

# Neuroprotective effects of N-acetyl cysteine on primary hippocampus neurons against hydrogen peroxide-induced injury are mediated via inhibition of mitogen-activated protein kinases signal transduction and antioxidative action

WEI WU<sup>1\*</sup>, BAO-HUA LIU<sup>1\*</sup>, CHENG-LONG XIE<sup>2</sup>, XIAO-DONG XIA<sup>3</sup> and YAN-MEI ZHANG<sup>4</sup>

<sup>1</sup>Department of Brain Center, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325027; <sup>2</sup>Department of Neurology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000; <sup>3</sup>Department of Respiratory Medicine, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325027; <sup>4</sup>Department of Neurology, Inner Mongolia People's Hospital, Hohhot, Inner Mongolia 010017, P.R. China

Received July 10, 2016; Accepted May 11, 2017

DOI: 10.3892/mmr.2018.8699

**Abstract.** N-acetyl cysteine (NAC) has been extensively reported to exert neuroprotective effects on the central nervous system. Oxidative stress may contribute to the underlying mechanisms causing Alzheimer's disease (AD). The effect of NAC against oxidative stress injury was investigated in a cellular model of AD in the present study and the underlying mechanisms were revealed. The neuroprotective action of NAC (1, 10, 100 and 1,000  $\mu\text{mol/l}$ ) on a cellular model of AD [hydrogen peroxide ( $\text{H}_2\text{O}_2$ )-induced (3, 30 and 300  $\mu\text{mol/l}$ ) toxicity in primary rat hippocampus neurons] demonstrated the underlying mechanisms. Cytotoxicity was measured using the MTT assay, and light microscopy and the dichloro-dihydro-fluorescein diacetate method were used to detect the reactive oxygen species (ROS) levels. Furthermore, the levels of mitogen-activated protein kinases (MAPKs) signal transduction and tau protein phosphorylation were measured via western blotting. NAC (100  $\mu\text{mol/l}$ ) protected hippocampus neurons against  $\text{H}_2\text{O}_2$ -mediated toxicity, as evidenced by enhanced cell viability. Using MTT assay and light microscopy for the observation of cell death, NAC ameliorated cell viability, which was induced by  $\text{H}_2\text{O}_2$  injury ( $P<0.05$ ). NAC was found to mitigate the excessive production of ROS ( $P<0.05$ ). Another mechanism involved in the

neuroprotective action of NAC may be its ability to inhibit MAPK signal transduction following  $\text{H}_2\text{O}_2$  exposure. In addition, NAC may protect cells against  $\text{H}_2\text{O}_2$ -induced toxicity by attenuating increased tau phosphorylation. Thus, the protective ability of NAC is hypothesized to result from inhibition of oxidative stress and downregulation of MAPK signal transduction and tau phosphorylation.

## Introduction

The global prevalence of Alzheimer's disease (AD), which is characterized by progressive deterioration in cognition and behavior, particularly memory loss, places a considerable burden on society (1). The neuropathological hallmarks of AD include extracellular senile plaques composed of  $\beta$ -amyloid ( $\text{A}\beta$ ) deposits, intracellular neurofibrillary tangles and cerebral atrophy (2). Pharmacological treatment of AD currently primarily focus on cholinesterase inhibitors and N-methyl-D-aspartic acid receptor antagonists. Unfortunately, according to previous studies, treatment with these two classes predominantly provided symptomatic benefits without counteracting the progression of the disease (3). Therefore, investigating compounds that target the underlying mechanisms of disease is of utmost importance for the development of novel therapeutic agents against AD (4).

The pathology of AD is complex and multifactorial; aggregated  $\text{A}\beta$  elicits neurotoxicity and induces oxidative-stress and inflammation in the brain of patients with AD (5). Emerging evidence has indicated that oxidative stress is important in the mechanisms associated with  $\text{A}\beta$ -induced neurotoxicity and cell loss, and is proposed as one of the basic mechanisms that contributes to the process of AD (6). In this regard, various studies have focused on the use of antioxidants for the management of AD (7). Additionally, A previous study demonstrated that increased p38 mitogen-activated protein kinase (MAPK) activity was associated with the neuropathology of AD. For example, p38 MAPK and its upstream

*Correspondence to:* Dr Yan-Mei Zhang, Department of Neurology, Inner Mongolia People's Hospital, 20 Zhaowuda Road, Hohhot, Inner Mongolia 010017, P.R. China  
E-mail: zhangyanmei2277@sina.com

\*Contributed equally

**Key words:** hydrogen peroxide, Alzheimer's disease, oxidative stress

kinase mitogen-activated protein kinase kinase 6 (MKK6) were activated in AD brain tissue samples, as demonstrated by immunohistochemistry (8). Activation of p38 MAPK signaling was also reported in AD-relevant animal models (9). Furthermore, c-Jun N-terminal kinase (JNK) MAPK activation was localized to amyloid deposits in AD models and this activation was coincident with the age-dependent increase in amyloid deposition, tau phosphorylation, and loss of synaptophysin (10).

In previous years, N-acetyl cysteine (NAC) had been extensively reported to exert neuroprotective effects on the central nervous system and may be effective against neurological conditions by rescuing severely compromised cells from an unremitting burden of oxidative stress (11). However, the protective effects of NAC on oxidative stress-induced cell death and the underlying mechanisms are unclear in primary hippocampus neurons. Therefore, in the present study, the protective effect of NAC against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-mediated damage of hippocampus neurons was investigated by measuring the cellular viability and reactive oxygen species (ROS) levels. Furthermore, the mechanisms underlying these neuroprotective effects were investigated by targeting MAPK signal transduction.

## Materials and methods

**Approval.** All experimental protocols were reviewed and approved by the Ethical Committee of Wenzhou Medical University (Wenzhou, China).

**Primary rat hippocampus neurons culture and treatments.** Primary cultures of hippocampus neurons were obtained and cultured according to previously described protocol (12). Briefly, primary rat hippocampus samples were prepared from Sprague-Dawley rat brains at embryonic days 1-3, which were purchased from the Experimental Animal Center of China Medical University (Beijing, China) and were dissected in calcium- and magnesium-free Hank's balanced salt solution (Beyotime Institute of Biotechnology, Haimen, China), following incubation with a 0.25% trypsin solution for 30 min at 36°C in order to obtain primary hippocampus neuron cells. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/high glucose, horse serum containing 10% fetal bovine serum, 1% L-glutamine (3.6 mM), and 1% penicillin antibiotics and were grown in a 5% CO<sub>2</sub> atmosphere at 37°C. The primary rat hippocampus cells were cultured on plates which were coated in the fetal bovine serum (Beyotime Institute of Biotechnology) and were cultured at 37°C in humidified air. Two-thirds of the growing medium was changed every 2-3 days and the cells were subcultured roughly once a week. On day 12 of culturing, the incubation media were replaced with media with H<sub>2</sub>O<sub>2</sub> (3, 30 and 300  $\mu$ mol/l) to achieve oxidative stress injury. Following incubation for 30 min, NAC was added to the media at concentrations of 1, 10, 100 or 1,000  $\mu$ mol/l.

**Measurement of cytotoxicity by MTT assay and light microscopy.** Cell viability was measured using the MTT assay (Beyotime Institute of Biotechnology), which is based on the conversion of MTT to formazan crystals by mitochondrial

dehydrogenases (13). MTT is absorbed by viable cells and then converted to formazan by the enzyme, succinate dehydrogenase in the mitochondria. The quantity of produced formazan thus correlates with the number of living cells. Cells were seeded in 24-well polystyrene plates with  $\sim 3 \times 10^3$  cells per well. Plates were incubated at 37°C for 24 h to allow the cells to attach. After treatment with H<sub>2</sub>O<sub>2</sub> for 0.5 h at 37°C followed by NAC for 24 h, the same volume of medium was added to the control cultures. Cell viability was determined using an MTT toxicity assay by adding 10  $\mu$ l of 5 mg/ml MTT to each well. After 4 h of incubation at 37°C in humid air, formazan crystals were solubilized in 200  $\mu$ l dimethyl sulfoxide. The optical density was measured at a wavelength of 570 nm with background correction at 655 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The mean averages of optical density from six replicate wells were used for each experimental sample and the control sample. Cell viability was calculated with a reference to the absorbance of control wells not challenged with H<sub>2</sub>O<sub>2</sub> (assumed as 100% protection). Analyzed by light microscopy, viable cells displayed normal nuclear size and dark brown granules, whereas toxic cells exhibited condensed chromatin. The number of residual viable cells was counted.

**Measurement of glutathione (GSH) and lipid peroxide.** To assess the enzymatic activity of GSH-peroxidase and lipid peroxide in primary hippocampus neuron culture after H<sub>2</sub>O<sub>2</sub> injury, the cultures were washed with ice-cold phosphate-buffered saline (PBS) and then pooled and homogenized in 0.1 mol/l PBS containing 0.05 mmol/l ethylenediaminetetraacetic acid according to previous protocol (14). GSH-peroxidase activity was assessed using a GSH assay kit (Beyotime Institute of Biotechnology) by quantifying the rate of oxidation of reduced GSH to oxidized GSH. To investigate the effect of NAC on anti-oxidative stress in the AD cell model, the biomarkers of oxidative stress, including GSH and GSH disulfide (GSSG) were assessed. The level of GSH activity by the means of GSH/GSSG ratio. GSSG was obtained by determining the absorbance of 5-thio-2-nitrobenzoic acid produced from the reaction of the reduced GSH with DTNB. The level of maleic dialdehyde (MDA), a product of lipid peroxidation, was measured using an MDA assay kit (Beyotime Institute of Biotechnology) based on the thiobarbituric acid method (15).

**Determination of intracellular ROS by dichloro-dihydro-fluorescein diacetate (DCFH-DA).** The fluorescent probe DCFH-DA was used to monitor intracellular accumulation of ROS (16). Hippocampus neurons were seeded in collagen-coated 24-well plates at a density of  $4 \times 10^5$  cells/ml and incubated for 72 h. Cells were incubated with 300  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub>, a mixture of 1, 10 or 100  $\mu$ mol/l NAC, or 300  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub> alone at 37°C for 9 h. The cells were collected and washed with PBS three times. DCFH-DA was diluted in fresh DMEM to a final concentration of 5  $\mu$ M and incubated with the cells for 30 min at 37°C. The chemicals were then removed and the cells were washed three times with PBS. Fluorescence emission was measured at excitation and emission wavelengths of 485 and 520 nm, respectively using fluorescence microplate. ROS production was expressed as a percentage of the control sample.

**Western blot analysis.** The primary rat hippocampus neurons were homogenized in protein extraction solution comprised of 20 mM Tris-HCl (pH 7.4), containing 1 mM NaF, 150 mM NaCl, 1% Triton X-100 and freshly-added protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and 100  $\mu$ M phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology). The supernatant contained total and membrane-enriched proteins. The Bicinchoninic Acid protein determination method was employed for the concentration of the proteins. Then, the proteins (30-50  $\mu$ g) were separated by 8-12% sodium dodecyl polyacrylamide gels at 80 V for 50 min followed by 120 V for 40 min and electrophoretically transferred to a polyvinylidene fluoride membrane (PVDF) at 300 mA; the duration of electrophoresis depended on the molecular weight of the proteins. The PVDF membrane was blocked with freshly prepared Tris-buffered saline with Tween-20 (0.1%) containing 5% non-fat dry milk for 30-60 min at room temperature with constant agitation. Subsequently, the membrane was incubated with polyclonal rabbit anti-phospho-p38 immunoglobulin G (IgG; 1:1,000; cat. no. 2729, Cell Signaling Technology, Inc., Danvers, MA, USA), monoclonal rabbit anti-phospho-JNK IgG (1:1,000; cat. no. 4671, Cell Signaling Technology, Inc.), polyclonal rabbit anti-phospho-extracellular regulated kinase (ERK; 1:1,000; cat. no. 4370, Cell Signaling Technology, Inc.), polyclonal mouse anti-phospho-tau IgG (1:100; cat. no. 9632, Cell Signaling Technology, Inc.), monoclonal rabbit anti-p38 IgG (1:1,000; cat. no. 8690, Cell Signaling Technology, Inc.), polyclonal rabbit anti-JNK IgG (1:1,000; cat. no. 5136, Cell Signaling Technology, Inc.), polyclonal rabbit anti-ERK IgG (1:1,000; cat. no. 8544, Cell Signaling Technology, Inc.), polyclonal rabbit anti-tau IgG (1:500; cat. no. T7951, Sigma-Aldrich; Merck KGaA) and monoclonal mouse anti- $\beta$ -actin IgG (1:1,000; cat. no. AA128, Beyotime Institute of Biotechnology) overnight at 4°C. The membrane was then incubated with anti-rabbit or anti-mouse horseradish peroxidase IgGs (1:1,000; A0208 or A0216, respectively, Beyotime Institute of Biotechnology) for 1-2 h at room temperature. Immunoreactive bands were visualized using an Enhanced Chemiluminescent Western Blotting Substrate (cat. no. 32106, Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and quantified using Quantity One software 3.0 (Image Lab, Bio-Rad Laboratories, Inc.).

**Statistical analysis.** Data were expressed as the mean  $\pm$  standard deviation. Comparisons between different groups were performed by one-way analysis of variance followed by least significant difference post-hoc comparisons when appropriate.  $P < 0.05$  was considered to indicate a statistically significant difference. All analyses were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA).

## Results

**Effects of NAC on cell viability in  $H_2O_2$ -induced primary hippocampus neuron injury.** The viabilities of primary hippocampus neurons exposed to different concentrations of  $H_2O_2$  (3, 30 or 300  $\mu$ mol/l) were detected after 24 h of  $H_2O_2$  incubation. The  $H_2O_2$  reduced cell viabilities in a dose-dependent manner ( $P < 0.05$  vs. control group; Fig. 1A). The survival rate of the hippocampus neurons was  $\sim 78\%$  when the neurons were

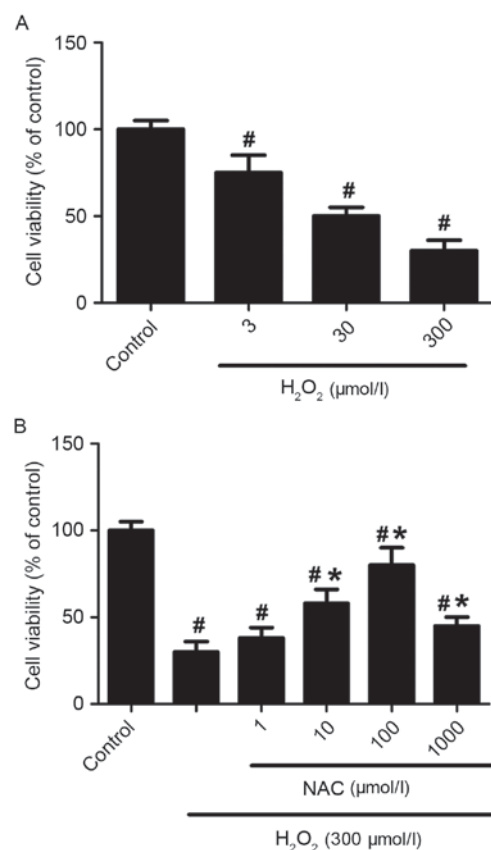


Figure 1. NAC treatment ameliorated  $H_2O_2$ -induced cell death in hippocampus neurons. (A)  $H_2O_2$  (3, 30 and 300  $\mu$ mol/l) concentration induced cell death in a dose-dependent manner. (B) NAC attenuated  $H_2O_2$ -induced cell death when compared with  $H_2O_2$  alone. Cell viability was assessed by MTT analysis. Data are expressed as percentage of survived neurons in relation to the control treatment. # $P < 0.05$  vs. control culture; \* $P < 0.05$  vs. 300  $\mu$ mol/l  $H_2O_2$  alone. NAC, N-acetyl cysteine;  $H_2O_2$ , hydrogen peroxide.

incubated with 3  $\mu$ mol/l of  $H_2O_2$  for 24 h. However, the survival rate of neurons reduced to  $\sim 31\%$  when treated with 300  $\mu$ mol/l of  $H_2O_2$  ( $P < 0.01$  vs. control group; Fig. 1A). Exposure of cells to NAC (1, 10, 100 or 1,000  $\mu$ mol/l) significantly improved cell viability ( $P < 0.05$  vs. control group; Fig. 1B), although treatment with 1  $\mu$ mol/l NAC was not significantly different when compared with  $H_2O_2$  alone ( $P > 0.05$ ; Fig. 1B). In addition, 100  $\mu$ mol/l NAC almost completely saved neurons from 300  $\mu$ mol/l  $H_2O_2$ -induced cell deaths (82% survival rate compared with the control group). Analysis under a light microscope demonstrated that  $H_2O_2$ -induced neuron death in 50% of cells and significantly reduced neurite length. The pretreatment of cells with 100  $\mu$ mol/l NAC tended to overcome these detrimental effects of  $H_2O_2$  incubation ( $P < 0.01$ ; Fig. 2). Treatment with 100  $\mu$ mol/l NAC significantly reduced  $H_2O_2$ -induced cell death ( $P < 0.05$ ; Fig. 2), indicating that NAC treatment elicited a potent protection effect on  $H_2O_2$ -induced cell viability.

**Effects of NAC on MDA and GSH activity.** In the present study, the MDA level as a measure of lipid peroxidation were significantly increased in the  $H_2O_2$  group (300  $\mu$ mol/l) compared with the control group ( $P < 0.05$ ; Fig. 3A). The MDA levels were significantly reduced in the NAC-low (L; 10  $\mu$ mol/l, low-concentration of NAC) and NAC-H (100  $\mu$ mol/l, high



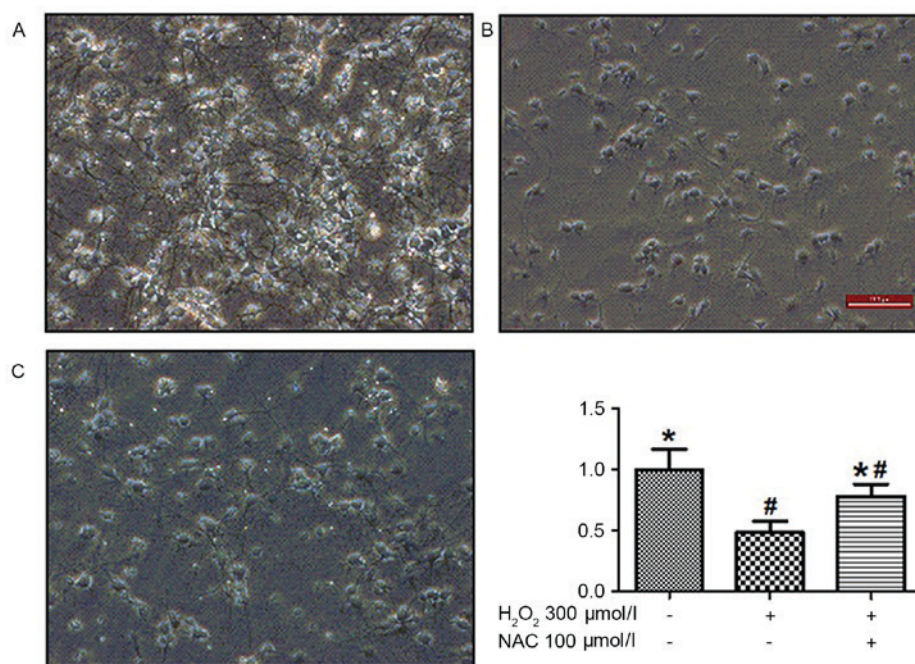


Figure 2. NAC protected primary hippocampus neurons against H<sub>2</sub>O<sub>2</sub>-induced cell death. Cells were treated with either H<sub>2</sub>O<sub>2</sub> or NAC. Residual viable cells were then observed and counted (magnification, x100). (A) Control hippocampus neurons; (B) 300 μmol/l H<sub>2</sub>O<sub>2</sub> exposure for 24 h; (C) 300 μmol/l H<sub>2</sub>O<sub>2</sub> + 100 μmol/l NAC for 24 h. The bar graph summarizes the number of residual viable neurons from three independent experiments, expressed as means ± standard deviation. \*P<0.05 vs. control culture; #P<0.05 vs. 300 μmol/l H<sub>2</sub>O<sub>2</sub> incubation alone group. NAC, N-acetyl cysteine; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

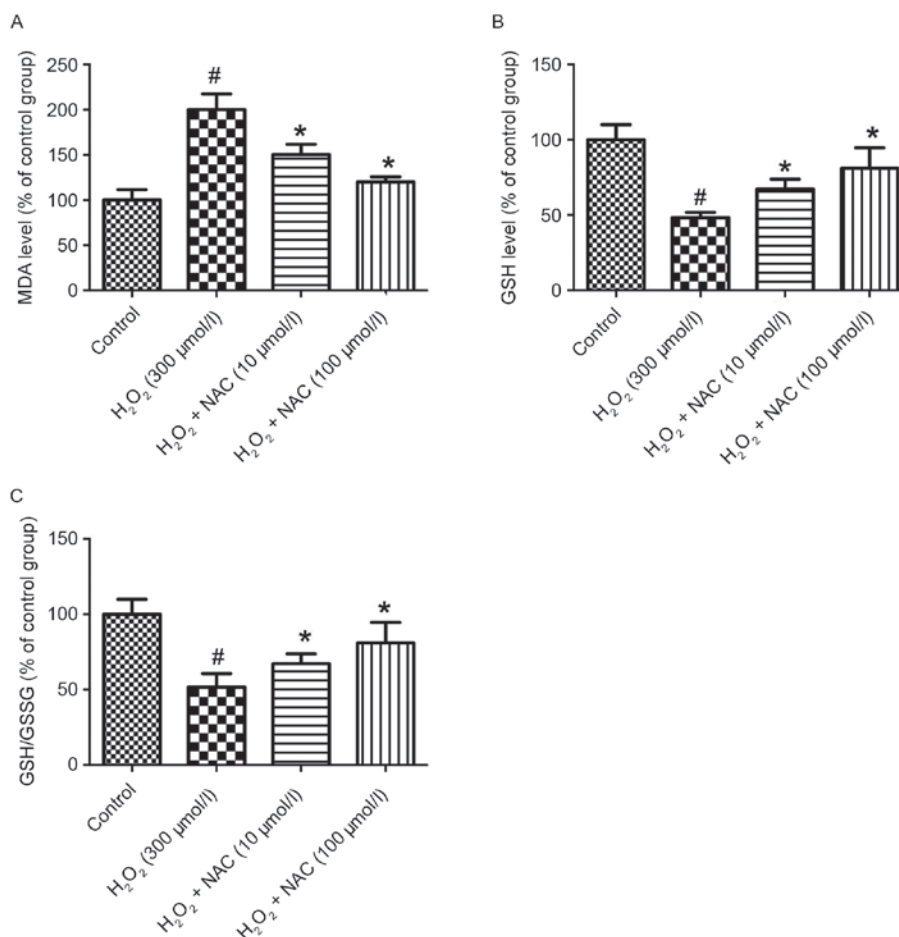


Figure 3. Effects of α-lipoic acid on MDA and GSH levels. (A) MDA levels as a measure of lipid peroxidation; (B) GSH levels in neurons; (C) GSH/GSSG levels in neurons. Data are presented as means ± standard error of the mean. \*P<0.05 vs. control group; #P<0.05 vs. 300 μmol/l H<sub>2</sub>O<sub>2</sub> group (n=4 per group; one-way analysis of variance followed by least significant difference post-hoc analysis). NAC, N-acetyl cysteine; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; MDA, maleic dialdehyde; GSH, glutathione; GSSG, GSH disulfide.

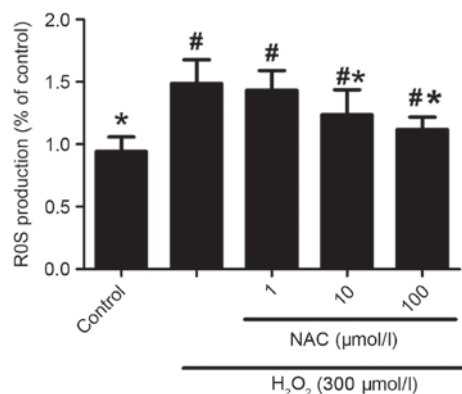


Figure 4. Treatment with N-acetyl cysteine reduced the rate of intracellular ROS accumulation in hippocampus neurons exposed to H<sub>2</sub>O<sub>2</sub>. The fluorescence density was determined at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. ROS production was expressed as a percentage of the control. Results are presented as means  $\pm$  standard deviation. \*P<0.05 vs. controls; #P<0.05 vs. 300  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub> alone group. ROS, reactive oxygen species; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

concentration of NAC) treatment groups vs. the H<sub>2</sub>O<sub>2</sub> group (P<0.05; Fig. 3A). Similarly, no significant difference between the NAC-L and NAC-H groups was identified with regard to reducing the MDA level (P>0.05; Fig. 3A). To investigate the effect of NAC on anti-oxidative stress in the AD cell model, the biomarkers of oxidative stress, including GSH and GSH disulfide (GSSG) were assessed. The level of GSH activity by the aid of GSH/GSSG in the H<sub>2</sub>O<sub>2</sub> group (300  $\mu$ mol/l) was significantly decreased compared with the control group (P<0.05; Fig. 3B and C). Additionally, treatment with NAC significantly alleviated GSH activity compared with the H<sub>2</sub>O<sub>2</sub> group (P<0.05; Fig. 3B and C). Furthermore, the NAC-H group demonstrated significantly increased GSH activity compared with the rats receiving NAC-L (P<0.05; Fig. 3B and C).

**NAC ameliorates H<sub>2</sub>O<sub>2</sub>-induced cell impairment by decreasing ROS production.** Oxidative stress is crucial in the pathogenesis of AD. In the current study, by using ROS fluorescent dye, DCFH-DA, the results demonstrated that intracellular DCF fluorescence was significantly increased in the presence of 300  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub> compared with the control sample, which was abolished by treatment with 10 and 100  $\mu$ mol/l NAC (P<0.05; Fig. 4), but not 1  $\mu$ mol/l NAC (P>0.05; Fig. 4). Taken together, these data indicate that the neuroprotective effects of NAC against H<sub>2</sub>O<sub>2</sub>-induced neurotoxicity involve limiting oxidative stress injury.

**NAC ameliorates H<sub>2</sub>O<sub>2</sub>-induced injury by inhibition of MAPK signal transduction.** Enhanced levels of phosphorylated (p)-p38 were detected in the presence of 300  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub> injury (P<0.05 vs. control; Fig. 5A), while treatment with 100  $\mu$ mol/l NAC caused the decline of p-p38 levels (P<0.05 vs. H<sub>2</sub>O<sub>2</sub> incubation alone; Fig. 5A). Thus, the protective effects of NAC involved the attenuation of p38 protein phosphorylation. Furthermore, the levels of JNK protein phosphorylation were analyzed in primary hippocampus neurons in the absence or presence of NAC. p-JNK levels were significantly increased in cells exposed

to H<sub>2</sub>O<sub>2</sub> (P<0.05 vs. control; Fig. 5B) and significantly decreased following the addition of 100  $\mu$ mol/l NAC to the culture (P<0.05 vs. H<sub>2</sub>O<sub>2</sub> alone; Fig. 5B). Similarly, p-ERK expression levels were increased in H<sub>2</sub>O<sub>2</sub>-induced hippocampus neurons (P<0.05 vs. control; Fig. 5C), indicating that ERK activity had increased. In addition, NAC (100  $\mu$ mol/l) significantly reduced the induction of p-ERK following H<sub>2</sub>O<sub>2</sub> incubation when compared with the control group (P<0.05; Fig. 5C).

**NAC decreased tau phosphorylation induced by H<sub>2</sub>O<sub>2</sub> injury.**

In the present study, western blot analyses demonstrated that an increased level of p-tau was observed in the presence of 300  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub> compared with control hippocampus neurons (P<0.05; Fig. 5D), while the expression levels of p-tau were decreased in the NAC (100  $\mu$ mol/l) treatment group compared with the H<sub>2</sub>O<sub>2</sub> alone group (P<0.05; Fig. 5D). This clearly indicated that H<sub>2</sub>O<sub>2</sub> resulted in tau phosphorylation in the hippocampus neurons and that NAC ameliorates the levels of p-tau.

## Discussion

In the present study, NAC was demonstrated to protect differentiated primary rat hippocampus neurons against H<sub>2</sub>O<sub>2</sub>-mediated toxicity as evidenced by enhanced cell viability. While H<sub>2</sub>O<sub>2</sub> (300  $\mu$ mol/l) markedly decreased cell viability, exposure of cells to NAC (100  $\mu$ mol/l) overcame the negative effect of oxidative stress on cell survival, and increased it by ~3-fold when compared with the intact control cells. The results demonstrated that treatment with NAC reduced the percentage of cell death that was observed following incubation with H<sub>2</sub>O<sub>2</sub>. Using MTT assay and light microscopy for the observation of cell viability, NAC appeared to ameliorate cell death events induced by H<sub>2</sub>O<sub>2</sub> injury. H<sub>2</sub>O<sub>2</sub>-induced cells displayed decreases in neurite number and their length; NAC treatment was demonstrated to restore the number of neurites and significantly augment their length (Fig. 2). In addition, NAC was observed to mitigate the excessive production of ROS, indicating that the neuroprotective effects of the compounds in this AD-like cellular model were probably associated with inhibition of H<sub>2</sub>O<sub>2</sub>-induced oxidative stress injury. Furthermore, NAC reduced H<sub>2</sub>O<sub>2</sub>-induced MDA over-expression and upregulated the level of GSH. In the current study, another mechanism underlying the neuroprotective action of NAC likely includes its ability to inhibit MAPK signal transduction following H<sub>2</sub>O<sub>2</sub> exposure. In addition, the present study demonstrated for the first time, to the best of our knowledge, that NAC protects cells against H<sub>2</sub>O<sub>2</sub>-mediated toxicity by attenuating the increase in tau phosphorylation. These results indicate that NAC may serve as a neuroprotective agent for H<sub>2</sub>O<sub>2</sub>-associated injury.

Emerging evidence has suggested that oxidative stress damage is closely associated with neurodegeneration, including AD (6). Although whether oxidative stress is involved in the onset of AD remains unclear, oxidative stress is pivotal in disease progression, particularly in cellular and tissue damage (17). During the process of the oxidative stress reaction, A $\beta$  passes through the neuronal membrane, resulting

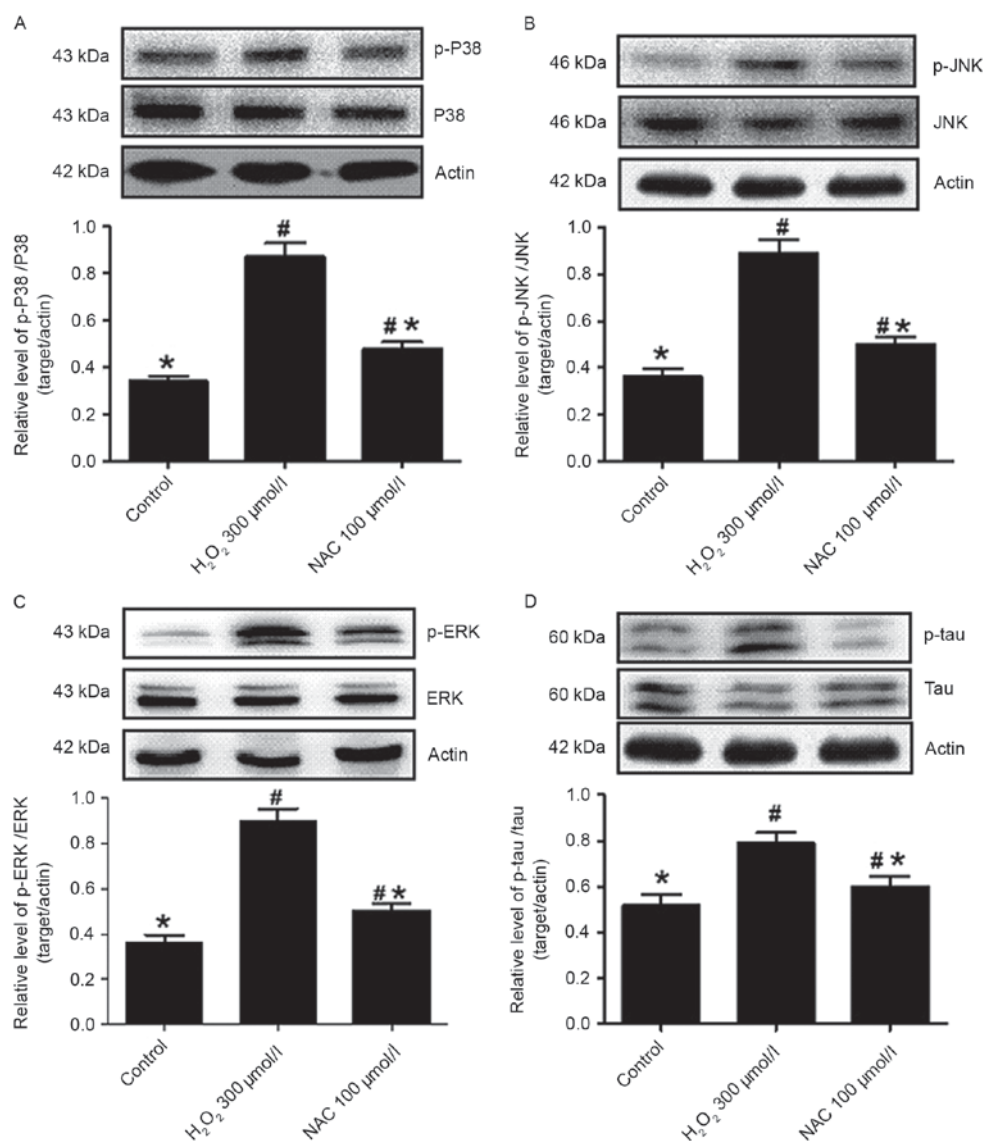


Figure 5. NAC attenuated H<sub>2</sub>O<sub>2</sub>-induced activation of p38, JNK, ERK and tau protein phosphorylation in primary hippocampus neurons. (A) p-p38 level expressed relative to the actin level; (B) p-JNK level expressed relative to actin level; (C) p-ERK level expressed relative to actin level; (D) p-tau level expressed relative to the actin level. The western blots are representative of three independent experiments with similar results. The bar graphs represent the quantification of immunoblot band densities, expressed as a percentage of actin, and data are presented as means  $\pm$  standard deviation. #P<0.05 vs. the control group; \*P<0.05 vs. 300  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub> alone group. NAC, N-acetyl cysteine; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; JNK, c-Jun N-terminal kinase; ERK, extracellular regulated kinase; p, phosphorylated.

in the overproduction of ROS, which may destroy various classes of biological molecules, such as lipids, proteins and DNA (18). Therefore, the ROS levels were used to evaluate the extent of oxidative stress damage. In the present study, NAC markedly reduced the excessive production of ROS levels in a dose-dependent manner (10 and 100  $\mu$ mol/l). These results indicate that the neuroprotective effects of NAC in this cellular model may be associated with antioxidant properties. In addition, the data were in accordance with previous findings, which reported the neuroprotective action of NAC in various *in vivo* and *in vitro* studies (11).

The mammalian family of MAPKs include ERK, p38, and JNK, with each MAPK signaling pathway consisting of at least three components (19). These signaling pathways regulate a variety of cellular activities, including cell proliferation, differentiation, survival and death. The activated

MAPK signaling pathways are proposed to contribute to AD pathogenesis via various mechanisms, including induction of neuronal death (20), and transcriptional and enzymatic activation of  $\beta$ - and  $\gamma$ -secretases (21). In addition, under conditions of oxidative stress, JNK and p38 are activated and induce the expression of the  $\beta$ -secretase gene, suggesting a pivotal role in cell viability in AD (22). Meanwhile,  $\gamma$ -secretase activity was found to be blocked by a JNK inhibitor, thus implicating the JNK signaling pathway in the regulation of  $\gamma$ -secretase activity. Furthermore, a previous study demonstrated that the detrimental effects of ERK resulted from promoting oxidative stress (23). In the current study, NAC was shown to protect cells against H<sub>2</sub>O<sub>2</sub>-induced toxicity by attenuating the increased levels of p38, JNK and ERK phosphorylation. These results indicated that inhibition of MAPK signal transduction by NAC was crucial in the survival against oxidative stress in



primary rat hippocampus neurons. It is well known that extensively phosphorylated tau protein forms pathologic inclusions, containing fibrillar aggregates, and are present in AD (24). Tau is proposed as one of the microtubule stabilizing proteins exerting a crucial role in the facilitation of tubulin assembly into microtubules, thus contributing to maintenance of normal cellular morphology (1). Abnormally hyperphosphorylated-tau possesses lower affinity for microtubules, which promotes cytoskeleton rearrangements with consequent impairments of axonal transport and intracellular trafficking (25). Results from the present study indicated that abrogation of tau hyperphosphorylation by 100  $\mu$ mol/l NAC may eventually contribute to restoration and even improvement of cell morphology.

From previous studies, NAC maintained intracellular GSH levels and may be beneficial for a range of neuronal cell types against various oxidative stress stimuli *in vitro* (26). In addition, Hart *et al* (27) indicated that NAC may reduce neuronal death by blocking attempted entry into the cell cycle, by improving free radical surveillance and scavenging ROS levels, or by preserving mitochondrial function, regenerating endogenous antioxidants and repairing oxidative damage (27). Recently, Adair *et al* (28) performed a controlled clinical trial where NAC or placebo was administered in a double-blind fashion to patients with probable AD. The authors observed that NAC exerted a positive effect on nearly every outcome measure, although significant differences were obtained only for a subset of cognitive tasks (28). In the current study, the findings indicated that NAC attenuated H<sub>2</sub>O<sub>2</sub>-induced injury by inhibition of MAPK signal transduction and antioxidative action.

In conclusion, NAC exerted a neuroprotective effect against H<sub>2</sub>O<sub>2</sub>-induced toxicity in primary hippocampus neurons. The protective ability of NAC most likely results from inhibition of oxidative stress and from reducing cell death. Another potential mechanism by which this compound protects cells from oxidative stress toxicity may be associated with the downregulation of MAPK signal transduction and tau phosphorylation.

### Acknowledgements

Not applicable.

### Funding

The current study was supported by the Projects of Wenzhou City Committee of Science and Technology (Wenzhou, China; Y20100146).

### Availability of data and materials

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

### Authors' contributions

WW and YMZ conceived and designed the experiment. CLX and XDX performed the experiments and acquisition of data. BHL and YMZ analyzed and interpreted the data. YMZ wrote the article. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

All experimental protocols were reviewed and approved by the Ethical Committee of Wenzhou Medical University (Wenzhou, China).

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### References

1. Giacobini E and Gold G: Alzheimer disease therapy-moving from amyloid- $\beta$  to tau. *Nat Rev Neurol* 9: 677-686, 2013.
2. Steel K: Alzheimer's disease. *N Engl J Med* 362: 1844-1845, 2010.
3. Schneider LS, Dagerman KS, Higgins JP and McShane R: Lack of evidence for the efficacy of memantine in mild Alzheimer disease. *Arch Neurol* 68: 991-998, 2011.
4. Lang AE: Clinical trials of disease-modifying therapies for neurodegenerative diseases: The challenges and the future. *Nat Med* 16: 1223-1226, 2010.
5. Pimplikar SW: Reassessing the amyloid cascade hypothesis of Alzheimer's disease. *Int J Biochem Cell Biol* 41: 1261-1268, 2009.
6. Butterfield DA, Swomley AM and Sultana R: Amyloid  $\beta$ -peptide (1-42)-induced oxidative stress in Alzheimer disease: Importance in disease pathogenesis and progression. *Antioxid Redox Signal* 19: 823-835, 2013.
7. Tavakkoli M, Miri R, Jassbi AR, Erfani N, Asadollahi M, Ghasemi M, Saso L and Firuzi O: Carthamus, Salvia and Stachys species protect neuronal cells against oxidative stress-induced apoptosis. *Pharm Biol* 52: 1550-1557, 2014.
8. Ando K, Uemura K, Kuzuya A, Maesako M, Asada-Utsugi M, Kubota M, Aoyagi N, Yoshioka K, Okawa K, Inoue H, *et al*: N-cadherin regulates p38 MAPK signaling via association with JNK-associated leucine zipper protein: Implications for neurodegeneration in Alzheimer disease. *J Biol Chem* 286: 7619-7628, 2011.
9. Savage MJ, Lin YG, Ciallella JR, Flood DG and Scott RW: Activation of c-Jun N-terminal kinase and p38 in an Alzheimer's disease model is associated with amyloid deposition. *J Neurosci* 22: 3376-3385, 2002.
10. Ferrer I, Gomez-Isla T, Puig B, Freixes M, Ribé E, Dalfó E and Avila J: Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. *Curr Alzheimer Res* 2: 3-18, 2005.
11. Unnithan AS, Jiang Y, Rumble JL, Pulugulla SH, Posimo JM, Gleixner AM and Leak RK: N-acetyl cysteine prevents synergistic, severe toxicity from two hits of oxidative stress. *Neurosci Lett* 560: 71-76, 2014.
12. Vedunova MV, Mitroshina EV, Sakharnova TA, Bobrov MY, Bezuglov VV, Khaspekov LG and Mukhina IV: Effect of N-arachidonoyl dopamine on activity of neuronal network in primary hippocampus culture upon hypoxia modelling. *Bull Exp Biol Med* 156: 461-464, 2014.
13. Garrido J, Gaspar A, Garrido EM, Miri R, Tavakkoli M, Pourali S, Saso L, Borges F and Firuzi O: Alkyl esters of hydroxycinnamic acids with improved antioxidant activity and lipophilicity protect PC12 cells against oxidative stress. *Biochimie* 94: 961-967, 2012.
14. Yu Y, Wang JR, Sun PH, Guo Y, Zhang ZJ, Jin GZ and Zhen X: Neuroprotective effects of atypical D1 receptor agonist SKF83959 are mediated via D1 receptor-dependent inhibition of glycogen synthase kinase-3  $\beta$  and a receptor-independent anti-oxidative action. *J Neurochem* 104: 946-956, 2008.
15. Mihara M and Uchiyama M: Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem* 86: 271-278, 1978.
16. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC and Thomas M: Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. *J Immunol* 130: 1910-1917, 1983.

17. Melo A, Monteiro L, Lima RM, Oliveira DM, Cerqueira MD and El-Bachá RS: Oxidative stress in neurodegenerative diseases: Mechanisms and therapeutic perspectives. *Oxid Med Cell Longev* 2011; 467180, 2011.
18. Dumont M and Beal MF: Neuroprotective strategies involving ROS in Alzheimer disease. *Free Radic Biol Med* 51: 1014-1026, 2011.
19. Raman M, Chen W and Cobb MH: Differential regulation and properties of MAPKs. *Oncogene* 26: 3100-3112, 2007.
20. Marques CA, Keil U, Bonert A, Steiner B, Haass C, Muller WE and Eckert A: Neurotoxic mechanisms caused by the Alzheimer's disease-linked Swedish amyloid precursor protein mutation: Oxidative stress, caspases, and the JNK pathway. *J Biol Chem* 278: 28294-28302, 2003.
21. Shen C, Chen Y, Liu H, Zhang K, Zhang T, Lin A and Jing N: Hydrogen peroxide promotes Abeta production through JNK-dependent activation of gamma-secretase. *J Biol Chem* 283: 17721-17730, 2008.
22. Tamagno E, Guglielmotto M, Giliberto L, Vitali A, Borghi R, Autelli R, Danni O and Tabaton M: JNK and ERK1/2 pathways have a dual opposite effect on the expression of BACE1. *Neurobiol Aging* 30: 1563-1573, 2009.
23. Sawe N, Steinberg G and Zhao H: Dual roles of the MAPK/ERK1/2 cell signaling pathway after stroke. *J Neurosci Res* 86: 1659-1669, 2008.
24. Takei Y, Teng J, Harada A and Hirokawa N: Defects in axonal elongation and neuronal migration in mice with disrupted tau and map1b genes. *J Cell Biol* 150: 989-1000, 2000.
25. Alonso AC, Zaidi T, Grundke-Iqbal I and Iqbal K: Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proc Natl Acad Sci USA* 91: 5562-5566, 1994.
26. Munoz AM, Rey P, Soto-Otero R, Guerra MJ and Labandeira-Garcia JL: Systemic administration of N-acetylcysteine protects dopaminergic neurons against 6-hydroxydopamine-induced degeneration. *J Neurosci Res* 76: 551-562, 2004.
27. Hart AM, Terenghi G, Kellerth JO and Wiberg M: Sensory neuroprotection, mitochondrial preservation, and therapeutic potential of N-acetyl-cysteine after nerve injury. *Neuroscience* 125: 91-101, 2004.
28. Adair JC, Knoefel JE and Morgan N: Controlled trial of N-acetylcysteine for patients with probable Alzheimer's disease. *Neurology* 57: 1515-1517, 2001.