

The protective effect of curcumin on A β induced aberrant cell cycle reentry on primary cultured rat cortical neurons

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Abstract. – Objectives: Alzheimer's disease (AD) is a neurodegenerative disorder marked by progressive loss of memory and impairment of cognitive ability. One current hypothesis for AD pathogenesis is that neuronal death is linked to aberrant cell-cycle re-entry. In AD, neurons have been shown to enter the cell cycle inappropriately without the ability to complete it fully and the aberrant re-entry leads to its death. Curcumin has been reported as having a neural protective effect on the AD model, and could modulate the proliferation of tumor cells through the regulation of cyclin D1 and c-myc cell signaling pathways. In this study, we first observed the protective action of curcumin on A β -induced neuron damage, and then investigated whether this protective effect was a result of the inhibition of cell cycle advance.

Materials and Methods: We used MTT assay and TUNEL assay to observe the effect of curcumin on A β -induced neuron death, and then examined the activated caspase-3 protein level to further confirm the protective effect of curcumin against A β -induced neuron toxicity. Next, we further investigate whether the inhibition of cell cycle reentry was mediated by the therapeutic effect of curcumin on A β induced primary cultured neuron damage by Brdu label assay and western blot assay.

Results: The results showed that administration of curcumin (1-10 μ M) could inhibit A β ₂₅₋₃₅ (40 μ g/ml) induced primary cultured rat cortical neuron death, down-regulating activated caspase-3 protein expression. Furthermore, treatment with curcumin could inhibit abnormal activated cyclin D1 protein level, and decrease the Brdu positive cells in proportion to the A β ₂₅₋₃₅ treatment neurons.

Conclusions: All the results suggest that curcumin has a protective effect against A β -induced toxicity in cultured rat cortical neurons, the inhibition of cell cycle re-entry at least partly mediating the therapeutic effect of curcumin in the AD model *in vitro*.

Key Words:

Alzheimer's disease, Cell cycle, Curcumin, Rat cortical neurons.

Introduction

Alzheimer's disease (AD) is a progressive and fatal neurodegenerative disease that is clinically characterized by dementia and neurobehavioral deterioration¹. It is the leading cause of senile dementia in aging populations, and a report has shown that it affects 15% of people over 65 and almost 50% of those over 85². The two major diagnostic markers for AD are amyloid plaques and neurofibrillary tangles; neuronal loss and dysfunction also characterize the disease. However, the precise pathogenesis of AD has not been fully elucidated. One current hypothesis for AD pathogenesis is that neuronal death is linked to aberrant cell cycle re-entry. Traditionally, neurons in the normal brain are viewed as being quiescent and in G0³. In AD, neurons have been shown to enter the cell cycle inappropriately without the ability to complete it fully⁴. Although various cyclins, CDKs, and other mitotic factors are expressed in the AD brain⁵⁻⁹, no evidence of actual mitosis has ever been found¹⁰, suggesting that these neurons are arrested at a point or points prior to the actual event of cellular division. Therefore, given the lack of evidence for successful completion of the cell cycle, it is likely that the re-activation of cell cycle machinery in postmitotic neurons leads to their death. The study of aberrant cell cycle regulation may provide extremely important insights into the therapeutic approaches of AD.

Curcuma longa is a member of the ginger family and has been used as folk medicine as well as dietary supplement in China and South-east Asia for thousands of years; curcumin is derived from the rhizome of this plant¹¹. Curcumin possesses a variety of pharmacological activities, including anti-inflammatory action¹², and possible protection against heart attacks¹³. In addition, curcumin modulates proliferation of tumor cells through regulation cyclin D1 and c-myc cell signaling pathways^{14,15}, and many clinical trials have been performed to elucidate curcumin's effects on cancers¹⁶. Furthermore, some reports have suggested the potential protective effects of curcumin on experimental models of Alzheimer's disease¹⁷⁻²⁰. However, it remains unclear whether the inhibition of cell cycle reentry mediates the therapeutic effect of curcumin in AD model. In this study, we first observed the protective action of curcumin in the treatment of A β -induced neurons damage, and then investigated whether the inhibition of cell cycle advance resulted in this protective effect.

Materials and Methods

Materials

Dulbecco's modified eagle's medium (DMEM)/ F12 culture medium, B27 components (Gibco BRL, Grand Island, NY, USA); A β ₂₅₋₃₅, MTT, Cytosine arabinoside, DAPI (Sigma, St Louis, MO, USA); Curcumin (National Institute for the control of pharmaceutical and biological products, Beijing, China); Trypsin (Invitrogen, Carlsbad, CA, USA); MAP-2 monoclonal rabbit antibody (Millipore, Billerica, MA, USA); CY-3 Goat-anti-Rabbit secondary antibody, GOAT-anti-Rabbit HRP (Zhongshan, Beijing, China); Cell Death Detection Kit (Roche Biochemicals, Germany); RIPA lysis buffer (Solarbio, China); BCA Protein Assay Kit (Galen, Beijing, China); polyclonal mouse anti- β -actin antibody (Santa Cruz, CA, USA); monoclonal rabbit anti-cleaved caspase-3 antibody (CST, Beverly, MA, USA); Anti-CyclinD1 (Ab-3) Mouse mAb (DCS-6) (CC12) (Calbiochem, San Diego, CA, USA); polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA); Cell Proliferation ELISA, BrdU (colorimetric) (Roche Biochemicals, Germany); 96-well plate reader (Bio-Rad Hercules, CA, USA); Bio imaging system (SYNGENE).

Neuron Culture

Primary cultures of rat cortical neurons were prepared from the brains of embryonic 16 day rats, as described previously^{21,22}, with some modifications. Briefly, the cerebral cortices were dissected in calcium-and magnesium-free Hank's balanced salt solution, incubated with 0.125% trypsin solution for 5 min at 37°C and filtered through nylon meshes to obtain a single-cell suspension. Cells were plated at 6 \times 10⁵ cells/ml on poly-d-lysine pre-coated plastic plates or coverslips. Cultures were maintained in serum-free 1:1 mixture of DMEM/F12 supplemented with B27 components in a humidified atmosphere (95% air, 5% CO₂) at 37°C. Cytosine arabinoside (2.5 μ M) was supplemented 3 days after plating to inhibit glial cell growth. Cortical neurons were chosen for experiments on culture between 7 and 10 days.

Drug Treatment and Groups

The culture neurons were treated with A β at the final concentration of 40 μ g/ml. Curcumin was dissolved in dimethyl sulfoxide (DMSO), with a concentration of DMSO not exceeding 0.1% of the total volume, and the curcumin was administered with the A β . The primary cultured cortical neurons were assigned to the control, A β ₂₅₋₃₅, Curcumin 1 μ M (A β +Ccu 1 μ M), Curcumin 5 μ M (A β +Cur 5 μ M), Curcumin 10 μ M (A β +Cur 10 μ M) group.

Immunocytochemistry

Cortical neurons were cultured for 7 day and then fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS). After blocking with 5% BSA in PBS, the cortical neurons were incubated overnight with MAP-2 (1:200) monoclonal antibody at 4°C. The cortical neurons were washed with PBS, treated for 1h with CY-3 secondary antibody (1:1000), and then examined under a fluorescence microscope equipped with digital camera.

Cell Viability

Cell viability was determined by methyl thiazolyl tetrazolium (MTT) assay. Cortical neurons were plated in 96-well plates for 7 days, then treated with A β and different concentrations of curcumin, according to the group, for 36h. After washing twice with PBS, the neurons were incubated with Neurobasal medium containing 0.5 mg/ml MTT for 4h at 37°C. Subsequently, the

medium was replaced by 100 μ l DMSO/per well to dissolve the formazan crystals. Absorbance at 570 nm was detected in a 96-well plate reader. The results were presented as a percentage of control.

TUNEL assay

Apoptosis of culture cortical neurons was measured after treatments by TUNEL assay following the manufacture's specifications with minor modifications. Briefly, neurons were plated in coverslips for 7 days, and then treated with A β and different concentrations of curcumin according the group for 36h. Then, 36h later, slides were fixed with 4% paraformaldehyde at room temperature and rinsed with PBS, pH 7.4. Next, they were permeabilised with 0.1% Triton X-100, 0.1% of sodium citrate in PBS for 2 min at 4°C. The slides were then incubated with TUNEL mixture solution at 37°C for 1h in the dark. The slides were rinsed three times in PBS and then counterstained with 10 mg/ml 4,6-diamidino-2-phenylindole (DAPI). First, the total number of cells per field stained with DAPI (blue) were counted, then the number of cells with green fluorescence (TUNEL positive cells) were counted. The percentage of cell apoptosis was determined by the ratio of the number of TUNEL-positive cells to the total of cells in one count.

Western Blot Assay

Primary cultured neurons were washed twice with cold PBS and extracted on ice with RIPA lysis buffer. Lysates were centrifuged at 12,000 g for 10 min at 4°C and then the supernatant was collected. The protein concentration in each sample was quantified using a BCA Protein Assay Kit. Denatured protein samples diluted with loading buffer were loaded equally to each lane and separated by 12% SDS-PAGE gels. After electrophoresis, the separated proteins were electrically transferred onto polyvinylidene difluoride membranes and were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% non-fat dried milk for 1h at room temperature. Subsequently, the membranes were incubated with the appropriate primary antibodies overnight at 4°C. Following five washes with TBST, the blots were incubated with the secondary antibody at room temperature for 1h. The blots were washed again five times by TBST buffer and the immunoreactive bands were detected using the enhanced chemiluminescence

method. Immunoblots were developed in the presence of enhanced chemiluminescence reagents, and the images detected on X-ray films were quantified by ImageJ (National Institutes of Health).

BrdU Label Assay

Neurons were cultured in DMEM/F12 medium according to the manufacturer's instructions. For the ELISA, cells were seeded in a 96-well plate in the medium at a density of 1×10^5 cells/well and cultured for 7 days. Following the manufacture's specifications with minor modifications, Bromodeoxyuridine BrdU (10 μ L/well) was added with A β and different concentration curcumin for 12h. Next, the medium was replaced with FixDenat (200 μ l/well) to incubate at 25-30°C for 30 min. After incubating, FixDenat was replaced by anti-BrdU-POD solution (100 μ /well) at 25-30°C for 90 min. Then, the cells were washed 3 times in washing solution, the solution was replaced and 100 μ l substrate solution was added at 15-25°C for 20 min. Colorimetric analysis was performed with an ELISA plate reader.

Statistical Analysis

Data were analyzed using SPSS 11.5 (SPSS Inc. Chicago, IL, USA) software and were expressed as mean \pm S.D (the n indicates the number of experiments in separated cultures). One-way analysis of variance was applied for comparison of means among groups. A value of $p < 0.05$ was considered significant.

Results

Protective Effect of Curcumin on A β Induced cell Damage

Immunofluorescence staining (Figure 1) showed that the primary cultured cells from rat cortices had more than 95% were labeled with Microtubule associate protein-2 (MAP-2), a neuron marker, mainly expressed in the body of central nervous system neurons and dendritic neurons.

Firstly, we used the MTT method to examine the effect of curcumin treatment against A β 25-35 induced toxicity on cell viability. As shown in Figure 2, the cell viability decreased significantly ($n=6$; $p < 0.01$) after treatment with 40 μ g/ml A β 25-35 for 36h compared to the control group,

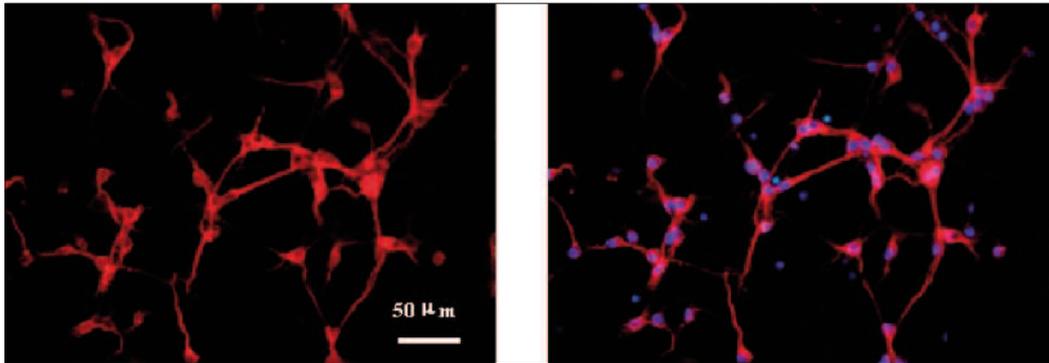


Figure 1. Immunofluorescence staining of Map-2. There are more than 95% primary cultured cells were labeled by Map-2, Red: Map-2, a neuron marker, mainly expressed in the body of the central nervous system neurons and dendritic neurons; Blue: DAPI.

as determined by MTT assay. The results showed that curcumin significantly protected the neurons against A β 25-35 induced cytotoxicity (n=6; $p < 0.01$) compared to the A β 25-35 treatment group.

To evaluate further the protective effect of curcumin against A β 25-35 induced toxicity in cultured rat cortical neurons, we used the TUNEL assay to measure the apoptosis of cortical neurons. As shown in Figure 3, the total number of cells per field were stained with DAPI (blue), and the cells with green fluorescence were the TUNEL positive cells. Figure 4 showed that the percentage of TUNEL positive cells increased significantly when treated with A β 25-35. On administration of curcumin, the percentage of A β 25-35 induced cell apoptosis decreased significantly (n=6; $p < 0.01$) compared to the A β 25-35 treated group. These data further confirm the MTT assay results that curcumin has a protective effect against A β -induced cell toxicity in rat primary cultured cortical neurons.

We then examined activated caspase-3, the apoptotic protein, by Western blot assay, to further investigate the protective effect of curcumin against A β 25-35 induced cytotoxicity in cultured rat cortical neurons. Figure 5 shows that treatment with A β 25-35 36h induced abnormal activated caspase-3 in cultured rat primary cortical neurons, added to curcumin, could inhibit abnormal activation.

The Inhibition of Curcumin on A β Induced cell Cycle Aberrant Re-Entry

To further investigate whether the inhibition of cell cycle reentry was mediated by the therapeutic effect of curcumin on A β induced primary cultured neuron damage, we first observed the cyclinD1 protein expression by Western blot assay (Figure 6) showed that A β treatment for 4h induced cyclinD1 protein up-regulation on primary cultured cortical neurons. The application of curcumin could inhibit this aberrant expression.

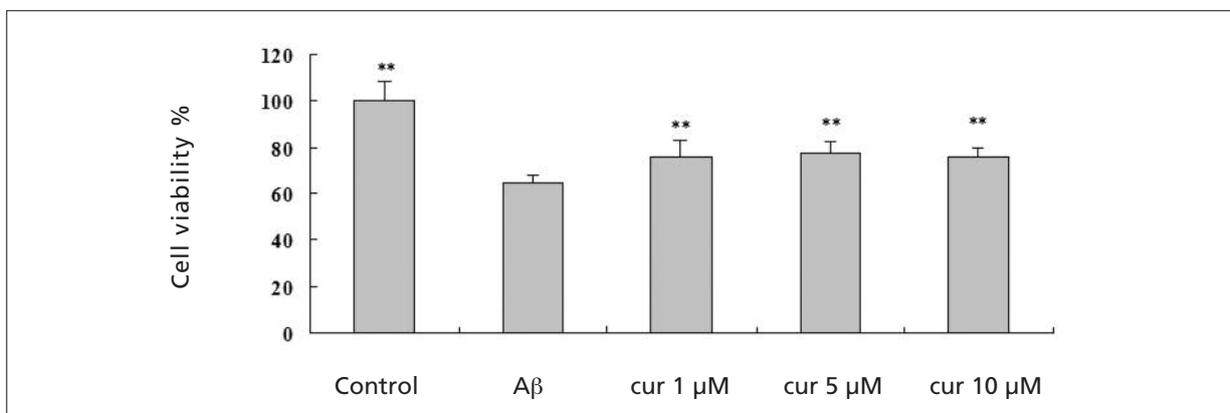


Figure 2. The effect of curcumin on A β induced cell viability. MTT assay results showed the influence of curcumin (1-10 μ m) on cell viability compared with A β treatment for 36h (n=6) $^{**}p < 0.01$ compared with the A β group.

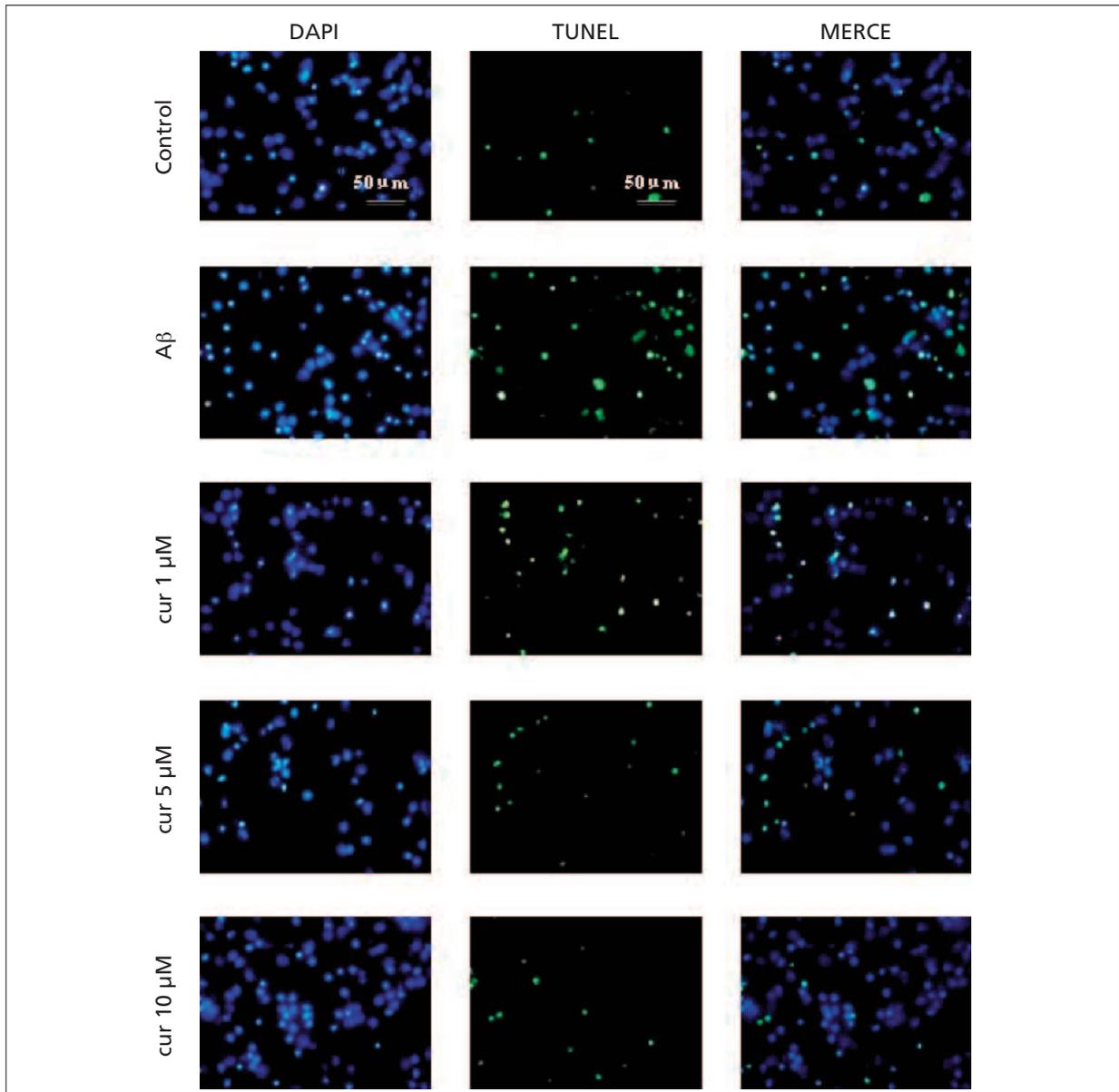


Figure 3. Effect of Curcumin on A β induced neural apoptosis. TUNEL assay show the protective effect of curcumin against A β -induced cell apoptosis on cultured rat cortical neurons. Blue: DAPI; Green: TUNEL positive cells.

To investigate further the inhibition of curcumin on A β induced cell cycle aberrant expression, we used a Brdu Elisa assay to observe the cells of re-entry cell cycle. The result was described by the ratio of OD (control group OD consider as 1), and showed that Brdu positive cells increased significantly when treated with A β . Figure 7 showed that on administration of curcumin, the Brdu positive cells decreased significantly ($n=6$; $p < 0.01$) compared to the A β treated group.

Discussion

Alzheimer's disease is characterized by neurodegeneration and cognitive impairment and is the leading cause of senile dementia. The hallmark features of AD, neurofibrillary tangles and senile plaques, are crucial to its development. The major protein component of senile plaques is a 4.2 kDa polypeptide termed amyloid- β , which is derived from a larger precursor, the A β protein precursor (APP), encoded on chromo-

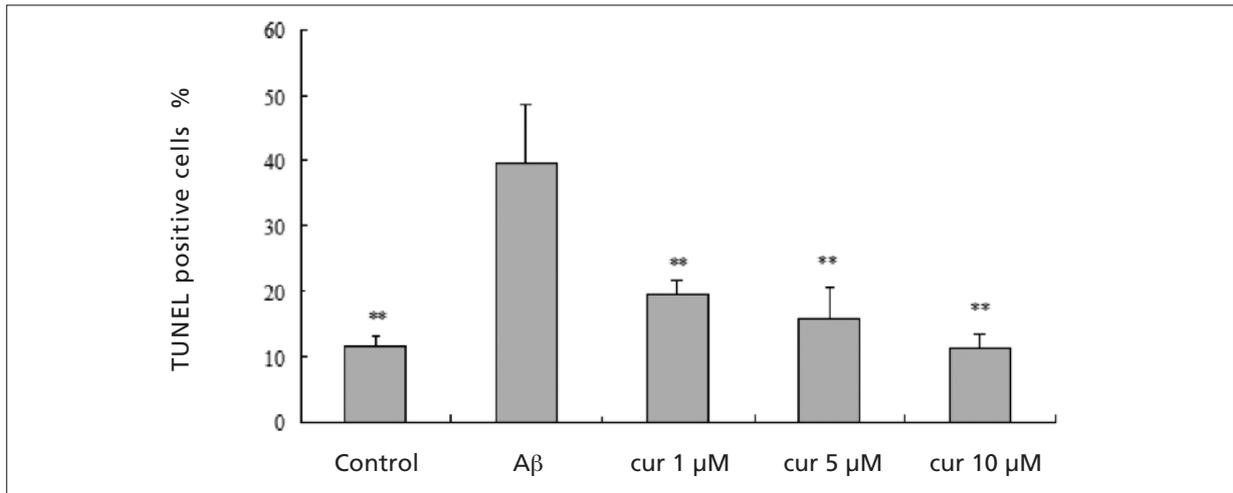


Figure 4. Effect of curcumin on Aβ induced cell apoptosis. TUNEL assay was used to measure the apoptosis of cortical neurons. The results showed that on administration of curcumin, the percentage of Aβ induced cell apoptosis decreased significantly ($n=6, p < 0.01$) compared to the Aβ treated group. $**p < 0.01$ compared with Aβ group.

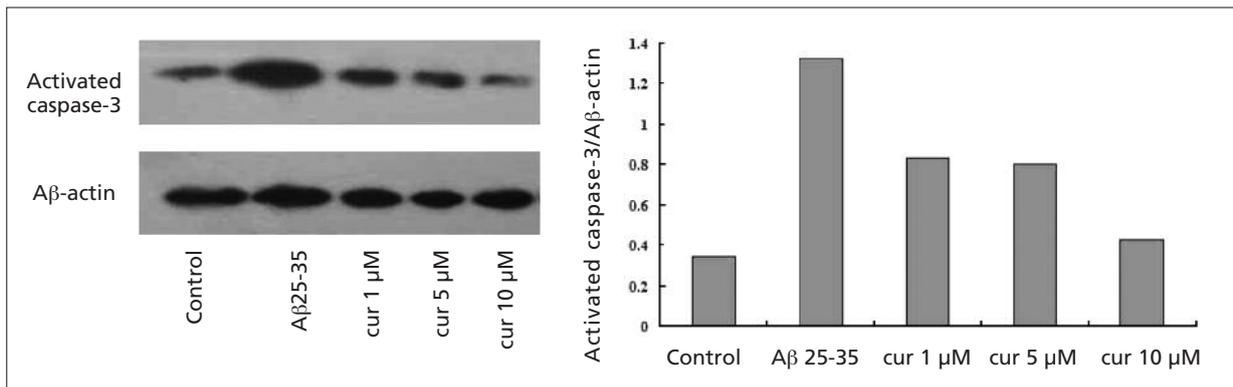


Figure 5. The level of activated caspase-3 protein expressed under different treatments. Western blot assay showed that treatment with Aβ25-35 36h induced abnormal activated caspase-3 in cultured rat primary cortical neurons, added to curcumin (1-10 μM), could inhibit abnormal activation.

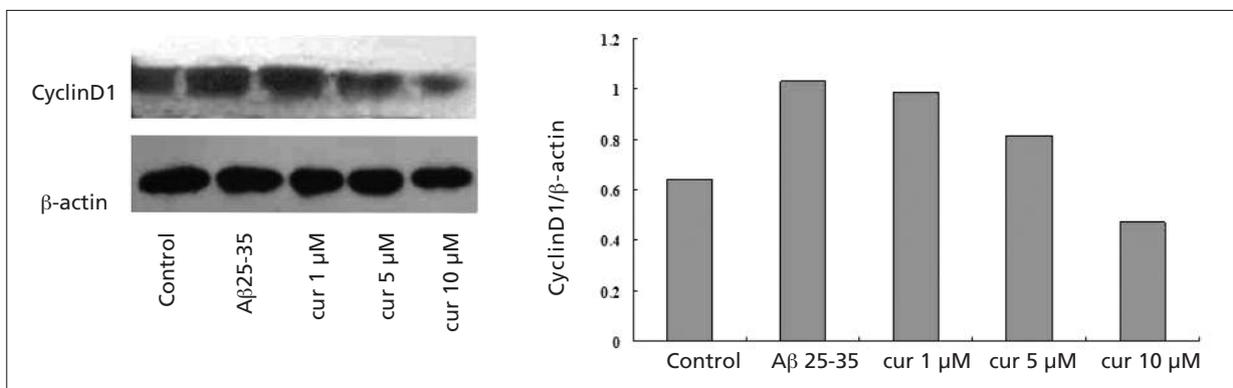


Figure 6. The level of CyclinD1 protein expressed under different treatments. The result showed that Aβ treatment for 4h induced cyclinD1 protein up-regulation on primary cultured cortical neurons. The application of curcumin (1-10 μM), could inhibit this aberrant expression.

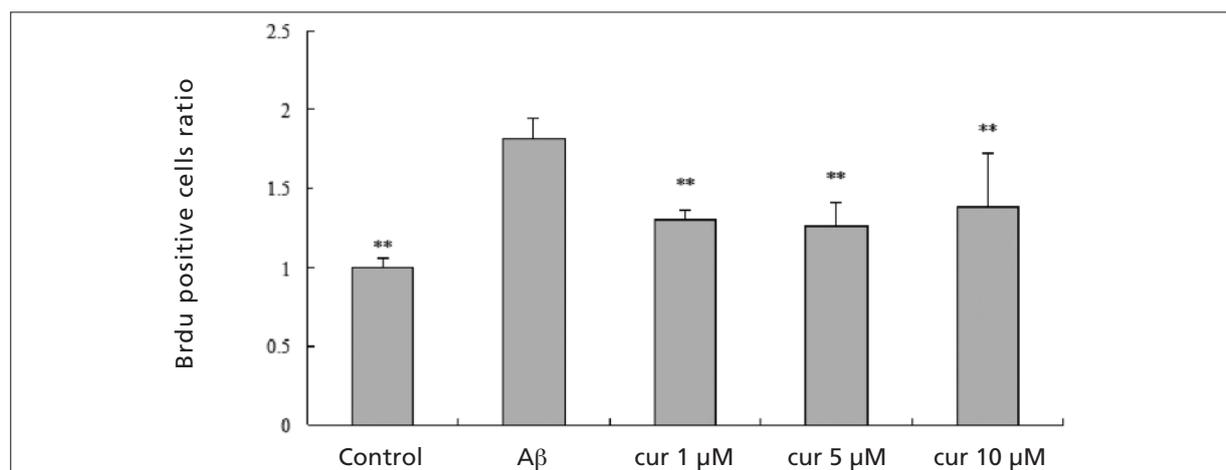


Figure 7. The result of the Brdu Elisa assay. The result was described by the ratio of OD (control group OD considered as 1). It showed that Brdu positive cells increased significantly when treated with. On administration of curcumin, the Brdu positive cells decreased significantly ($n=6$; $p < 0.01$) compared to the A β treated group. ** $p < 0.01$ compared to the A β group.

some 21^{23,24}. Reports have shown that A β is at least one of the originating causes of AD^{25,26} and affects an extensive array of neural and glial functions, leading to neural cell death²⁷. Moreover, aggregations of A β have been proven to be toxic to cell membranes and to elicit inflammatory responses from glial cells²⁸.

Therefore, in this study we first used the MTT and TUNEL assay to investigate whether curcumin has a protective effect against A β induced cytotoxicity in primary cultured rat cortical neurons. The results showed that administration of curcumin significantly increased the cell viability and reduced the cell apoptosis of primary cultured rat cortical neurons compared with treatment with A β ($p < 0.01$), confirming the protective effect of curcumin against A β ₂₅₋₃₅ induced neuron death. A β may drive cells into apoptosis when they are associated with several receptors activating the cell death signal pathway^{29,30}, including caspase-3. Caspase-3 is a type of apoptotic protein and its precursor exists in the cytoplasm under normal conditions. Once caspase-3 is activated, cell apoptosis is inevitable³¹. Caspase-3 is not only involved in APP processing consistent with the elevation of A β formation in neurons of AD patients³², but also mediates APP activation in neurons³³. Abnormal concentrations of activated caspase-3 and A β formation could have long-term pathological co-accumulation³⁴. Therefore, we examined the activated caspase-3 protein expression in this study. As shown in the results, curcumin significantly decreases the content of apoptotic protein, activated caspase-3. All

above results propose that curcumin has a protective effect against A β -induced toxicity on cultured rat cortical neurons.

Traditionally, neurons in the normal brain have been viewed as being quiescent and in G0³⁵. They lose their capacity for cell division and differentiation, and never enter the cell cycle. The cell cycle of eukaryotic cells comprises four main successive phases: G1 phase (first gap), S phase (DNA synthesis), G2 phase (second gap) and M phase (mitosis). Transition between the different phases and subsequent progression through the mitotic cycle is driven by a group of protein kinases whose activity is central to this process, namely, the cyclin-dependent kinase (CDKs), and requires the binding of their activating partners, cyclins, whose levels of expression vary throughout the cycle.

Recent studies have described aberrant neuronal expression and localization of cell-cycle proteins in postmortem tissue from AD patients. Several positive regulators of the G1/S and G2/M cell-cycle transitions are aberrantly expressed or localized, including cyclins and Cdks³⁶, the S-phase marker proliferating cell nuclear antigen (PCNA³⁷), and the M-phase marker phosphohistone-3 (PH3³⁸). In addition, one study has demonstrated that neurons in AD replicate their DNA prior to dying³⁹. However, no studies have demonstrated the neurons of AD brain completion of M-phase³⁸. It seems that these cells are unable to complete the cell cycle as a result of inadequate control and halted protein expression. Importantly, the cells become committed to divi-

sion and lack the ability to return to G0. It is likely that the reactivation of cell cycle machinery in postmitotic neurons leads to their death^{40, 41}.

Reports demonstrated that A β itself is mitogenic *in vitro*^{42,43} and, therefore, may play a direct role in the induction and/or propagation of cell cycle-mediated events in AD. Additionally, A β -mediated cell death, at least *in vitro*, is dependent on the presence of various cell cycle-related elements⁴⁴. Most importantly, the ectopic re-entry of neurons into the cell cycle was recently shown to lead to cell death, gliosis, and cognitive deficits—all cardinal features of AD⁴⁵. Taking all these data into consideration, we suggest that curcumin could inhibit the A β -induced neurons' re-entry into the cell cycle and may, therefore, result in a protective effect. In this study, we first observed the cyclinD1 protein expression by Western blot assay. The result showed that A β enhanced the ectopic expression of cyclinD1 in primary culture rat cortical neurons, and that administration with curcumin could reverse abnormal up-regulation. Furthermore, as the report showed that the ectopic cell cycle re-entry of neurons could occur through S-phase in AD⁴⁶, we investigated the effect of curcumin on an AD model *in vitro* by Brdu label assay. The results showed that A β evoked the Brdu positive cells of neurons, but this activation was inhibited by treatment with curcumin. All above results suggest that the application of curcumin could inhibit A β -induced cell cycle aberration in primary cultured rat cortical neurons. Moreover, these data taken together may indicate that the application of curcumin could inhibit re-entry cell cycle and result in a protective effect against A β -induced neuron damage in primary cultured cortical neurons.

Alzheimer's disease is likely to result from the complex interplay of genetics, environment and aging that affect cellular metabolism, mechanistic pathways and stress response, resulting in abnormal protein deposition, mitochondrial dysfunction, and severe synaptic and neuronal loss. The notion that AD neurodegeneration is a cell cycle-driven process opens up a world of new possibilities for potential AD therapies. Curcumin might be one of the most promising compounds in the attempt to halt the development of AD, as has been reported in several models *in vitro* and *in vivo*. In the present study, our finding suggested that cell cycle regulation may provide new insights into the investigation of the neuroprotective effect of curcumin.

Conclusions

The key finding of the present study is that the application of curcumin could inhibit A β -induced aberrant cell cycle reentry in primary cultured rat cortical neurons, which mediates the neuroprotective effect of curcumin.

Ethical Approval

Experiments were performed with the approval of the Animal Ethics Committee of Tianjin University of TCM in China.

Acknowledgements

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