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# Protective effects of melatonin on cortico-hippocampal neurotoxicity induced by amyloid beta-peptide 25 - 35[1]

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**KEY WORDS** melatonin; neurons; amyloid beta-protein; lactate dehydrogenase

## ABSTRACT

**AIM**: To study the effects of melatonin on primary rat cortico-hippocampal neurotoxicity induced by amyloid beta-peptide 25 - 35. **METHODS**: The neuronal morphology was observed by *phase-contrast microscopy*. The neurotoxicity was quantitatively estimated by measuring lactate dehydrogenase (LDH) released into the culture medium from the damaged neurons. The neuronal metabolic state was quantified by the reduction of 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT). **RESULTS**: Treatment of primary rat cortico-hippocampal neurons with amyloid beta-peptide 25 - 35 (20 μmol/L) for 24 h caused a significant decrease in neurocyte viability ($P < 0.01$, compared with control). Melatonin (1 or 10 μmol/L) reduced the neurotoxicity induced by amyloid beta-peptide 25 - 35. **CONCLUSION**: Amyloid beta-peptide 25 - 35 could exert direct cytotoxicity on rat cortico-hippocampal neurocytes and melatonin concentration-dependently rescued cultured neurons from exposure to amyloid beta-peptide 25 - 35 induced injury.

## INTRODUCTION

Amyloid β protein (Aβ), a spontaneously aggregating peptide of 39 - 43 amino acids, is the primary protein component of senile plaques, the pathological hallmarks of Alzheimer's disease (AD) *in inflicted brains*. This peptide is derived from proteolytic processing of the amyloid precursor protein (APP), a type Ⅰ transmembrane glycoprotein that is expressed in a variety of cells during normal cellular metabolism[1]. An increasing amount of experimental as well as genetic evidence supports a causal role for Aβ in the pathogenesis of AD. Briefly, senile plaques containing Aβ as a core protein are often closely associated with degenerating neurons[2]. And longer species of the Aβ peptide (ie, 1 - 42 and 1 - 43) are preferentially produced and deposited in the brain of AD patients. At present there is no effective treatment to prevent or ameliorate the progression of Alzheimer's disease, which is the commonest cause of dementia in the elderly.

A number of studies have shown that high concentrations of Aβ are toxic and damage biological macromolecules. Its degenerative effects include an alteration in $Ca^{2+}$ homeostasis in the cell[3], apoptosis[4], and an inhibition of cellular redox activity[5], and these actions are believed to be dependent on the peptide assembly state such as its aggregation state[6]. The effects of Aβ are localised to amino acid residues 25 - 35 of the full-length peptide[7]. The Aβ peptide fragment 25 - 35 ($Aβ_{25-35}$) has been shown to be directly toxic to neurocytes[6,7] and be able to increase the vulnerability of neurons to other insults[8].

Melatonin (*N*-acetyl-5-methoxytryptamine) is synthesized by the pineal gland of vertebrates including humans. Melatonin participates in several important physiological functions, including the circadian rhythm regulation and normal patterns of sleep. The original interest in melatonin derives from the suggestion that melatonin may act as a key regulator in aging and senescence[9]. The level of melatonin in the pineal gland declines progressively with age, such that in old animals and elderly humans the level of melatonin available to the organism is a fraction of that of young individuals[10]. Recent studies suggest that melatonin exerts a neuroprotective role, and accelerates the early process of neuronal differentiation[11]. The present study was designed to determine whether melatonin may protect

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primary cultures of cortico-hippocampal neurons from the toxicity *in vitro* induced by $A\beta_{25-35}$.

## MATERIALS AND METHODS

**Materials** $A\beta_{25-35}$ (Sigma Chemical Co, USA) was dissolved in sterile deionized water, and stored at −20 ℃. To obtain the neurotoxic form of $A\beta_{25-35}$, the peptide solution was placed in an incubator at 37 ℃ for 7 d[6]. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and melatonin, were purchased from Sigma Chemical Co (USA). Melatonin was dissolved in 0.9 % saline with ethanol (less than 0.01 %, v/v) and stored at − 20 ℃. Dulbecco's modified Eagle's medium (DMEM, Gibco) was supplemented with HEPES 25 mmol/L, sodium pyruvate 1 mmol/L, 2-mercaptoethanol 50 μmol/L, *L*-glutamine 2 mmol/L, benzylpenicillin 100 kU /L, streptomycin 100 mg/L, 10 % fetal bovine serum, and 10 % heat-inactivated horse serum.

**Rat cortico-hippocampal cultures** All procedures were performed in sterile conditions. Brains were removed from 16 − 18 d-old foetal Sprague-Dawley rats (Clean grade, Certificate № 005, supplied by Shanghai Experimental Animal Centre, Chinese Academy of Science) into $Ca^{2+}$ and $Mg^{2+}$-free Hanks' solution (D-Hanks'). The cortex and hippocampus were dissected out and dissociated cells were prepared as described previously[12] with minor modifications. Briefly, the tissue pieces from cortico-hippocampal regions were chopped into less than 0.5 $mm^2$ pieces and then resuspended in 10 mL of serum-free DMEM solution containing 0.025 % − 0.06 % trypsin and incubated for 10 − 20 min at 37 ℃ in a shaking water bath. Immediately after incubation serum was added to stop the action of trypsin. The contents were filtered through a 200 μm nylon mesh and centrifuged (1300 × *g* for 2 s). The cortical-hippocampal cells were resuspended in DMEM containing 10% fetal bovine serum and 10 % heat-inactivated horse serum at a concentration of $1 \times 10^9$ cells/L. Cells were plated onto 24-well plastic plates coated with poly-*L*-lysine (0.5 g/L in D-Hanks') for 2 h and then washed 3 times. The cultures were incubated at 37 ℃ in humidified 5 % $CO_2$ for 2 weeks. The cultured cells were exposed to cytosine arabinoside (5 mg/L) for 24 h (between 6 and 7 d after plating) to inhibit the proliferation of non-neuronal cells. At the time of experiment, cells were first changed into low serum (5 %) medium, then different concentrations of $A\beta_{25-35}$ and melatonin were added simultaneously for 24 h.

**Photographic methods** Morphological changes in the cells were monitored throughout the course of the experiment with an inverse phase-contrast microscope. The number of damaged neurons was estimated by trypan-blue staining. The number of neuronal cells bodies were counted in five fields. The percent of neuronal death was calculated for each photographic field.

**MTT assay** After the neurons were treated with different concentrations of $A\beta_{25-35}$ and melatonin for 24 h, MTT solution (0.5 g/L) was added to each culture well. After incubation at 37 ℃ for an additional 4 h, the formazan crystals were dissolved in 120 μL isobutanol (contaning HCl 0.04 mol/L) and the absorbance was measured at 570 nm using an ELISA plate reader.

**LDH release assays** LDH activity in the extracellular medium was measured using a commercial kit (Jiancheng Institute of Biotechnology, Nanjing, China), wherein the colorimetric assay measured the pyruvate-mediated conversion of 2,4-dinitrophenylhydrazine into a visible hydrazone precipitate following the kit instructions.

**Statistical analysis** Statistical analysis of the data for multiple comparisons was performed by ANOVA followed by Dunnett's test. For single comparisons, the significance of differences between means was determined by *t*-test.

## RESULTS

**Effect of melatonin on the morphology of neurons treated with $A\beta_{25-35}$** The cortico-hippocampal cells were plated in the absence or presence of aged $A\beta_{25-35}$ (20 μmol/L). The morphology of neurons was observed 2 weeks after culture. The general health of cultures was evaluated by monitoring morphological changes through microscopic examination. Neurons plated in the absence of $A\beta_{25-35}$ appeared normal, exhibiting large vacuole-free cell bodies with elaborate networks of neurites. The synapse connections between neurons could be seen. As early as 24-h exposure of cultures to $A\beta_{25-35}$ resulted in a decrease in the total number of cells remaining on the plate, and the neurites of partial neurons disappeared (Fig 1B). These changes were counteracted by melatonin (1 μmol/L and 10 μmol/L) (Fig 1C, D).

**Effect of melatonin on the proliferation of**

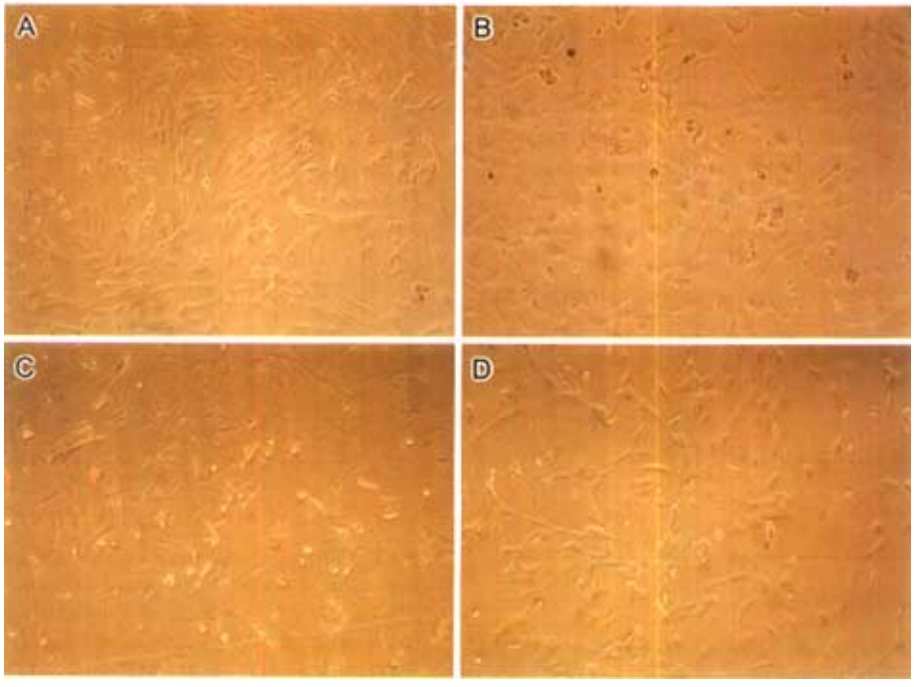


**Fig 1. Phase-contrast micrographs of rat cortico-hippocampal cell cultures after exposure for 24 h to control medium (A); medium containing $A\beta_{25-35}$ (20 μmol/L) (B); medium containing $A\beta_{25-35}$ (20 μmol/L) and melatonin (1 μmol/L) (C) or melatonin (10 μmol/L) (D). ×100.**

**cortico-hippocampal neurons treated with $A\beta_{25-35}$**

In order to correlate changes in morphology and cell number with the general metabolic state of the cell, MTT reduction was assayed. Fig 2 showed that incubation with $A\beta_{25-35}$ (0.01 – 10 μmol/L) resulted in a significant decline in the ability of cells to reduce MTT in concentration-dependent manner ($r = -0.897$, $P < 0.05$). Melatonin (1 or 10 μmol/L) completely blocked the decrease of neuronal proliferation induced by $A\beta_{25-35}$ (Fig 3).

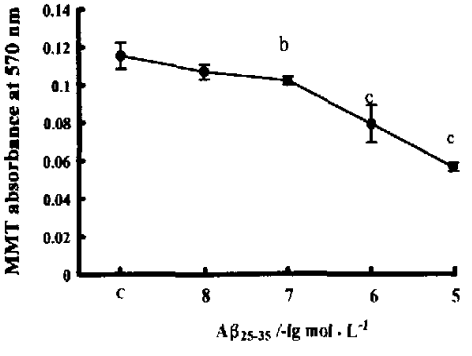


**Fig 2. The effect of $A\beta_{25-35}$ on cell viability. $n = 11$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control. C: control.**

**Effect of melatonin on neuronal death induced by exposure to $A\beta_{25-35}$** Above morphological observations were confirmed by microscope. In the

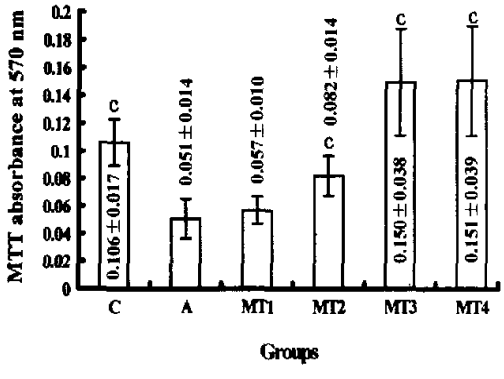


**Fig 3. The protective effect of melatonin on the cell viability treated with $A\beta_{25-35}$. $n = 11$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* $A\beta_{25-35}$. C: control; A: $A\beta_{25-35}$ (20 μmol/L); MT: melatonin (MT1: 0.01, MT2: 0.1, MT3: 1, MT4: 10 μmol/L).**

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present study, the measurement of LDH releasing into the extracellular bathing medium was used for quantitative assessment of injury or death of cultured neurons. When cortico-hippocampal neurons were exposed to $A\beta_{25-35}$ (0.01 – 10 μmol/L), it produced a marked increase in LDH release into the medium at 24 h after treatment with this peptide (Fig 4). The effect of $A\beta_{25-35}$ on the LDH release of neurons was concentration-dependent ($r$ = 0.755, $P < 0.05$). Melatonin (1 μmol/L and 10 μmol/L) also blunted $A\beta_{25-35}$-induced release of LDH (Fig 5).

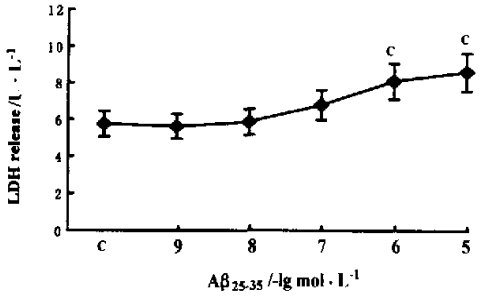


**Fig 4. Effects of $A\beta_{25-35}$ on the LDH release from the cultured cortico-hippocampal neurons. $n = 11$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. C: control.**

It was also found that the number of neurons stained by trypan-blue was increased by the addition of $A\beta_{25-35}$ (20 μmol/L). Melatonin (0.01 – 1 μmol/L) protected cortico-hippocampal cells from the death induced by $A\beta_{25-35}$ (Fig 6, 7).

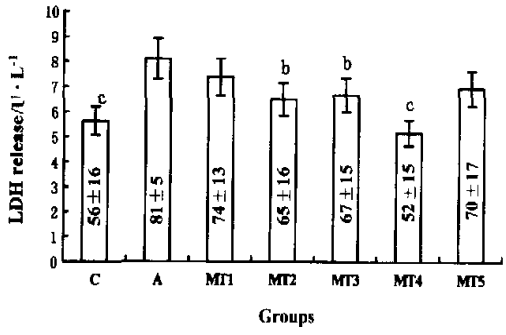


**Fig 5. Effects of melatonin on the LDH release from the cultured cortico-hippocampal neurons caused by $A\beta_{25-35}$. $n = 11$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* A. C: control; A: $A\beta_{25-35}$ 20 μmol/L; MT: melatonin (MT1: 0.001, MT2: 0.01, MT3: 0.1, MT4: 1, MT5: 10 μmol/L).**

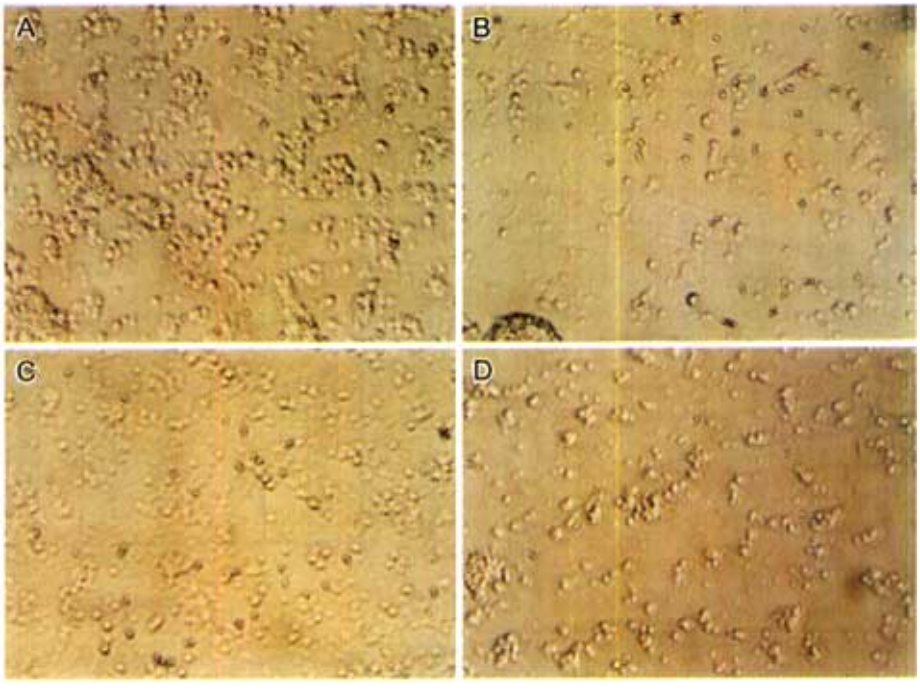


**Fig 6. Effects of melatonin on toxicity of cultured rat cortico-hippocampal cells after 24 h exposure to control medium (A); medium containing $A\beta_{25-35}$ (20 μmol/L) (B); medium containing $A\beta_{25-35}$ (20 μmol/L) and melatonin (1 μmol/L) (C), or melatonin (10 μmol/L) (D). The death cells were stained by trypan blue. × 100.**

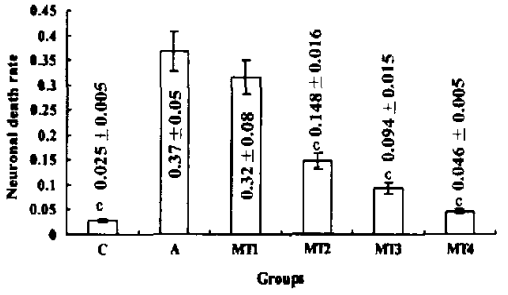


**Fig 7. Effects of melatonin on the viability of cultured cortico-hippocampal neurons treated with $A\beta_{25-35}$.** $n=6$. $\bar{x} \pm s$. $^cP<0.01$ *vs* $A\beta_{25-35}$. **C: Control; A: $A\beta_{25-35}$ (20 μmol/L); MT: melatonin (MT1: 0.01, MT2: 0.1; MT3: 1, MT4: 10 μmol/L).**

## DISCUSSION

Aβ toxicity has been suggested to be responsible for at least some of the neurodegeneration observed in post-mortem brains of AD patients. In our study, MTT and LDH release assay were used to measure the viability of cotico-hippocampal neurons. When cortico-hippocampal neurons were exposed to $A\beta_{25-35}$, it produced a marked change in the morphology of cultured neurons and increased neuronal death at 24 h after treatment. The result was further confirmed by a decrease in the neuronal proliferation and increase in the LDH release in cultured medium. These results agree with earlier observations[13] with continuous treatment with $A\beta_{25-35}$ or $A\beta_{1-40}$ also produced a significant increase in degeneration of cultured cortical neurons.

The aim of this study was to observe the effect of melatonin on neurotoxicity caused by $A\beta_{25-35}$. The present study provides clear evidence that $A\beta_{25-35}$-induced injury to rat cortico-hippocampal cultures can be prevented by melatonin. These results are in accordance with the findings of Pappolla *et al*[14], who demonstrated improved cell survival when neuroblastoma cells were exposed to the same neurotoxic Aβ fragment in the presence of melatonin.

Strong evidence has shown that free radicals play a very important role in Aβ-induced neurotoxicity[15,16]. Melatonin is considered to be an effective free radical scavenger and antioxidant[17]. Being lipophilic, melatonin can move into the lipid bilayer of cell membranes, where it prevents the peroxidation of phospholipids. Melatonin receptors have now been found to be associated with various regions of the brain[18]. These receptors may be involved in the production of free radical scavengers and participate in the process of neuroprotection. This study further suggests that the progression of Aβ toxicity in AD may be reduced by melatonin supplementation. However, further studies are needed to clarify the pathophysiologic role and therapeutic potential of melatonin in aging and age-related illness.

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## 褪黑激素对β-淀粉样多肽25-35片段所致皮层海马神经细胞毒性损伤的防护作用

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**关键词** 褪黑激素; 神经元; 淀粉样β蛋白; 乳酸脱氢酶

**目的**: 观察褪黑激素对β-淀粉样多肽25-35片段($A\beta_{25-35}$)所致神经细胞毒性作用的影响. **方法**: 用相衬倒置显微镜观察原代培养的神经细胞的形态; 用噻唑兰(MTT)法、乳酸脱氢酶(LDH)测定及台盼兰染色观察神经细胞的存活情况. **结果**: $A\beta_{25-35}$ (20 μmol/L)可引起培养的神经细胞数量减少, 部分突起消失; 台盼兰着色细胞数增加; 神经细胞增殖能力降低; 培养上清液中LDH释放量增加; 褪黑激素(1, 10 μmol/L)可抑制$A\beta_{25-35}$诱导的上述毒性损伤. **结论**: $A\beta_{25-35}$对原代培养的皮质-海马神经细胞有直接的细胞毒作用, 褪黑激素对$A\beta_{25-35}$所致神经细胞毒性损伤具有剂量依赖性的防护作用.

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