

Experimental study on the neurotoxic effect of β -amyloid on the cytoskeleton of PC12 cells

LAI WANG^{1*}, JINGJING CAO^{1*}, ZHENYU SHI¹, WENJUAN FAN¹, HONGLIANG LIU¹,
JINBO DENG¹ and JIEXIN DENG²

¹Institute of Neurobiology, College of Life Sciences, Henan University; ²College of Nursing, Henan University, Kaifeng, Henan 475004, P.R. China

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Abstract. The aim of the present study was to establish a cell model of Alzheimer's disease (AD) and investigate the neurotoxic effects of β -amyloid ($A\beta$) on the cytoskeleton. PC12 cells were cultured and treated with $A\beta_{25-35}$, and cell survival was analyzed with the MTT assay. Cell apoptosis was visualized using 4',6-diamidino-2-phenylindole staining and the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. Immunocytochemistry and phalloidin staining were used to label the cytoskeleton of PC12 cells. $A\beta_{25-35}$ was found to induce PC12 cell apoptosis in a dose-dependent manner ($P < 0.05$). Moreover, $A\beta_{25-35}$ also caused dose-dependent disintegration of the cytoskeleton ($P < 0.05$). Therefore, the PC12 cell cytoskeleton was found to be sensitive to $A\beta_{25-35}$ neurotoxicity. The disintegration of the cytoskeleton is likely an important pathological alteration in AD, and $A\beta$ is a key molecule involved in AD pathogenesis.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, and its pathological lesions often appear in the neocortex and, particularly, the hippocampus. An estimated ~36 million individuals suffer from AD worldwide. In the UK, AD-related mortality is ranked no. 6 in the population aged >65 years, while in the United States it is ranked no. 5 (1). In China, the total number of AD patients is >5 million (2). Therefore, the prevention and treatment of AD represent major challenges for scientists and

clinicians. The mechanism of AD pathogenesis has not yet been fully elucidated. There are numerous hypotheses, but the most widely accepted hypothesis is the amyloid cascade reaction. This hypothesis suggests that neurotoxic β -amyloid ($A\beta$) protein is deposited in the brain and triggers a series of pathological alterations, including amyloid plaques and neurofibrillary tangles (3). Previous studies confirmed that $A\beta$ neurotoxicity causes formation of amyloid plaques in the neocortex through a variety of mechanisms (4); however, the association between the formation of neurofibrillary tangles and cytoskeletal alterations is not well understood. The cytoskeleton is a fibrous network mainly composed of microtubules, microfilaments and intermediate filaments. Previous studies demonstrated that, during cell apoptosis, the cytoskeleton exhibits major changes (5); therefore, the structure and function of the neuronal cytoskeleton may play an important role in the pathogenesis of neurodegenerative diseases (6). In the present study, the neurotoxic effect of $A\beta_{25-35}$ on the cytoskeleton were investigated in a PC12 cell culture, aiming to elucidate the role of the cytoskeleton in the pathogenesis of AD to provide novel insight and treatment strategies.

Materials and methods

PC12 cell culture. The PC12 adrenal pheochromocytoma cell line was purchased from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The present study used differentiated PC12 cells, as they share several characteristics with nervous cells and are widely accepted as a neuronal model. In addition, compared with primary neurons, the procedures of cell culturing are simple, with efficient cell proliferation. For the cell culture, PC12 cells removed from liquid nitrogen were thawed at 37°C, and were then seeded and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; 12100-038) with 10% fetal calf serum (FCS; 140714) (both from Gibco; Thermo Fisher Scientific, Grand Island, NY, USA) and 1% penicillin and streptomycin. PC12 cells in the adherent culture started to grow and differentiate and, after 24 h, spindle-like and polygon-like cells were observed, with clear boundaries and a neuron-like shape. When cultured PC12 cells reached a confluence of 70-80%, cell passaging was performed. Following digestion with 0.25% trypsin, the cells were collected and seeded for the following experiments. Some cells were cryopreserved in liquid nitrogen.

Correspondence to: Dr Jinbo Deng, Institute of Neurobiology, College of Life Sciences, Henan University, 1 Jinming Road, Kaifeng, Henan 475004, P.R. China
Email: jinbo_deng@henu.edu.cn

Dr Jiexin Deng, College of Nursing, Henan University, 1 Jinming Road, Kaifeng, Henan 475004, P.R. China
E-mail: dengjiexin@yahoo.com

*Contributed equally

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A β_{25-35} protein treatment and experimental grouping. A β accumulation in brain is the main event in the pathogenesis of AD; therefore, instead of toxic oligomers of the conventional 1-40 or 1-42 subtypes, A β has been used as a inducer for the AD cell model (7). Naturally occurring A β protein exhibits only weak neurotoxicity *in vivo* or *in vitro*; however, the aggregated A β protein and its fragments may be severely neurotoxic. This process is referred to as 'aging'. According to Pike *et al.* (8), aged A β_{25-35} (A4559; Sigma-Aldrich; Merck, KGaA, St. Louis, MO, USA) was prepared as follows: A β_{25-35} protein (1 mg) was dissolved in 1 ml sterile water (1 mmol/l). Subsequently, 1 mmol/l A β_{25-35} was incubated at 37°C for 4-7 days, and the aged A β_{25-35} was used for further neurotoxicity experiments. In the present study, cultured PC12 cells were divided into treatment and control groups. In the control group, the cells were cultured in high-glucose DMEM with 10% FCS for 48 h. In the experimental groups, the cells were cultured in the same medium as the control group, but the medium was supplemented with aged A β_{25-35} at various concentration (10, 50, 90, 180 and 360 μ mol/l). Subsequent experiments were performed in the control and treatment groups.

Apoptotic assays

MTT assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reacts with succinate dehydrogenase in mitochondria and is reduced into water-insoluble blue-violet formazan crystals. Formazan is deposited in living rather than dead cells; therefore, formazan colorimetry may be used to evaluate cell viability. After reaching the logarithmic growth phase, 100 μ l PC12 cells at a concentration of 3×10^4 cells/ml were seeded in a 96-well culture plate and incubated for 24 h at 37°C with 5% CO₂. After changing the medium (100 μ l), PC12 cells in both the control (n=5) and experimental groups (n=5) continued to be cultured for another 48 h, followed by the addition of 20 μ l MTT (5 mg/ml). The cells were incubated with MTT for another 4 h, then disrupted with 150 μ l dimethyl sulfoxide. After shaking for 10 min, the absorbance (OD) values of the sample were read using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 570 nm (n>3).

4',6-Diamidino-2-phenylindole (DAPI) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. PC12 cells in the logarithmic growth phase (1×10^5 cells/ml) were used for DAPI and TUNEL assays. The cells in the control and experimental groups were fixed with 4% paraformaldehyde (pH 7.4) at 4°C for 30 min. After washing 3 times with 0.01 mol/l phosphate-buffered saline (PBS), the cells were coverslipped with DAPI-glycerin (DAPI:65% glycerol = 1:1,000) and examined with a fluorescent microscope (BX61; Olympus, Tokyo, Japan). With DAPI staining, the cell nuclei and apoptotic bodies (condensed and fragmented nuclei) were visualized. TUNEL staining was also performed using a TUNEL assay kit (G3250; Promega, Madison, WI, USA). Cell collection and fixation were performed as described above. Subsequently, the cells were treated with 0.2% Triton X-100 to increase cell transparency. After rinsing with 0.01 mol/l PBS three times, TUNEL reaction solution was added, followed by incubation at 37°C for 60 min. The reaction was then stopped with termination solution. The sections were coverslipped with DAPI-glycerin and examined with an epifluorescence micro-

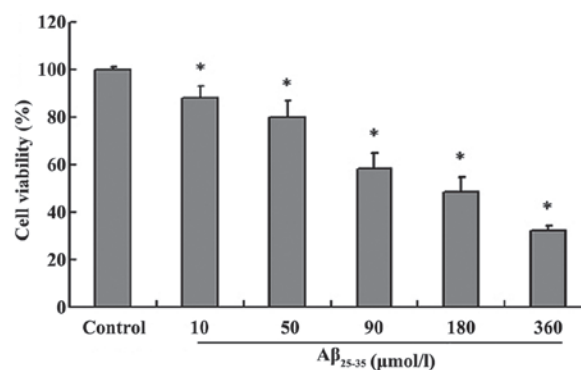


Figure 1. Viability of PC12 cells following β -amyloid (A β)₂₅₋₃₅ treatment (MTT method). Values are presented as mean \pm standard deviation (n=15). Following treatment with A β_{25-35} , the viability of PC12 cells decreased in a dose-dependent manner compared with the control group. *P<0.05 vs. control group. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

scope (BX61; Olympus) following excitation with rhodamine or ultraviolet (UV) light.

Cytoskeleton visualization with phalloidin staining and immunocytochemistry

Phalloidin staining. F-actin is the main component of microfilaments, and is capable of binding with phalloidin. Therefore, phalloidin may be used to stain microfilaments in the cell. Cells in the control and experimental groups were collected as described above and fixed in 4% paraformaldehyde for 30 min. Subsequently, 0.2 μ g/ml phalloidin (A12380; Invitrogen; Thermo Fisher Scientific; Carlsbad, CA, USA) was diluted with preparation solution (0.1% NaN₃, 3% BSA, 0.3% Triton X-100 in 0.01 M PBS). After washing with 0.01 M PBS, the cells were incubated with phalloidin solution overnight and coverslipped with DAPI-glycerin. The cells were examined under a fluorescence microscope following rhodamine or UV excitation.

Immunocytochemistry. The main component of microtubules is β -II-tubulin, which was used as a marker in the present study to visualize microtubules. The primary antibody used was rabbit anti- β -II-tubulin polyclonal antibody (1:100, AB151318; Abcam, Cambridge, MA, USA). The cells were incubated with the primary antibody at 4°C overnight. After washing with 0.01 M PBS 3 times, secondary antibody (Alexa Fluoro 488 donkey anti-rabbit IgG, 1:500, A21206; Invitrogen; Thermo Fisher Scientific) was added and incubated at room temperature for 3 h. The cells were coverslipped with DAPI-glycerin, observed and photographed with a fluorescence microscope.

Measurements and statistical tests. The MTT assay was used to evaluate the viability of PC12 cells as follows: Cell survival rate (%) = (OD₅₇₀ in the experimental group - OD₅₇₀ in the blank group)/(OD₅₇₀ in the control group - OD₅₇₀ in the blank group) \times 100%. The remaining cultured cells were considered as non-viable, including necrotic and apoptotic cells. Using the cell survival rate, the ratio of non-viable cells was calculated using the formula: 1 - cell survival rate (%). ImageJ 1.48 software was used to quantify the apoptotic cells and disintegrated cytoskeleton as follows: Apoptotic rate (DAPI or TUNEL staining) = (apoptotic cells/total cells) \times 100%. Cytoskeleton disintegration = (number of cells with disintegrated cytoskeleton/total cell number) \times 100%.

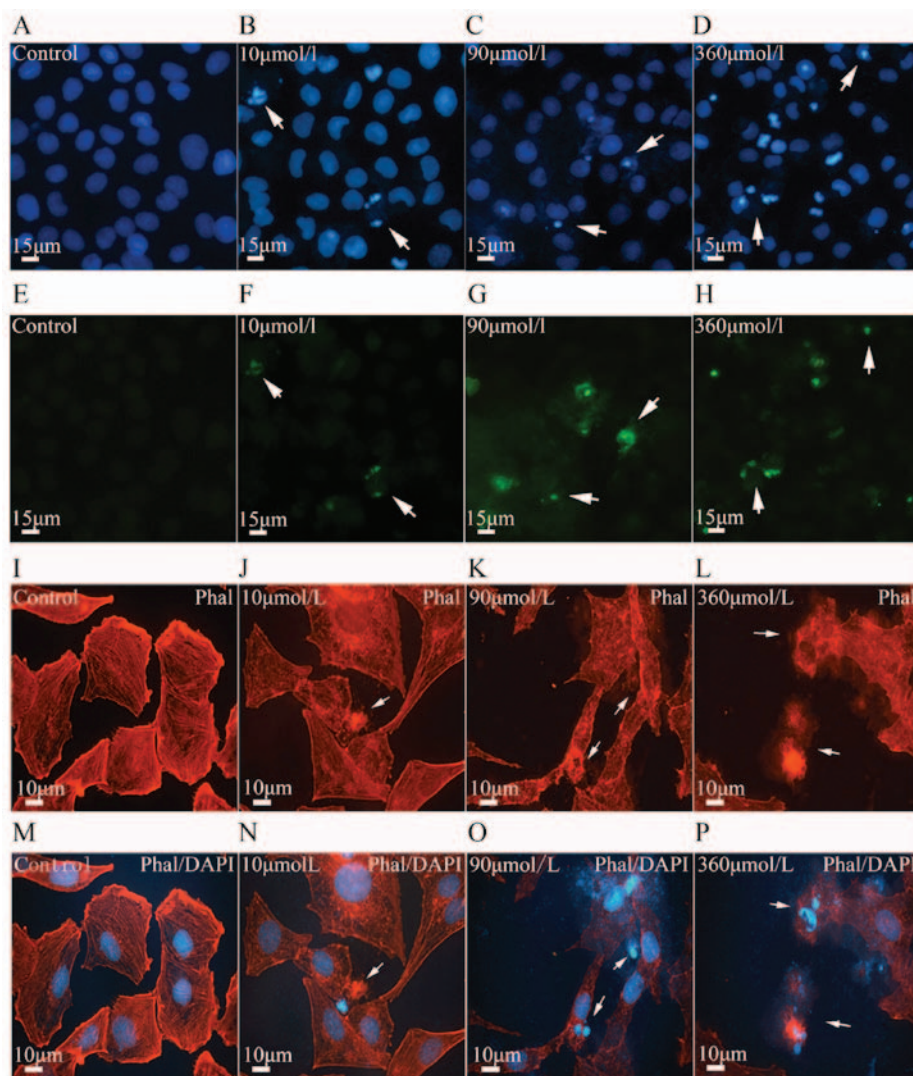


Figure 2. Cell apoptosis and microfilament alterations following β -amyloid ($A\beta$)₂₅₋₃₅ treatment (phalloidin staining and DAPI counterstaining). (A-D) Nuclear condensation and apoptotic bodies (arrows) were visualized with DAPI staining (blue) in the control and treatment groups. (E-H) Cell apoptosis determined with the TUNEL assay. $A\beta$ ₂₅₋₃₅ induced PC12 cell apoptosis (green, arrows) in a dose-dependent manner. (I-P) $A\beta$ ₂₅₋₃₅ neurotoxicity and microfilament alterations (phalloidin staining and DAPI counterstaining). Following $A\beta$ ₂₅₋₃₅ treatment, the microfilaments (red) in PC12 cells appeared to disintegrate (arrows). The disintegrated microfilaments (J and L, arrows) were often observed in apoptotic cells with condensed nuclei or apoptotic bodies (N and P, arrows). With increasing $A\beta$ ₂₅₋₃₅ dose, the number of cells with disintegrated microfilaments gradually increased. Scale bars, 15 μ m in (A-H) and 10 μ m in (I-P). TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; DAPI, 4',6-diamidino-2-phenylindole; Phal, phalloidin.

In order to exclude the interference of necrosis, we also estimated the proportional contribution of apoptosis to the viable reduction according to the formula: Neurapoptosis/non-viable cells = apoptotic rate/(1 - cell survival rate) (%).

Statistical analysis. All data were recorded as mean \pm standard deviation. Using SPSS 11.5 statistical software (SPSS, Inc., Chicago, IL, USA), comparisons of the abovementioned parameters were performed between the experimental and control groups with one-way analysis of variance. $P < 0.05$ was considered to indicate statistically significant differences, and $P < 0.01$ was considered as highly statistically significant.

Results

$A\beta$ ₂₅₋₃₅ neurotoxicity and neuroapoptosis in PC12 cells. The MTT assay was used to detect cell viability following exposure to $A\beta$ ₂₅₋₃₅ toxicity (9). The results revealed that $A\beta$ ₂₅₋₃₅

decreased cell viability in a dose-dependent manner compared with the control group. Cell survival was gradually reduced with increasing concentrations of $A\beta$ ₂₅₋₃₅ (10, 50, 90, 180 and 360 μ mol/l; $P < 0.05$, Fig. 1).

In order to determine whether the reduction in cell survival was caused by cell apoptosis, TUNEL assay with DAPI counterstaining was performed. DAPI stains cell nuclei with blue color. The PC12 cells in the control group appeared to grow well, with uniform brightness, and their nuclei had a smooth outline with homogeneous chromatin distribution. However, the cells in the experimental group displayed pyknotic nuclear chromatin, which was often marginated along the nuclear edge, conferring an appearance of half moon-like nuclei, with occasional formation of typical apoptotic bodies (Fig. 2A-D). With increasing $A\beta$ ₂₅₋₃₅ dose, the number of apoptotic cells gradually increased in a dose-dependent manner ($P < 0.05$; Fig. 3A). TUNEL assay confirmed the results of DAPI staining. Apoptotic cells were hardly found in the control group; however, following $A\beta$ ₂₅₋₃₅

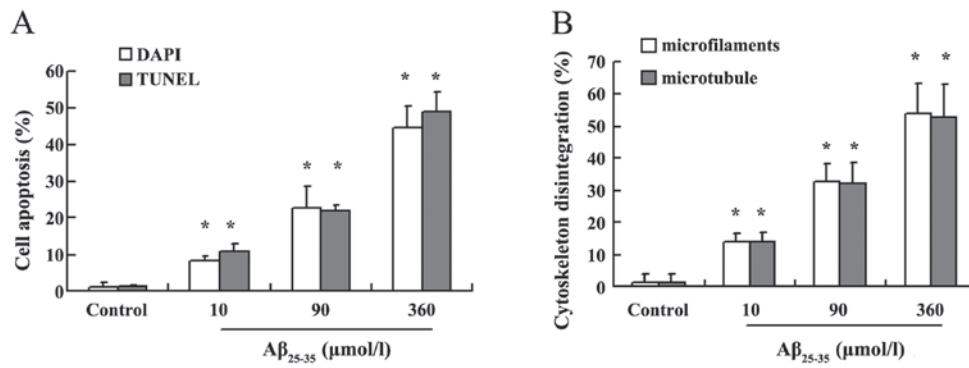


Figure 3. Histograms of cell apoptosis and cytoskeletal disintegration among various groups (mean \pm standard deviation) (n=5). (A) The apoptotic cells were visualized with DAPI and TUNEL staining, and the apoptotic rate was measured and calculated with ImageJ software. Compared with the control group, A β_{25-35} induced cell apoptosis dose-dependently. *P<0.05 vs. control group. (B) The number of cells with disassembled microfilaments and microtubules was also compared between the treatment and control groups. *P<0.05 vs. control. TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; DAPI, 4',6-diamidino-2-phenylindole.

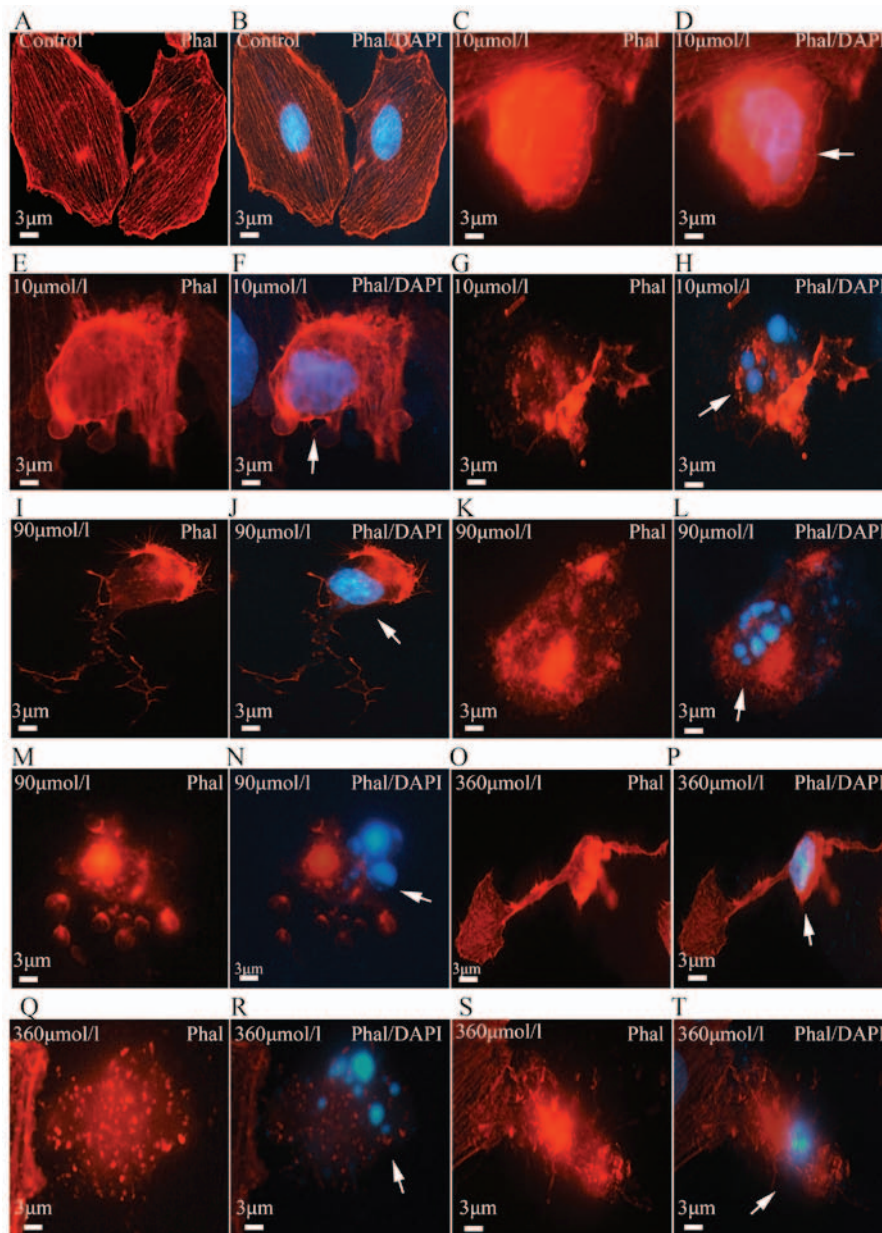


Figure 4. Neurotoxicity and microfilament alterations induced by β -amyloid (A β)₂₅₋₃₅ under high magnification (phalloidin staining and DAPI counterstaining). (A and B) In the control group, numerous microfilaments were found in the cytoplasm. (C-T) However, in the treatment groups, the microfilaments collapsed and disintegrated in a dose-dependent manner. (C and T, arrows) The cells with disintegrated microfilaments were usually apoptotic cells with condensed and fragmented nuclei. Scale bar, 3 μ m. Phal, phalloidin staining; DAPI, 4',6-diamidino-2-phenylindole.

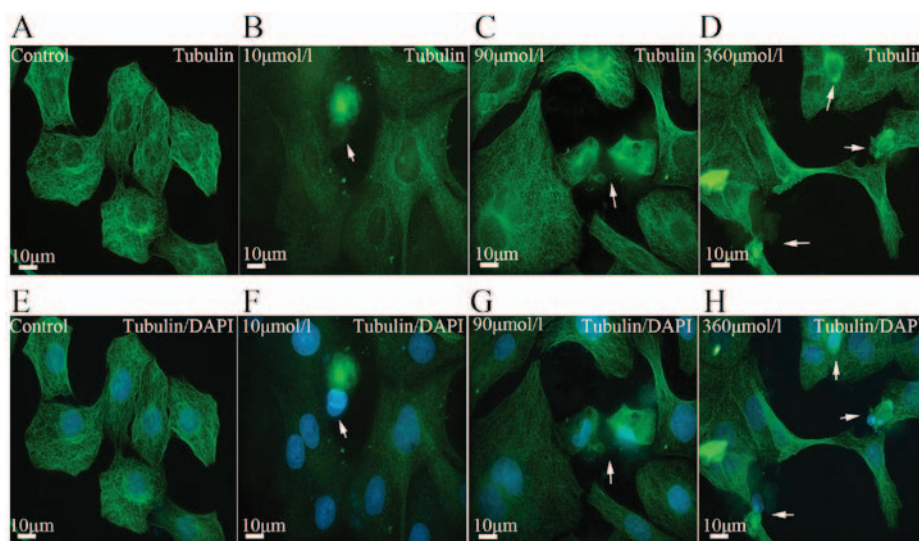


Figure 5. (A-H) Neurotoxicity and microtubule disintegration induced by β -amyloid ($A\beta$)₂₅₋₃₅ (β -II-tubulin immunolabeling and DAPI counterstaining). Microtubules were labeled with β -II-tubulin immunolabeling (green), and cell nuclei were counterstained with DAPI (blue). (A and H) Following $A\beta$ ₂₅₋₃₅ treatment, the microtubules in PC12 cells disintegrated (arrows) in a dose-dependent manner. Scale bar, 10 μ m. DAPI, 4',6'-diamidino-2-phenylindole.

treatment, the number of apoptotic cells identified with TUNEL staining increased in a dose-dependent manner ($P < 0.05$; Fig. 3A). The apoptotic cells observed with DAPI staining and the TUNEL-positive cells usually overlapped (Fig. 2A-H). In the present study, neuroapoptosis/non-viable cells were calculated under various $A\beta$ concentrations; at $A\beta$ ₂₅₋₃₅ concentrations of 10, 50, 90, 180 and 360 μ mol/l, neuroapoptosis/non-viable cells was 95.41, 87.18, 83.61, 82.87 and 84.51%, respectively, suggesting neuroapoptosis was the main cause of cell death.

$A\beta$ ₂₅₋₃₅ neurotoxicity and cytoskeletal alterations in PC12 cells.

The cytoskeleton is an important component of eukaryotic cells, and it plays a key role in the maintenance of cell shape, intracellular substance transportation and cell amoeba-like movement. Recent studies reported that there is a close association between cytoskeletal abnormalities and cell apoptosis. It was previously demonstrated that, during cell division, suppressed microtubule assembly is followed by protein kinase activation and induction of cell apoptosis (10). In yeast and certain types of animal cells, such as HL-60 cells, depolymerization of microfilaments may be induced in apoptotic cells (11). In the present study, major changes were observed in the cytoskeleton following $A\beta$ ₂₅₋₃₅ treatment. The cells in the control group contained numerous microfilaments with intensive fluorescence and a clear outline. Microfilaments of varying lengths exhibited a rod-like appearance and were parallelly arranged. Following $A\beta$ ₂₅₋₃₅ treatment, the microfilaments in the apoptotic cells appeared vague and started to disintegrate (Figs. 2I-P and 4). Statistical analysis revealed that the microfilament disintegration rate increased in $A\beta$ ₂₅₋₃₅ treatment groups in a dose-dependent manner ($P < 0.05$; Fig. 3B). Furthermore, a neurotoxic effect of $A\beta$ ₂₅₋₃₅ on microtubules was also observed. In the control group, the fibrous microtubules formed a web structure around the nucleus. In the experimental groups, the microtubules started to disintegrate and even aggregate in dense lumps (Fig. 5). These pathological alterations were often observed in apoptotic cells (Fig. 6E-T). Statistical analysis revealed that $A\beta$ ₂₅₋₃₅ induced microtubule disintegration in a dose-dependent manner compared with the control group ($P < 0.05$; Fig. 3B).

Discussion

$A\beta$ ₂₅₋₃₅ induces neuroapoptosis in PC12 cells. Apoptosis plays a crucial role during cell differentiation, organ development and pathogenesis (12,13); it is also physiologically important for neural pathfinding and synaptogenesis during brain development. Neuroapoptosis may also occur during cell aging, neuronal injury and neural degenerative diseases. AD is one of the most common neurodegenerative diseases, and the mechanism underlying its pathogenesis is extremely complex. Scientists believe that $A\beta$ is a key factor in the development of AD. $A\beta$ is cleaved from amyloid precursor protein by β - or γ -hydrolase. $A\beta$ consists of 39-43 amino acid residues and it has three types, namely $A\beta$ ₂₅₋₃₅, $A\beta$ ₁₋₄₂ and $A\beta$ ₁₋₄₀, of which $A\beta$ ₂₅₋₃₅ is considered to be the most toxic. In the present study, the neurotoxic effect of $A\beta$ ₂₅₋₃₅ on PC12 cells was assessed. As Loo *et al* (14) and Kim *et al* (15) previously reported, $A\beta$ induces neuronal apoptosis and necrosis. The present study demonstrated that $A\beta$ ₂₅₋₃₅ reduced cell viability, as determined by the MTT assay. In addition, $A\beta$ ₂₅₋₃₅ also caused PC12 cell apoptosis in a dose-dependent manner. It was also determined that apoptosis was the main contributor to cell death in non-viable cells, indicating that neuroapoptosis is a key factor in AD pathogenesis. The mechanism underlying $A\beta$ neurotoxicity is complex, and likely involves cell oxidative stress and intracellular calcium deregulation. Deposition of $A\beta$ triggers the oxidative stress response in cells, and calcium balance is disturbed. These events then trigger cell apoptosis through the endoplasmic reticulum and death receptor pathways (7,16). In the present study, $A\beta$ clearly induced neuroapoptosis; however, the findings require confirmation by further studies.

$A\beta$ ₂₅₋₃₅ induced disintegration of the cytoskeleton. Microfilaments and microtubules are crucial for cell functions. If the cytoskeleton is disintegrated, inevitable pathological alterations will occur. Previous studies reported that the cytoskeletal malformations during the pathogenesis of Huntington's chorea, Parkinson's disease and AD (17). As a key risk factor of AD pathogenesis, $A\beta$ induces neuronal cytoskeleton abnormalities (18,19). In the present study, following $A\beta$ ₂₅₋₃₅ treatment, the cytoskeleton

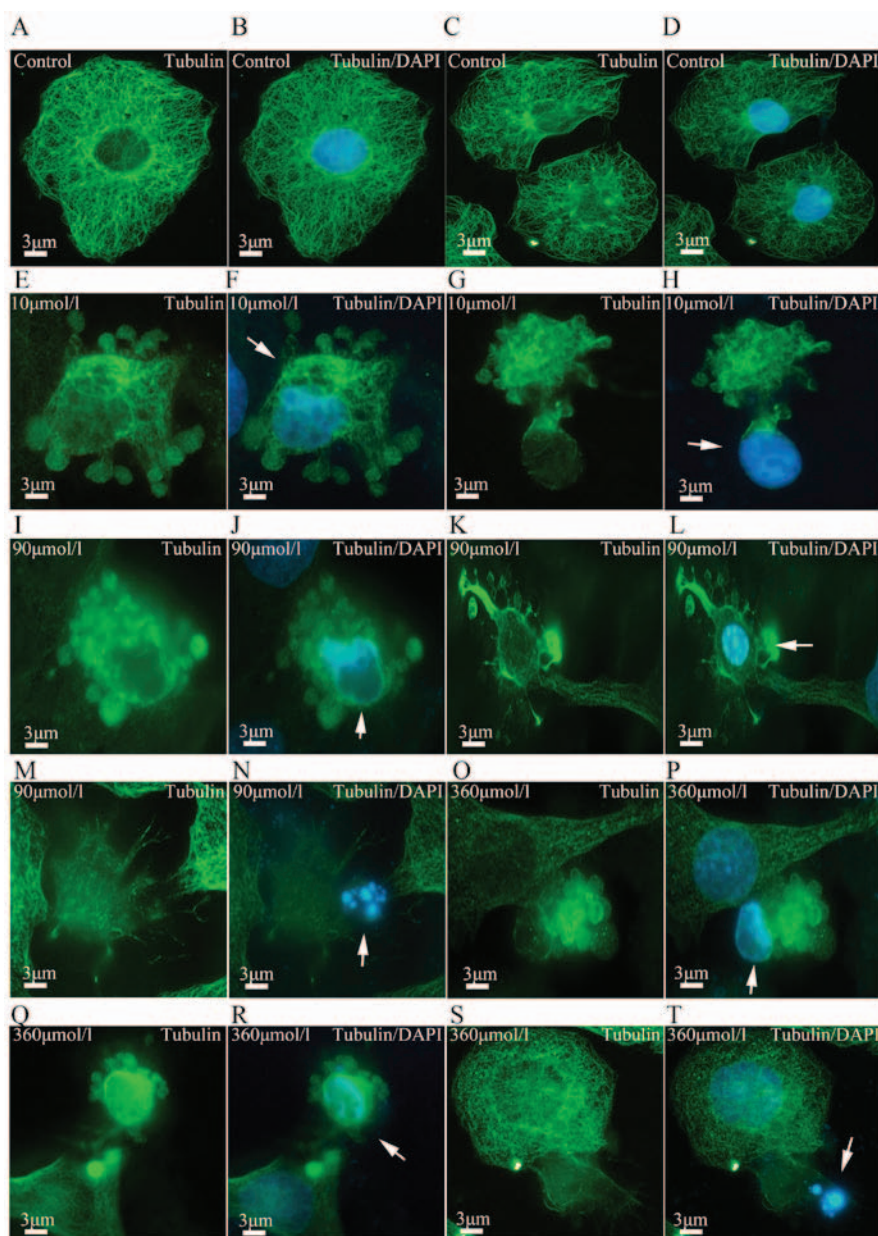


Figure 6. Neurotoxicity and microtubule alterations induced by β -amyloid ($A\beta$)₂₅₋₃₅ in cells under high magnification (β -II-tubulin immunolabeling and DAPI counterstaining). (A-D) The microtubules were arranged in a web-like formation around the nucleus in the control group. (E-T) In the $A\beta$ ₂₅₋₃₅ treatment groups, collapsed and disintegrated microtubules were observed in apoptotic cells with fragmented nuclei (arrows). Scale bar, 3 μ m. DAPI, 4',6'-diamidino-2-phenylindole.

collapsed and disintegrated in a dose-dependent manner. Cytoskeletal disintegration was often observed in apoptotic cells. The changes in the treatment groups were similar to those reported in the brain of an AD patient, exhibiting nerve inflammation and destruction of the cytoskeleton caused by $A\beta$ neurotoxicity and Tau protein hyperphosphorylation (20). It is known that $A\beta$ may trigger certain signaling pathways and cause rearrangement of the microfilament and microtubule network, leading to neuronal function disorders (21,22). The microtubule-associated proteins regulate the arrangement of the microtubules, and the Tau protein promotes microtubule assembly; therefore, $A\beta$ neurotoxicity by cytoskeletal disintegration is probably mediated by the activity of cytoskeleton-related proteins; however, the detailed mechanism requires further investigation.

Cytoskeletal disintegration is an important pathological event in AD. According to the AD cell model constructed by

Ostrovskaya *et al* (23), the neurotoxicity of $A\beta$ ₂₅₋₃₅ was investigated in PC12 cells in an attempt to elucidate the association between $A\beta$ neurotoxicity and AD pathogenesis. The results demonstrated that $A\beta$ ₂₅₋₃₅ induced PC12 cell apoptosis and cytoskeletal disintegration. Previous studies also demonstrated that cell apoptosis is accompanied by cytoskeletal destruction (24). In the present study, $A\beta$ ₂₅₋₃₅ induced cell apoptosis and cytoskeletal disintegration, which is likely the main pathological basis of neurofibrillary tangle formation in AD. According to the amyloid cascade hypothesis, $A\beta$ may promote abnormal phosphorylation of the Tau protein (25), cell apoptosis and cytoskeletal disintegration. The present study suggests that $A\beta$ ₂₅₋₃₅ induces not only cell apoptosis, but also cytoskeletal disintegration.

In conclusion, $A\beta$ ₂₅₋₃₅ decreased the viability of PC12 cells and induced cell apoptosis in a dose-dependent manner. The cytoskeleton is also very sensitive to the neurotoxic effects of

A β_{25-35} , leading to cytoskeletal disintegration. As indicated by the present study, this A β -induced cytoskeletal disintegration is likely an important event in the pathological base of neurofibrillary tangle formation during AD pathogenesis. These findings will hopefully provide a new approach to the prevention and treatment of AD.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LW, JC, ZS and JinD designed the study, and performed the analyses. WF and HL analyzed data. JinD and JieD were involved in the conception of the study and wrote the manuscript. The final version of the manuscript has been read and approved by all authors.

Ethics approval and consent to participate

The protocol was approved by the Committee of Medical Ethics and Welfare for Experimental Animals, Henan University School of Medicine (HUSOM:2015-87).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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