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Research Report

Protective effect of *N*-acetyl-L-cysteine on amyloid β-peptide-induced learning and memory deficits in mice

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ARTICLE INFO

Article history:
Accepted 15 June 2006
Available online 26 July 2006

Keywords:
Alzheimer's disease
Amyloid β-peptide
N-acetyl-L-cysteine
Neuroprotection

ABSTRACT

This study aimed to examine the effects of *N*-acetyl-L-cysteine (NAC) on protecting neurons function and improving learning and memory deficits in mice. Mice were intracerebroventricularly (icv) injected with the aggregated amyloid β-peptide (Aβ) to produce Alzheimer's disease (AD). Learning and memory functions in mice were examined by the step through test and the water maze performance. The results showed that the mice pretreated with NAC had significantly greater retention in the step through test and shorter latencies in the water maze performance. Biochemical studies showed the potential role of free radical toxicity and the damage of cholinergic neurons in the Aβ-treated mice. There was an increased lipid peroxidation as indicated by elevated malondehyde (MDA) and decrease of glutathione (GSH) levels. There was also an increase in acetylcholinesterase (AChE) activity and a reduction in the choline acetyltransferase (ChAT) activity and acetylcholine (ACh) levels. NAC pretreatment significantly reversed the elevated MDA, AChE and the reduced GSH, ChAT and ACh in the Aβ-model mice. The results of the present study suggest the potential usage of the neuroprotective action of NAC on AD.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive loss of cognitive ability and by neuropathological features including senile plaque, neurofibrillary tangles and neuronal loss in selective brain regions. AD is estimated to affect approximately 15 million people worldwide, and the incidence increases from 0.5% per year at the age of 65 years to 8% per year at the age of 85 years. As more people live to old age, AD is becoming a greater medical and social problem.

Oxidative stress is increasingly being implicated as an important causative factor in various neurodegenerative diseases (Matsuoka et al., 2001). Amyloid β-peptide (Aβ), the central constituent of senile plaques in AD brain, has been shown to be a source of free radical oxidative stress that may lead to neurodegeneration. Aβ, perhaps in concert with bound redox metal ions, initiates free radical processes resulting in protein oxidation, lipid peroxidation, reactive oxygen species (ROS) formation, cellular dysfunction leading to calcium ion accumulation and subsequent neuronal death. Antioxidants that prevent the detrimental consequences of Aβ are consequently considered to be a promoting approach to neuroprotection in AD brain (Peng et al., 2002).

N-acetyl-L-cysteine (NAC) is a small molecule containing a thiol group, which has antioxidant properties, and is freely filterable with a ready access to blood–brain barrier and intracellular compartments (Farr et al., 2003). Studies had reported that NAC could prevent apoptotic death of cultured

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doi:10.1016/j.brainres.2006.06.042

Table 1 – Reversal effects of NAC for AD model mice in behavioral experiments

	n	Passive avoidance test, latency (s)	Water maze test (s)		
			d1	d2	d3
Control	10	108±18	48±25	29±12	21±13
Aβ	8	11±9*	133±26*	91±16*	63±16*
Aβ+NAC (50 mg/kg)	8	52±34#	114±39	53±21	30±17#
Aβ+NAC (100 mg/kg)	9	74±36##	92±15	45±21#	22±12##
Aβ+NAC (200 mg/kg)	9	103±20##	31±16##	33±22##	11±9##

**P<0.01 compared with control group, #P<0.05, ##P<0.01 compared with Aβ model.

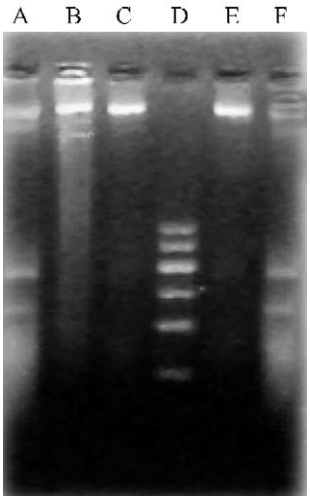

Fig. 1 – Agarose gel electrophoresis of brain DNA of NAC treated Aβ model mice. A=Aβ+NAC (50 mg/kg); B=Aβ+NAC (100 mg/kg); C=Aβ+NAC (200 mg/kg); D=DNA marker; E=control; F=Aβ-treated.

neuronal cells (Ferriari et al., 1995; Olivieri et al., 2001) and promote survival of PC12 cells as lack of neurotrophic factor, which was presumed that this protective effect was due to its ability to scavenge free radicals (Yan et al., 1995). In the present study, we are interested whether NAC can improve the cognitive and memory ability on Aβ-induced learning and memory deficits in mice.

2. Results

2.1. *Learning and memory test*

Mice were examined 11 days after icv injection of the aggregated Aβ, for the step through test, an index of long-term memory and, for the water maze performance, an index of spatial recognition memory. Post hoc analysis indicated that the mice which received Aβ had significantly shorter memory retention in the step through test ($p<0.01$) and longer latency in the water maze performance as compared with vehicle-treated mice (Table 1). These results suggest that icv administration of Aβ (22–35) caused impairments in memory and cognitive ability.

To determine the effect of NAC on AD model mice, we examined the reversal action ability of NAC on Aβ-induced amnesia. NAC was ip injected (50, 100, 200 mg/kg) to mice 7 days before each testing trial. The vehicle-treated mice were administered the same amount of saline. The results (Table 1) showed that the mice pretreated with NAC had significantly longer retention in the step through test and shorter latencies in the water maze performance ($p<0.01$ vs. Aβ model mice). It implies that NAC obviously improves the learning and memory of the Aβ model mice.

2.2. *Biochemistry assays*

Biochemical parameters were measured after the learning and memory tests. The levels of MDA were significantly higher in the Aβ group when compared with the control, and the GSH levels decreased after icv Aβ, statistically different from that of the control group, whereas NAC treatment reversed the effects (Table 2).

AChE activity was significantly elevated in the Aβ group, and the ChAT activity and ACh content decreased remarkably. However, they gradually reached the control levels when treated with NAC (Table 2).

2.3. *DNA agarose gel electrophoresis*

The agarose gel electrophoresis pattern of the DNA in the brains of the mice 11 days after the icv injection of Aβ showed obviously the DNA ladder (Fig. 1F). After pretreatment of different dosages of NAC from 50 to 200 mg/kg, it showed that the DNA ladders abated along with the increases of NAC (Figs.

Table 2 – Influence of NAC on the biochemical parameters in brains of AD model mice

Group	MDA (μmol/g protein)	GSH (mg/g protein)	AChE (mmol/min·g protein)	ChAT (nmol/h·g protein)	ACh (μmol/g protein)
Control	2.8±0.3	33.2±4.0	54.5±7.9	43.8±4.6	12.0±1.0
Aβ	3.8±0.2*	17.4±2.1*	93.4±2.0*	31.8±4.9*	8.4±0.9*
Aβ+NAC (50 mg/kg)	3.3±0.2	20.6±3.0	85.3±2.4#	38.2±5.6	11.2±0.6#
Aβ+NAC (100 mg/kg)	2.9±0.1##	25.4±3.8#	71.1±6.6##	41.7±7.9	11.0±0.5#
Aβ+NAC (200 mg/kg)	2.8±0.3##	32.1±0.4##	59.8±5.8.##	45.5±6.7#	12.5±1.1##

**P<0.01, compared with control group, #P<0.05, ##P<0.01, compared with Aβ group. $n=3$.

1A to C). The pattern of the DNA in the brain of the treated mouse at concentration of 200 mg/kg (Fig. 1C) was similar to that of the normal control (Fig. 1E).

2.4. *Histology*

Histological examination was carried out on HE-stained slices of mouse brain 11 days after the icv administrations. The slices showed that saline failed to produce any significant neuronal damage in the control animals, but the aggregated Aβ (25–35) produced a significant neuronal loss in the cerebral cortical layer and in the region of the hippocampus formation. Treatment of NAC could protect neurons from Aβ-induced damage (Fig. 2).

3. Discussion

AD is one of the most challenging brain disorders and has profound medical and social consequences. Neuronal cell dysfunction and oxidative cell death caused by Aβ contribute to the pathogenesis of AD (Boncristiano et al., 2002). Aβ is a 39–43 amino acid peptide that is formed by a much larger transmembrane protein, the amyloid precursor protein (APP). Two catabolic pathways have been identified for APP processing: the non-amyloidogenic pathway relying on α-secretase activity and resulting in secretion of soluble forms of APP, and the amyloidgenic pathway where β- and γ-secretase activities allow Aβ production (Ikezu et al., 2003). A close correlation existed between Aβ production and the neurodegenerative process of AD. The neurotoxic effects of Aβ have been studied by treating neurons with high concentration of Aβ that result in free radical damage, oxidative stress and cell death.

Various lengths of Aβ fragments such as 1–28, 12–20, 12–28, 18–28, 22–35, 25–35 and 31–35 also exhibited neurotoxic properties of the full-length sequence (Maurice et al., 1996). Incubation of Aβ fragment in water for several days produces a conformational transformation from random coil to β-sheet coinciding with an increase in peptide neurotoxic potency. A single acute icv administration of aggregrated Aβ active fragment induced marked amnesic effects in mice, evidenced as deficiencies in both short- and long-term memory (Yamaguchi and Kawashima, 2001). In our experiment, icv administration of the aggregated Aβ (22–35) significantly induced the neuronal loss and amyloid deposits in brain and caused impairments in memory function and cognitive ability in mice at day 11. These

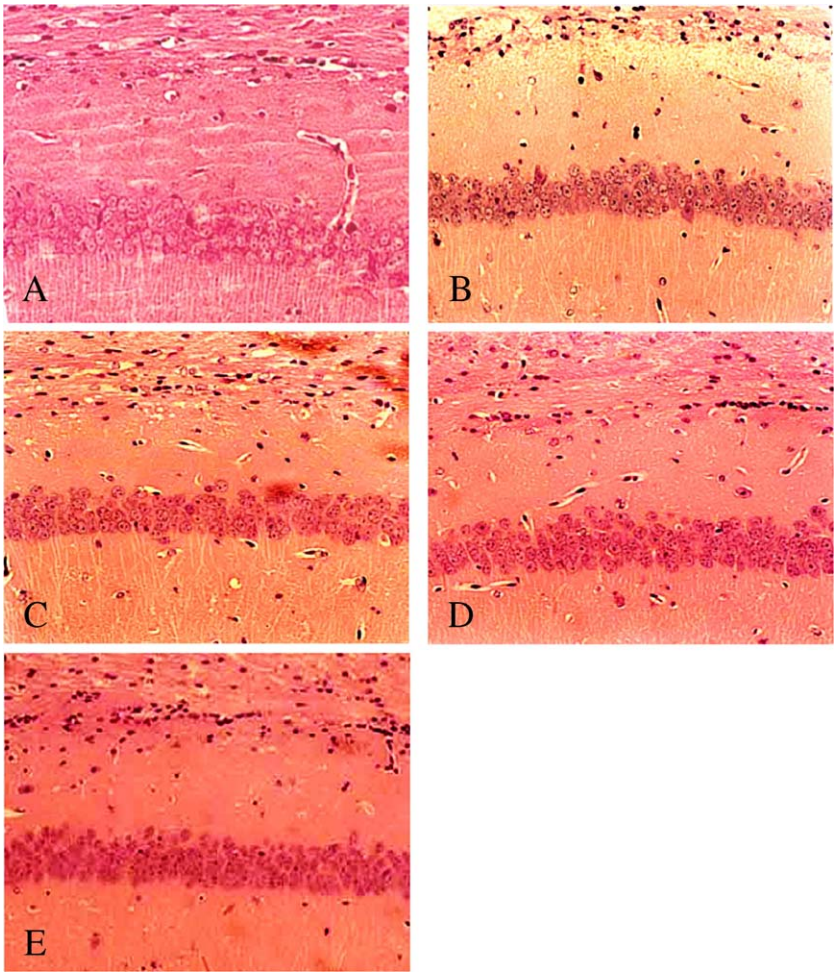

Fig. 2 – Representative photographs of slices of central cortical layer and hippocampus formation (HE staining). (A) Control; (B) Aβ-treated; (C) Aβ + NAC (50 mg/kg); (D) Aβ + NAC (100 mg/kg); (E) Aβ + NAC (200 mg/kg).

results proved that the Aβ (22–35) is suitable to use in the production of the amyloid toxicity model.

According to the oxidative stress hypothesis of AD, the Aβ inserts into the neuronal membrane bilayer and generates oxygen-dependent free radicals and then causes the lipid peroxidation and protein oxidation. The loss of membrane integrity leads to cellular dysfunction, such as loss of Ca^{2+} homeostasis, disruption of signal pathways and activation of nuclear transcription factors and apoptotic pathways. The neuronal death is the ultimate consequence of these cellular dysfunctions (Yatin et al., 1999). In the Aβ (22–35) model mice, MDA (an end product of lipid peroxidation) increased; GSH (the first line of defense against oxidative stress in the form of ROS) remarkably decreased; and the Aβ-mediated neuron apoptotic events appeared. It suggests that Aβ induces the free radical damages in neurons.

NAC is known to serve as cysteine donors. The beneficial effect of NAC administration on memory is related to its activity as a direct and potent free radical scavenger. First, NAC increases levels of intracellular cysteine, the limiting amino acid in GSH biosynthesis, and subsequently potentiates the natural antioxidative cellular defense mechanisms (Pocernich and La Fontaine, 2000). Second, NAC can act by direct reaction between its reducing thiol group and ROS. Third, it has been shown that NAC can prevent apoptosis in cultured neuronal cells. The other benefit of NAC as possible effective agents is the fact that the antioxidant seems to be less toxic compared with cysteine itself in the central nervous system (Park et al., 2004). In our study, the ip injected antioxidant NAC improved the cognitive function, and the Aβ-model mice showed significantly longer memory retention in the step through test and a shorter latency in the water maze performance as compared with that of the Aβ mice. As a result of the action of NAC on scavenging of the free radicals, the lipid peroxidation and the protein oxidation are prevented to a certain extent, thus the cholinergic neurons may get rid of the impairments of the membrane integrity, cellular dysfunction and apoptosis, manifesting restoration of the MDA, AChE, ChAT and ACh to their normal levels *in vivo*. The level of GSH in the cerebra also increased as expected, owing to the supplement of the precursor NAC.

In conclusion, NAC regulates cholinergic system, inhibits the cell apoptosis by scavenging the free radicals induced by the aggregated Aβ and leads the improvement of learning and memory deficits. Because of the ease of administration and low cost and low toxicity of NAC, it is of potential use in the therapy of AD.

4. Experimental procedures

4.1. *Materials*

The NAC and Aβ (22–35) were purchased from Sigma. Aβ (25–35) was dissolved in sterile saline (vehicle) at a concentration of 1 mM, sealed and incubated for 96 h at 37 °C to transform Aβ into an "aggregated phase". The other reagents were all of chemical grade.

4.2. *Animals and housing conditions*

Healthy mice, Kunming species (Grade II, Certificate NO. 01-3023), male, aged 3–4 weeks and weighing 18–20 g at the beginning of the experiments, provided by the Animal Breeding Center Affiliated to AMMS, China, were used throughout the study. Animals were housed in plastic cages, with free access to standard laboratory food and water, and kept in a regulated environment (23±1 °C). Animal studies were approved by the Animal Care and Use Committee, Ministry of Science and Technology, China (Document 545, License no. SYXK-2002-001).

4.3. *Experimental groups*

The mice were divided into 5 groups at random. (1) Aβ model group: Aβ model mice were made according to the method of Maurice (Maurice et al., 1996). Model mice were intracerebroventricularly (icv) injected with 4 μl (equivalent to 4 nmol) of Aβ. (2) Aβ+NAC (50 mg/kg body weight) group: Aβ model mice, intraperitoneally (ip) injected NAC 50 mg/kg, 7 days before the learning and memory text. (3) Aβ+NAC (100 mg/kg) group: injection of NAC 100 mg/kg. (4) Aβ+NAC (200 mg/kg) group: injection of NAC 200 mg/kg. (5) Control: normal mice, icv vehicle 4 μl.

4.4. *Examination of learning and memory ability*

4.4.1. *Step through test*

The step through test was performed 11 days after the icv administration of 4 nmol of Aβ in the JZZ94 multifunction passive avoidance apparatus (PA M1 O'Hara & Co. Ltd). The apparatus consisted of two compartments separated by a black wall with a hole in the lower middle part. One of the two chambers is illuminated and the other, dark. The test was conducted for 2 consecutive days including one training trial (d1), each mouse was placed in the illuminated compartment, facing away from the dark compartment and left for 5 min to habituate to the apparatus, and 1 h after the adaptation trial, the mouse received the training trial. The training trial is similar to the adaptation trial except that, when the mouse entered the dark compartment, it would receive the electric foot shock (30 V) through the stainless steel grid floor. In the testing trial (d2), the same test procedure was performed. The interval between the placement in the illuminated compartment and the entry into the dark compartment was recorded as memory retention. Mice were allowed to stay in the illuminated compartment for 30 s in the training trial and 120 s in the testing trial.

4.4.2. *Water maze test*

The water maze apparatus (Chinese Academy of Medical Sciences, China) consisted of a black Plexiglas rectangular tank and a transparent cover on its top. The tank included a starting point, a terminal platform and six non-exits. Near the platform was the safe region, and an invisible ladder was located for rest. The latencies of time were recorded from the starting point (the farthest non-exit to the end) to the terminal platform. The maze was filled with water to a depth of 25 cm and the temperature was kept at 20±1 °C. At training trial on d1, each mouse stayed on the terminal platform 30 s for

recognizing the location then placed at the starting point of the box facing the wall, and the latency to find the ladder was recorded up to 3 min. If the mouse did not find the ladder within 3 min, the swimming time was assigned as 3 min, and the mouse was manually led to ladder, left for 30 s and returned to its home cage. The testing trials were carried out 2 h after the training trial (d1), and on days 2 and 3 as well. The latency to reach the ladder from the starting point (swimming time) was recorded.

4.5. *Biochemistry analysis*

Mice were killed by decapitation. The cerebra were dissected and kept at −70 °C ready for use. Ten percent (1:10, w/v) cerebral homogenate in cold saline was prepared (10,000 rpm, 10 s for twice with 30 s interval) in ice bath.

The malondialdehyde (MDA) level was assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously (Buege and Aust, 1978). Glutathione (GSH) was determined by the spectrophotometric method based on the use of Ellman's reagent (Beutler et al., 1975). The choline acetyltransferase (ChAT) activity was determined spectrophotometrically according to Wolfgram (1972). The reaction mixture contained sodium phosphate buffer (pH 7.0), acetyl coenzyme A, chloride choline, physostigmine, NaCl, EDTA, hydrochloric creatinine and DTT. The mixture was preincubated at 37 °C for 5 min then mixed with the cerebral cortex homogenates, incubated at 37 °C for 20 min and finally stopped the reaction in boiling water. Sodium arsenate was added to each tube for precipitation. The supernatant was mixed with 3 nM 4-PDS and incubated at 25 °C for 15 min. Absorbances were read at 324 nm.

Acetylcholine (ACh) level was determined by using the method of Hestrin (Vincent et al., 1958). Briefly, the cerebral cortex homogenates were mixed with physostigmine and trichloroacetic acid. After centrifugation, the supernatant was added to basic hydroxylamine. The mixture was incubated and added HCl and $FeCl_3$. Absorbances were read at 540 nm. The acetylcholinesterase (AChE) activity was assayed by spectrophotometric method (Zhang et al., 2000), using acetylthiocholine iodide (ATCh-I) as substrate. The rate of hydrolysis was measured at the extent of light absorption (wavelength 412 nm). All reactions ran at room temperature in the presence of a butyrylcholinesterase inhibitor (ethopropazine, 10^{-4} M). Protein concentration was determined according to Lowry et al. (1951).

4.6. *DNA fragmentation*

DNA fragmentation was analyzed by electrophoresis. The total DNA was extracted from cerebra homogenate by the method described (Sun et al., 2000). Electrophoresis was performed on a 2% agarose gel at 60 V for 1 h. At the end of electrophoresis, the gel was stained in ethidium bromide (EB) 0.5 mg/L for 30 min and photographed using ultraviolet gel documentation.

4.7. *Histological analysis*

After decapitation of mice, the cerebra were dissected and placed in 10% (vol/vol) formalin solution and processed routinely by embedding in paraffin. Tissue sections (10 μm) were stained with hematoxylin and eosin (HE). An experienced histologist who was unaware of the treatment conditions made histological assessments.

4.8. *Statistical analysis*

Data were expressed as mean ± SEM. The step through tests were evaluated for statistical significance with non-parametric ANOVA followed by Duncan's multiple range tests, and the water maze tests were analyzed using repeated measure ANOVA.

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