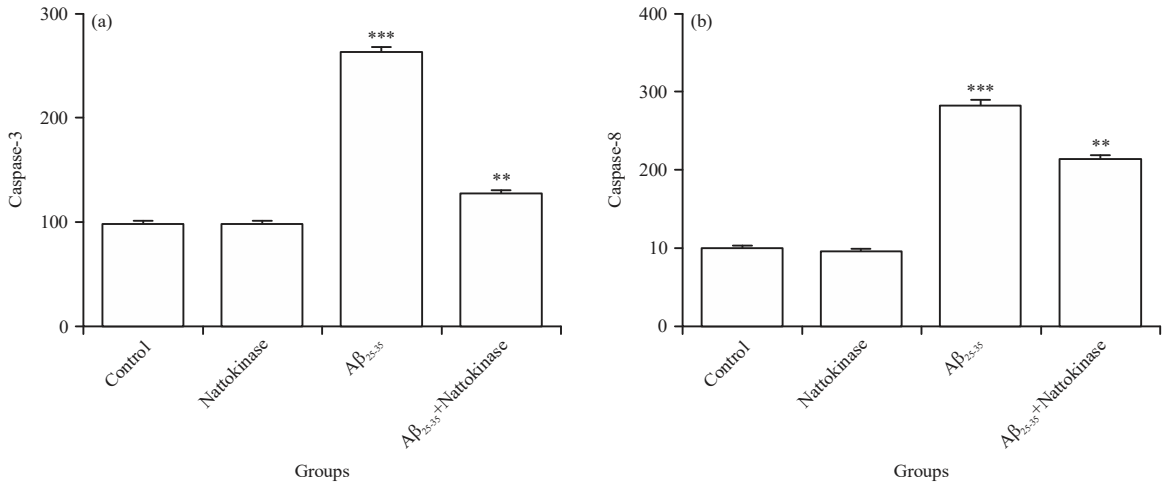

Fig. 5(a-b): Effect of nattokinase of (a) Caspase-3 and (b) Caspase-8

Control group contained no nattokinase and Aβ treatment, cells treated with nattokinase (20 μM) with or without treatment of Aβ, ***p<0.01, **p<0.01, compared with the $Aβ_{25-35}$ injury group compared by student's t-test (n = 3)

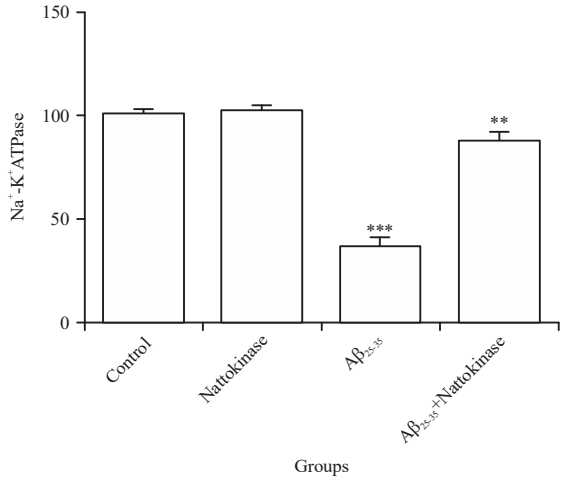

Fig. 6: Effect of nattokinase of the Na^+-K^+ ATPase activity

Cells treated with nattokinase (20 μM) with or without treatment of Aβ, ***p<0.01, **p<0.01, compared with the $Aβ_{25-35}$ injury group compared by student's t-test (n = 3)

$Aβ_{25-35}$ increased the activity of caspase 3/7 as compared to the control group and nattokinase treatment significantly reduced (35%) the activity of caspase 3/7 as compared to $Aβ_{25-35}$ (Fig. 4b).

Effect of nattokinase on caspase: The effect of nattokinase on the caspase-3 and caspase-8 showed in the Fig. 5a and b. Normal and nattokinase group showed an almost similar level. $Aβ_{25-35}$ treated group showed the increased activity of caspase-3, caspase-8 and nattokinase treatment showed the significant reduction of caspase-3 (Fig. 5a) and caspase-8 activity (Fig. 5b).

Effect of nattokinase upon mitochondrial membrane stability: Without treatment of nattokinase, the activity of Na^+-K^+ ATPase similar to control group. The Na^+-K^+ ATPase activity of $Aβ_{25-35}$ treated group showed the reduced activity and nattokinase treatment significantly ($p<0.001$) increased the activity of Na^+-K^+ ATPase (Fig. 6).

Effect of nattokinase on pro-inflammatory: Pro-inflammatory cytokines played an important role in the damage of brain cells. Figure 7a-c showed the effect of the nattokinase on the pro-inflammatory cytokines level of $Aβ_{25-35}$ treated group. Normal and nattokinase treated group showed the almost similar level of pro-inflammatory cytokines such as; IL-1β (Fig. 7a), IL-6 (Fig. 7b) and TNF-α (Fig. 7c). The $Aβ_{25-35}$ treated group showed the increased level of pro-inflammatory cytokines IL-1β (201.34±7.65), IL-6 (222.34±7.04) and TNF-α (254.5±9.34) and nattokinase significantly reduced the IL-1β, IL-6 and TNF-α as compared to $Aβ_{25-35}$ treated group.

Effect of nattokinase on apoptosis marker: The effect of nattokinase on the apoptosis marker showed on the Fig. 8a, b. Figure 8a demonstrated that the decreased BCl-2 level (0.56±0.04) (Fig. 8a) and increased level of Bax (1.03±0.09) (Fig. 8b) in the $Aβ_{25-35}$ treated group and nattokinase significantly increased the Bcl-2 level (3.46±0.13) and reduced the Bax level (1.03±0.03).

Effect of nattokinase on SIRT1 and ROCK1 expression: The effect of nattokinase on the ROCK1 and SIRT1 expression presented in the Fig. 9. The SIRT1 expression level decreased

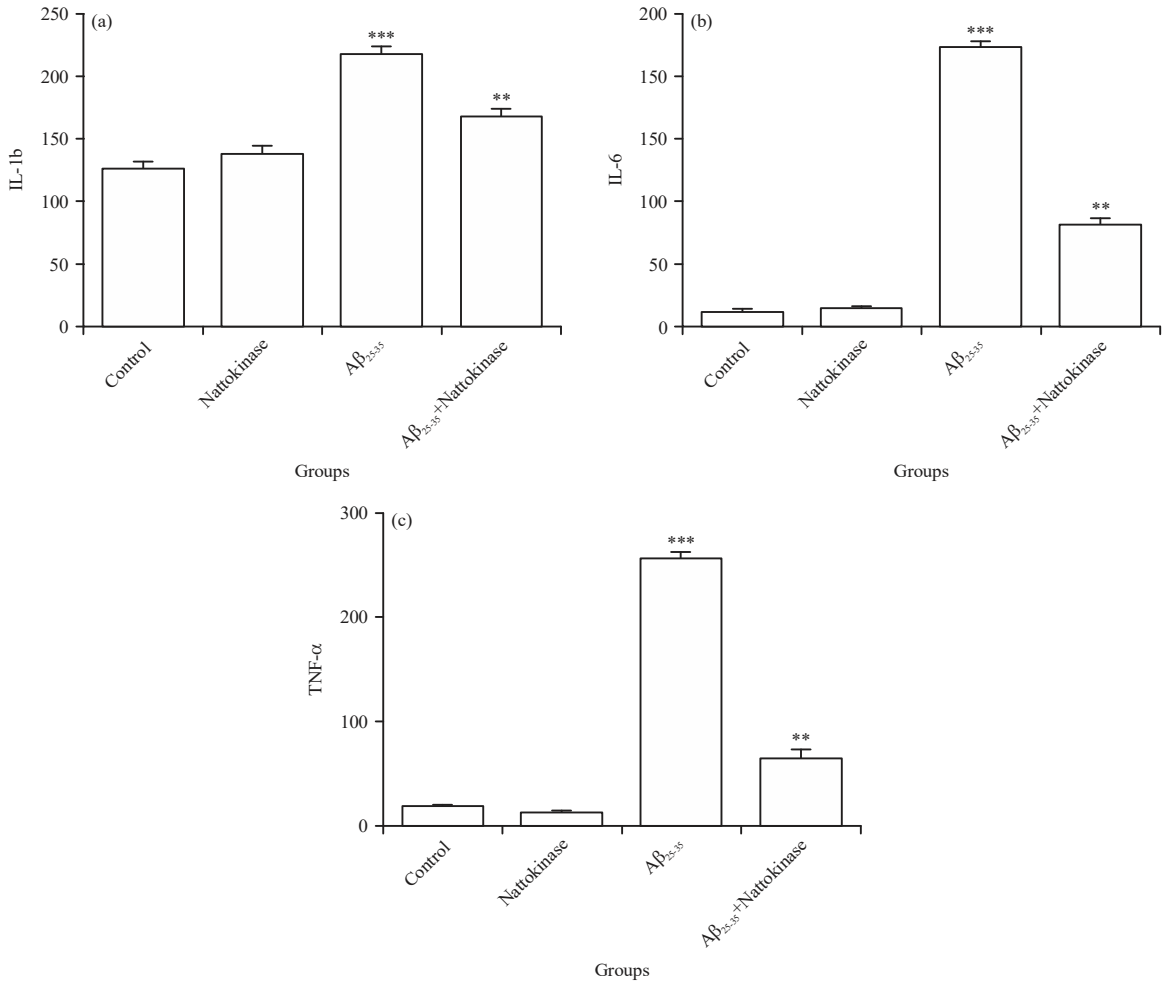

Fig. 7(a-c): Effect of nattokinase on the pro-inflammatory cytokines, (a) IL-1β, (b) Il-6 and (c) TNF-α

Control group contained no nattokinase and Aβ treatment, cells treated with nattokinase (20 μM) with or without treatment of Aβ, ***p<0.01, **p<0.01, compared with the $A\beta_{25-35}$ injury group compared by student's t-test (n = 3)

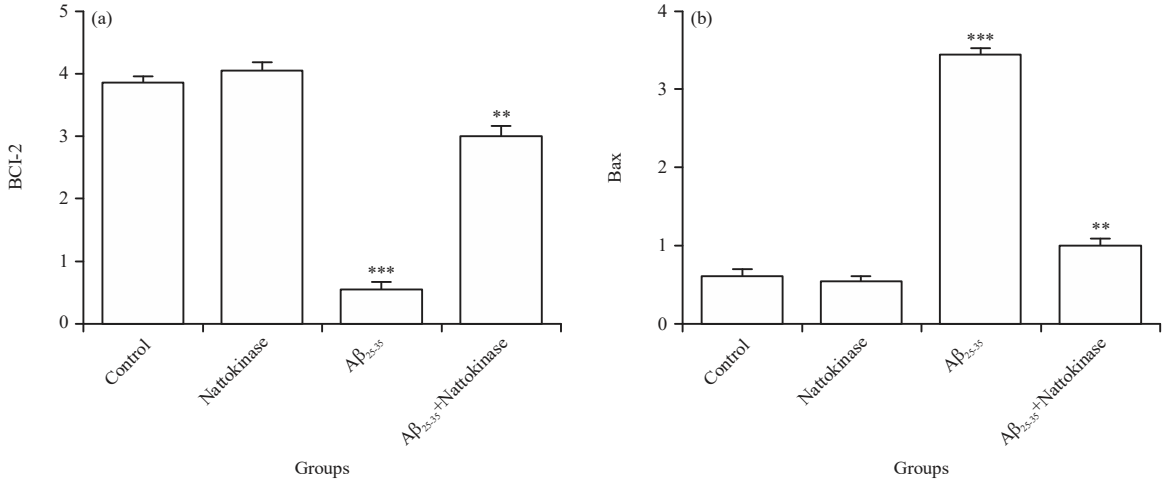

Fig. 8(a-b): Effect of nattokinase on the BCl-2 and Bax level, (a) BCl-2 and (b) Bax

Control group contained no nattokinase and Aβ treatment, cells treated with nattokinase (20 μM) with or without treatment of Aβ, ***p <0.01, **p <0.01, compared with the $A\beta_{25-35}$ injury group compared by student's t-test (n = 3)

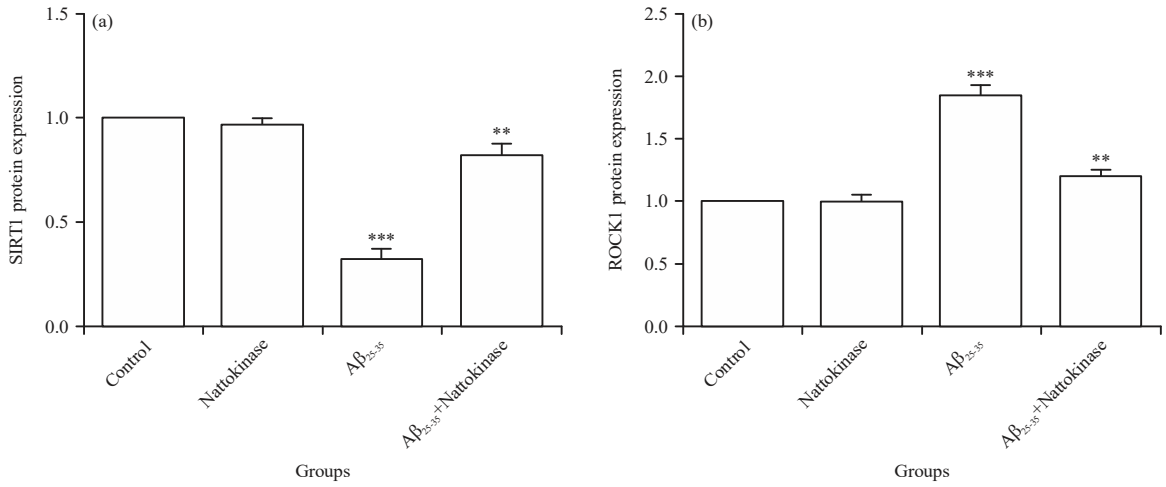

Fig. 9(a-b): Effect of nattokinase on (a) SIRT1 expression and (b) ROCK1 expression

Cells treated with different concentration of $A\beta_{25-35}$, ***p<0.01, **p<0.01, compared with the $A\beta_{25-35}$ injury group compared by student's t-test (n = 3)

(Fig. 9a) and ROCK1 expression (Fig. 9b) were increased observed in the $A\beta_{25-35}$ treated group and nattokinase treatment significantly increased the SIRT1 expression and decreased expression of ROCK1.

DISCUSSION

The AD is demoralizing and common neurodegenerative disorder and it's also the trademark of pathologic features are neurofibrillary tangles, neuron death and β-amyloid plaques[22,23,26-29]. Currently, increasing the aging patient populations, the frequency of AD is upward exponentially and it became the major burden on the patient's families and society[24]. Nevertheless, the pathogenesis of disease (AD) is not clearly described despite growth in basic research and their remnants a dearth of effective cures and treatments[24,26]. Several researchers believed that Aβ deposition plays an important role in the pathological of AD brain[29,30]. The Aβ-induced apoptosis in PC12 cell lines is a common *in vitro* model for estimation of cellular toxicity model[25,30-32]. Previous research suggested that the Aβ induced neurotoxicity included the various Aβ fragments. Current research targeted the cell apoptosis induced by Aβ to cure the AD[30,31].

In the current experimental study, *in vitro* neurotoxicity model of PC12 cells via using the $A\beta_{25-35}$ and scrutinize the possible role of nattokinase against Aβ neurotoxicity. Nattokinase showed the up-regulated of cell viability and reduced the intracellular calcium level and cell apoptosis. Experimental protocol, nattokinase altered the SIRT1 expression and suggested neuroprotective effects. Hence, the neuroprotective effect of nattokinase against $A\beta_{25-35}$ in PC12 cells was partially arbitrated via increasing the expression of SIRT1.

Bax and Bcl-2 were the pro-apoptotic and anti-apoptotic molecule and played a significant role in the apoptosis and consider as the marker of neuron survival[33,34]. Experimental protocol, nattokinase could penetrate into the NGF-differentiated PC12 cells and showed its potential effect against Aβ induced injury. Nattokinase reduced the expression of Bax, which in turn increased the ratio of Bcl-2/Bax ratio in Aβ induced injured cells. The current finding suggested that the nattokinase reduced the Aβ induced apoptotic damage by regulating pro-apoptotic molecules.

Previously published literature suggested that the ROS (an indicator of oxidative stress) play an imperative role in the expansion of neurological disease and brain aging[24,26]. Reports also suggested that the existence of diffuse ROS caused oxidative stress in the whole brain during AD cases[22,24]. In the current study, $A\beta_{25-35}$ enhance the ROS deposition and collapse the $\Delta\psi m$ in the PC12 cells. On the other hand, nattokinase treatment significantly reduced the ROS and $\Delta\psi m$ in PC12 cells. Nattokinase reduced cell viability and enhanced the LDH release induced by $A\beta_{25-35}$ and nattokinase treatment significantly increased the cell viability and reduced the LDH activity. Result suggested that nattokinase having the potential antioxidant potential contributed to its beneficial effects. Na^+-K^+-ATPase (transmembrane protein) played an important role in the exchange of intracellular Na^+ and extracellular K^+[35]. Previous research suggested that the reduced level of Na^+-K^+-ATPase

showed the crack of the mitochondrial membrane, which is further stimulated the apoptotic insult and disturbed the mitochondrial membrane[23,35].

In the current study, reduced activity of Na^+-K^+-ATPase was observed in Aβ induced damage cells, which further lead to crack the mitochondrial membrane and alter the functions of Na^+ and K^+ exchange. Moreover, nattokinase maintained the level of Na^+-K^+-ATPase activity, which further took part to maintain the mitochondrial membrane potential and also provided the beneficial effect of ion homeostasis. Caspase (caspase-3 and caspase-8) are the mediator of upstream initiator and downstream effector in caspase cascade[36,37]. Previous studies suggested that both the caspase normalize the mitochondrial arbitrated apoptotic pathway[36,38]. The studies also suggested that both the caspase act as apoptotic initiators to induced the apoptosis in PC12 cell because caspases directly circulate the morphological damage in the cells[36-38]. Aβ treatment showed the increased activity of caspase-3 and caspase-8, which directly initiated the apoptotic process in the cells[39,40]. Conversely, nattokinase treatment reduced the caspase-3 and 8 activity and suggested the protective effect on the neurotoxicity induced by Aβ in the cells. The result showed that the nattokinase reduced the caspase cascade by limiting the activity of downstream initiator. For examine the underlying mechanism of nattokinase, scrutinized the SIRT1 expression (a silent information regulator). The previous study suggested that the SIRT1 is closely related to Aβ accumulation and aging-related disorder[40-42].

The study also suggested that the low expression of SIRT1found in the cerebral cortex of AD[43]. In the current experimental study, $A\beta_{25-35}$ induced neurotoxicity showed decreased SIRT1 expression, which was recovered by the nattokinase. Current studies suggested that the α-secretase activity enhanced in SITR1 transgenic mice, which is associated with decrease ROCK1 expression. The same result was obtained in the current study, Aβ reduced the SIRTI and enhanced the ROCK1 expression and nattokinase treatment exhibited the increased SIRT1 level accompanied by a reduction of ROCK1 level, suggested that ROCK1 and SIRT1 play a crucial role in the anti-neurotoxicity effect of nattokinase. As predicted, nicotinamide reduced the expression of SIRT1 concurrently boosting the expression of ROCK1. An opposite result was obtained in the Y-27632 treatment, the expression of ROCK1 reduced and no or little effect was found on the SIRT1 expression. A recent study suggested that the SIRT1 also regulated the 2 apoptosis linked proteins[44] FOXO and P53. Furthermore, the current study suggested that the SIRT1 reduces the nuclear factor kappa B (NF-κB) signalling pathway and protects the neurons from damage[44-46]. Additionally, the result showed that the nattokinase directly bind to the Aβ-42 and restricted its accumulation, leading to and decreased Aβ cytotoxicity. Result suggested that the nattokinase play a synergistic role in the protection of neuronal effect, which further investigated.

CONCLUSION

Nattokinase showed the neuroprotective effect against the PC12 cells via multiple mechanisms. Nattokinase significantly down-regulated the ROS formation and suggested the antioxidant pathway in the Alzheimer disease. It was also significantly reduced the pro-inflammatory cytokines which are directly altered the NF-κB pathway and suggested the neuroprotective effect via an inflammatory pathway. Nattokinase also altered the SIRTI-ROCK1 and provided the neuroprotective effect via SIRTI-ROCK1 pathway in PC12 cells. For the confirmation of neuroprotective effect *in vivo* study should be performed at the molecular level.

SIGNIFICANCE STATEMENT

This study discovered the neuroprotective effect of nattokinase that can be beneficial for Alzheimer disease and this study will help the researchers to uncover the critical areas of Alzheimer disease and its related disorder that many researchers were not able to explore. Thus a new theory on Alzheimer disease may be arrived at in this experimental study.

REFERENCES

1. Aka, T.D., M.M.O. Rashid, S.C. Paul and M.A. Halim, 2019. A review on molecular neuropathology of Alzheimer's disease in association with aging. J. Res. Pharm., 23: 1-15.
2. Shirwany, N.A., D. Payette, J. Xie and Q. Guo, 2007. The amyloid beta ion channel hypothesis of Alzheimer's disease. Neuropsychiatric Dis. Treatment, 3: 597-612.
3. McKeith, I.G., D. Galasko, K. Kosaka, E.K. Perry and D.W. Dickson *et al*., 1996. Consensus guidelines for the clinical and pathologic diagnosis of Dementia with Lewy Bodies (DLB): Report of the consortium on DLB international workshop. Neurology, 47: 1113-1124.
4. Rajasekhar, K., C. Madhu and T. Govindaraju, 2016. Natural tripeptide-based inhibitor of multifaceted amyloid β toxicity. ACS Chem. Neurosci., 7: 1300-1310.

5. Ariga, T., M.P. McDonald and R.K. Yu, 2008. Role of ganglioside metabolism in the pathogenesis of Alzheimer's disease: A review. J. Lipid Res., 49: 1157-1175.
6. Hung, S.Y. and W.M. Fu, 2017. Drug candidates in clinical trials for Alzheimer's disease. J. Biomed. Sci., Vol. 24. 10.1186/s12929-017-0355-7.
7. Caselli, R.J., T.G. Beach, R. Yaari and E.M. Reiman, 2006. Alzheimer's disease a century later. J. Clin. Psychiat., 67: 1784-1800.
8. Oddo, S., A. Caccamo, J.D. Shepherd, M.P. Murphy and T.E. Golde *et al*., 2003. Triple-transgenic model of Alzheimer's disease with plaques and tangles: Intracellular Aβ and synaptic dysfunction. Neuron, 39: 409-421.
9. Sakono, M. and T. Zako, 2010. Amyloid oligomers: Formation and toxicity of Aβ oligomers. FEBS J., 277: 1348-1358.
10. Reitz, C. and R. Mayeux, 2014. Alzheimer disease: Epidemiology, diagnostic criteria, risk factors and biomarkers. Biochem. Pharmacol., 88: 640-651.
11. Christensen, R., A.B. Marcussen, G. Wortwein, G.M. Knudsen and S. Aznar, 2008. $A\beta_{(1-42)}$ injection causes memory impairment, lowered cortical and serum BDNF levels and decreased hippocampal $5\text{-}HT_{2A}$ levels. Exp. Neurol., 210: 164-171.
12. Lichtenthaler, S.F., 2012. Alpha-secretase cleavage of the amyloid precursor protein: Proteolysis regulated by signaling pathways and protein trafficking. Curr. Alzheimer Res., 91: 165-177.
13. Buoso, E., C. Lanni, G. Schettini, S. Govoni and M. Racchi, 2010. β-Amyloid precursor protein metabolism: Focus on the functions and degradation of its intracellular domain. Pharmacol. Res., 62: 308-317.
14. Li, M., L. Chen, D.H.S. Lee, L.C. Yu and Y. Zhang, 2007. The role of intracellular amyloid β in Alzheimer's disease. Prog. Neurobiol., 83: 131-139.
15. Selkoe, D.J. and J. Hardy, 2016. The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol. Med., 8: 595-608.
16. Killick, R., E.M. Ribe, R. Al-Shawi, B. Malik and C. Hooper *et al*., 2014. Clusterin regulates β-amyloid toxicity via Dickkopf-1-driven induction of the wnt-PCP-JNK pathway. Mol. Psychiat., 19: 88-98.
17. Lorenzo, A., M. Yuan, Z. Zhang, P.A. Paganetti and C. Sturchler-Pierrat *et al*., 2000. Amyloid β interacts with the amyloid precursor protein: A potential toxic mechanism in Alzheimer's disease. Nature Neurosci., 3: 460-464.
18. Shi, J., X. Wu, M. Surma, S. Vemula and L. Zhang *et al*., 2013. Distinct roles for ROCK1 and ROCK2 in the regulation of cell detachment. Cell Death Dis., Vol. 4.
19. Hartmann, S., A.J. Ridley and S. Lutz, 2015. The function of Rho-associated kinases ROCK1 and ROCK2 in the pathogenesis of cardiovascular disease. Front. Pharmacol., Vol. 6. 10.3389/fphar.2015.00276.
20. Mong, P.Y. and Q. Wang, 2009. Activation of Rho kinase isoforms in lung endothelial cells during inflammation. J. Immunol., 182: 2385-2394.
21. Lock, F.E., K.R. Ryan, N.S. Poulter, M. Parsons and N.A. Hotchin, 2012. Differential regulation of adhesion complex turnover by ROCK1 and ROCK2. Plos One, Vol. 7. 10.1371/journal.pone.0031423.
22. Zeng, Z., J. Xu and W. Zheng, 2017. Artemisinin protects PC12 cells against β-amyloid-induced apoptosis through activation of the ERK1/2 signaling pathway. Redox Biol., 12: 625-633.
23. Li, R.C., F. Pouranfar, S.K. Lee, M.W. Morris, Y. Wang and D. Gozal, 2008. Neuroglobin protects PC12 cells against β-amyloid-induced cell injury. Neurobiol. Aging, 29: 1815-1822.
24. Park, S.Y., H.S. Kim, E.K. Cho, B.Y. Kwon, S. Phark, K.W. Hwang and D. Sul, 2008. Curcumin protected PC12 cells against beta-amyloid-induced toxicity through the inhibition of oxidative damage and tau hyperphosphorylation. Food Chem. Toxicol., 46: 2881-2887.
25. Yao, Z.X., R.C. Brown, G. Teper, J. Greeson and V. Papadopoulos, 2002. *22R* Hydroxycholesterol protects neuronal cells from β amyloid induced cytotoxicity by binding to β amyloid peptide. J. Neurochem., 83: 1110-1119.
26. Zhang, H.Y., Y.H. Liu, H.Q. Wang, J.H. Xu and H.T. Hu, 2008. Puerarin protects PC12 cells against β amyloid induced cell injury. Cell Biol. Int., 32: 1230-1237.
27. Martin, D., M. Salinas, R. Lopez Valdaliso, E. Serrano, M. Recuero and A. Cuadrado, 2001. Effect of the Alzheimer amyloid fragment Aβ (25-35) on Akt/PKB kinase and survival of PC12 cells. J. Neurochem., 78: 1000-1008.
28. Paddon-Jones, D., K.R. Short, W.W. Campbell, E. Volpi and R.R. Wolfe, 2008. Role of dietary protein in the sarcopenia of aging. Am. J. Clin. Nutr., 87: 1562S-1566S.
29. Zhang, X. and W. Le, 2010. Pathological role of hypoxia in Alzheimer`s disease. Exp. Neurol., 223: 299-303.
30. Patel, N.S., D. Paris, V. Mathura, A.N. Quadros, F.C. Crawford and M.J. Mullan, 2005. Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease. J. Neuroinflam., Vol. 2. 10.1186/1742-2094-2-9.
31. Gold, M. and J. El Khoury, 2015. β-amyloid, microglia and the inflammasome in Alzheimer's disease. Semin. Immunopathol., 37: 607-611.
32. Edwards, 3rd, G., I. Moreno-Gonzalez and C. Soto, 2017. Amyloid-beta and tau pathology following repetitive mild traumatic brain injury. Biochem. Biophys. Res. Commun., 483: 1137-1142.
33. Adams, J.M. and S. Cory, 2007. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene, 26: 1324-1337.
34. Kang, M.H. and C.P. Reynolds, 2009. Bcl-2 inhibitors: Targeting mitochondrial apoptotic pathways in cancer therapy. Clin. Cancer Res., 15: 1126-1129.

35. Feng, X., N. Liang, D. Zhu, Q. Gao and L. Peng *et al*., 2013. Resveratrol inhibits β-amyloid-induced neuronal apoptosis through regulation of SIRT1-ROCK1 signaling pathway. Plos One, Vol. 8. 10.1371/journal.pone.0059888.
36. Cohen, G.M., 1997. Caspases: The executioners of apoptosis. A review. Biochem. J., 326: 1-16.
37. Pasinelli, P., D.R. Borchelt, M.K. Houseweart, D.W. Cleveland and R.H. Brown, Jr., 2000. Caspase-1 and -3 are sequentially activated in motor neuron death in Cu, Zn superoxide dismutase-mediated familial amyotrophic lateral sclerosis. Proc. Nat. Acad. Sci., 95: 15763-15768.
38. Chowdhury, I., B. Tharakan and G.K. Bhat, 2008. Caspases-An update. Comparat. Biochem. Physiol. Part B: Biochem. Mol. Biol., 151: 10-27.
39. Swanton, E., P. Savory, S. Cosulich, P. Clarke and P. Woodman, 1999. Bcl-2 regulates a caspase-3/caspase-2 apoptotic cascade in cytosolic extracts. Oncogene, 11: 1781-1787.
40. Furlong, H., C. Mothersill, F.M. Lyng and O. Howe, 2013. Apoptosis is signalled early by low doses of ionising radiation in a radiation-induced bystander effect. Mutat. Res./Fundam. Mol. Mech. Mutagenesis, 741: 35-43.
41. Julien, C., C. Tremblay, V. Emond, M. Lebbadi, N. Salem, Jr., D.A. Bennett and F. Calon, 2009. Sirtuin 1 reduction parallels the accumulation of tau in Alzheimer disease. J. Neuropathol. Exp. Neurol., 68: 48-58.
42. Lim, Y.Y., P. Maruff, R. Schindler, B.R. Ott and S. Salloway *et al*., 2015. Disruption of cholinergic neurotransmission exacerbates Aβ-related cognitive impairment in preclinical Alzheimer's disease. Neurobiol. Aging, 36: 2709-2715.
43. Hokama, M., S. Oka, J. Leon, T. Ninomiya and H. Honda *et al*., 2013. Altered expression of diabetes-related genes in Alzheimer's disease brains: The Hisayama study. Cerebral Cortex, 24: 2476-2488.
44. Peng, S.L., 2008. Foxo in the immune system. Oncogene, 27: 2337-2344.
45. Xiang, H.F., D.H. Cao, Y.Q. Yang, H.Q. Wang and L.J. Zhu *et al*., 2014. Isoflurane protects against injury caused by deprivation of oxygen and glucose in microglia through regulation of the Toll-like receptor 4 pathway. J. Mol. Neurosci., 54: 664-670.
46. Zeng, K.W., T. Zhang, H. Fu, G.X. Liu and X.M. Wang, 2012. Modified Wu-Zi-Yan-Zong prescription, a traditional Chinese polyherbal formula, suppresses lipopolysaccharide-induced neuroinflammatory processes in rat astrocytes via NF-κB and JNK/p38 MAPK signaling pathways. Phytomed., 19: 122-129.