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 µmol/l) on a cellular model of AD [hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-induced (3, 30 and 300 µmol/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 µmol/l) protected hippocampus neurons against H\textsubscript{2}O\textsubscript{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 H\textsubscript{2}O\textsubscript{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 H\textsubscript{2}O\textsubscript{2} exposure. In addition, NAC may protect cells against H\textsubscript{2}O\textsubscript{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 β-amyloid (Aβ) 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 Aβ 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 Aβ-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...
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 synaptofysin (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 neu- rological 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 (H₂O₂)-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% CO₂ 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 H₂O₂ (3, 30 and 300 µmol/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 µmol/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 ~3x10⁵ cells per well. Plates were incubated at 37°C for 24 h to allow the cells to attach. After treatment with H₂O₂ 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 µl 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 ml 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 H₂O₂ (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 H₂O₂ 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 4x10⁵ cells/ml and incubated for 72 h. Cells were incubated with 300 µmol/l H₂O₂, a mixture of 1, 10 or 100 µmol/l NAC, or 300 µmol/l H₂O₂ 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 µm 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 µM 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 μg) 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 rabbit anti-β-actin IgG (1:1,000; cat. no. 8690, Cell Signaling Technology, Inc.), monoclonal rabbit anti-β-actin (1:1,000; cat. no. 8690, Cell Signaling Technology, Inc.), monoclonal rabbit anti-phospho-JNK IgG (1:1,000; cat. no. 5136, Cell Signaling Technology, Inc.), polyclonal rabbit anti-JNK IgG (1:1,000; cat. no. 8544, Cell Signaling Technology, Inc.), polyclonal rabbit anti-tau IgG (1:1,000; cat. no. T7951, Sigma-Aldrich; Merck KGaA) and monoclonal mouse anti-β-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 ± 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 H₂O₂-induced primary hippocampus neuron injury. The viabilities of primary hippocampus neurons exposed to different concentrations of H₂O₂ (3, 30 or 300 µmol/l) were detected after 24 h of H₂O₂ incubation. The H₂O₂ reduced cell viabilities in a dose-dependent manner (P<0.05 vs. control group; Fig. 1A). The survival rate of the hippocampus neurons was ~78% when the neurons were incubated with 3 µmol/l of H₂O₂ for 24 h. However, the survival rate of neurons reduced to ~31% when treated with 300 µmol/l of H₂O₂ (P<0.01 vs. control group; Fig. 1A). Exposure of cells to NAC (1, 10, 100 or 1,000 µmol/l) significantly improved cell viability (P<0.05 vs. control group: Fig. 1B), although treatment with 1 µmol/l NAC was not significantly different when compared with H₂O₂ alone (P>0.05; Fig. 1B). In addition, 100 µmol/l NAC almost completely saved neurons from 300 µmol/l H₂O₂-induced cell deaths (82% survival rate compared with the control group). Analysis under a light microscope demonstrated that H₂O₂-induced neuron death in 50% of cells and significantly reduced neurite length. The pretreatment of cells with 100 µmol/l NAC tended to overcome these detrimental effects of H₂O₂ incubation (P<0.01; Fig. 2). Treatment with 100 µmol/l NAC significantly reduced H₂O₂-induced cell death (P<0.05; Fig. 2), indicating that NAC treatment elicited a potent protection effect on H₂O₂-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 H₂O₂ group (300 µmol/l) compared with the control group (P<0.05; Fig. 3A). The MDA levels were significantly reduced in the NAC-low (L; 10 µmol/l, low-concentration of NAC) and NAC-H (100 µmol/l, high...
Figure 2. NAC protected primary hippocampus neurons against $H_2O_2$-induced cell death. Cells were treated with either $H_2O_2$ or NAC. Residual viable cells were then observed and counted (magnification, x100). (A) Control hippocampus neurons; (B) 300 µmol/l $H_2O_2$ exposure for 24 h; (C) 300 µmol/l $H_2O_2$ + 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 $H_2O_2$ incubation alone group. NAC, N-acetyl cysteine; $H_2O_2$, 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 $H_2O_2$ group (n=4 per group; one-way analysis of variance followed by least significant difference post-hoc analysis). NAC, N-acetyl cysteine; $H_2O_2$, hydrogen peroxide; MDA, maleic dialdehyde; GSH, glutathione; GSSG, GSH disulfide.
concentration of NAC) treatment groups vs. the H_2O_2 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_2O_2 group (300 µmol/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_2O_2 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_2O_2-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 µmol/l H_2O_2 compared with the control sample, which was abolished by treatment with 10 and 100 µmol/l NAC (P<0.05; Fig. 4), but not 1 µmol/l NAC (P>0.05; Fig. 4). Taken together, these data indicate that the neuroprotective effects of NAC against H_2O_2-induced neurotoxicity involve limiting oxidative stress injury.

NAC ameliorates H_2O_2-induced injury by inhibition of MAPK signal transduction. Enhanced levels of phosphorylated (p)-38 were detected in the presence of 300 µmol/l H_2O_2 injury (P<0.05 vs. control; Fig. 5A), while treatment with 100 µmol/l NAC caused the decline of p-38 levels (P<0.05 vs. H_2O_2 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_2O_2 (P<0.05 vs. control; Fig. 5B) and significantly decreased following the addition of 100 µmol/l NAC to the culture (P<0.05 vs. H_2O_2 alone; Fig. 5B). Similarly, p-ERK expression levels were increased in H_2O_2-induced hippocampus neurons (P<0.05 vs. control; Fig. 5C), indicating that ERK activity had increased. In addition, NAC (100 µmol/l) significantly reduced the induction of p-ERK following H_2O_2 incubation when compared with the control group (P<0.05; Fig. 5C).

NAC decreased tau phosphorylation induced by H_2O_2 injury. In the present study, western blot analyses demonstrated that an increased level of p-tau was observed in the presence of 300 µmol/l H_2O_2 compared with control hippocampus neurons (P<0.05; Fig. 5D), while the expression levels of p-tau were decreased in the NAC (100 µmol/l) treatment group compared with the H_2O_2 alone group (P<0.05; Fig. 5D). This clearly indicated that H_2O_2 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_2O_2-mediated toxicity as evidenced by enhanced cell viability. While H_2O_2 (300 µmol/l) markedly decreased cell viability, exposure of cells to NAC (100 µmol/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_2O_2. Using MTT assay and light microscopy for the observation of cell viability, NAC appeared to ameliorate cell death events induced by H_2O_2 injury. H_2O_2-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_2O_2-induced oxidative stress injury. Furthermore, NAC reduced H_2O_2-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_2O_2 exposure. In addition, the present study demonstrated for the first time, to the best of our knowledge, that NAC protects cells against H_2O_2-mediated toxicity by attenuating the increase in tau phosphorylation. These results indicate that NAC may serve as a neuroprotective agent for H_2O_2-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, Aβ passes through the neuronal membrane, resulting...
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 µmol/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 β- and γ-secretases (21). In addition, under conditions of oxidative stress, JNK and p38 are activated and induce the expression of the β-secretase gene, suggesting a pivotal role in cell viability in AD (22). Meanwhile, γ-secretase activity was found to be blocked by a JNK inhibitor, thus implicating the JNK signaling pathway in the regulation of γ-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$_2$O$_2$-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

Figure 5. NAC attenuated H$_2$O$_2$-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 ± standard deviation. *P<0.05 vs. the control group; #P<0.05 vs. 300 µmol/l H$_2$O$_2$ alone group. NAC, N-acetyl cysteine; H$_2$O$_2$, hydrogen peroxide; JNK, c-Jun N-terminal kinase; ERK, extracellular regulated kinase; p, phosphorylated.
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 µmol/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$_2$O$_2$-induced injury by inhibition of MAPK signal transduction and antioxidative action.

In conclusion, NAC exerted a neuroprotective effect against H$_2$O$_2$-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.

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Availability of data and materials

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

Authors' contributions

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. YMY 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.

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