Dl-3-n-butylphthalide alleviates cognitive impairment in amyloid precursor protein/presenilin 1 transgenic mice by regulating the striatal-enriched protein tyrosine phosphatase/ERK/cAMP-response element-binding protein signaling pathway

YAN ZHAO[1*], WEN-QIANG YANG[1], LU YU[2], JING YANG[2], HAI-RONG ZHU[3] and LIN ZHANG[4*]

[1]Life Science Institution, Jinzhou Medical University; [2]Provincial Key Laboratory of Cardiovascular and Cerebrovascular Drug Basic Research, Jinzhou Medical University, Jinzhou, Liaoning 121001; [3]Department of Neurology, Affiliated Taizhou Hospital of Nanjing University of Chinese Medicine, Taizhou, Jiangsu 225300; [4]Department of Neurology, Affiliated Wenling Hospital of Wenzhou Medical University, Wenling, Zhejiang 317500, P.R. China

Received July 8, 2021; Accepted January 4, 2022

DOI: 10.3892/etm.2022.11248

Abstract. Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive impairment and the deposition of amyloid plaques in the brain. In a transgenic mouse model of AD, cognitive impairment and synaptic dysfunction were revealed to be associated with soluble amyloid oligomers and to occur prior to plaque formation. The results of our previous studies revealed that striatal-enriched protein tyrosine phosphatase $(STEP)_{61}$ negatively regulated the β-amyloid protein-mediated ERK/cAMP-response element-binding protein (CREB) signaling pathway. Dl-3-n-butylphthalide (NBP) is a synthetic compound approved by the Food and Drug Administration of China for the treatment of ischemic stroke in 2002. Studies have shown that the neuroprotective effects of NBP involve multiple mechanisms. The present study further explored the mechanism of NBP therapy in amyloid precursor protein (APP)/presenilin 1 (PS1) transgenic mice, and the involvement of the STEP/ERK/CREB signaling pathway. The results suggested that NBP treatment effectively ameliorated the spatial learning and memory impairment of the APP/PS1 transgenic mice, which was assessed using a Morris water maze. In addition, NBP reduced amyloid-induced activation of $STEP_{61}$ levels, while increasing phosphorylated (p)-ERK1/2 and p-CREB levels in the cerebral cortex and hippocampus of APP/PS1 transgenic mice by western blotting and immunostaining. In conclusion, the present study provided evidence to suggest that the new drug NBP improved amyloid-induced learning and memory deficits, likely through the regulation of the STEP/ERK/CREB pathway. The results revealed that NBP, as a multi-target drug, may exert a neuroprotective effect. Therefore, NBP may serve as an effective treatment for AD.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by various pathological markers in the brain, such as large numbers of amyloid plaques surrounded by neurons containing neurofibrillary tangles, vascular damage and neuronal cell loss (1,2). The pathogenesis of AD remains largely unknown and no effective pharmacotherapy is available to date.

The amyloid cascade hypothesis suggests that the deposition of amyloid plaques in the brain is the causative agent for AD pathogenesis, and that the neurofibrillary tangles, cell loss, vascular damage and dementia follow as a direct result of this deposition (3). β-Amyloid is generated from the sequential and proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. Individual amyloid β (Aβ)42 monomers form soluble oligomers of different molecular weights, which further aggregate to form insoluble Aβ fibrils and amyloid plaques (4). In recent years, soluble Aβ aggregates have been found to cause hippocampal synaptic plasticity impairment, induce progressive memory loss and be associated with cognitive impairment, both in AD mouse models and in humans (4,5). Synaptic plasticity is the neurophysiological

Correspondence to: Dr Lin Zhang, Department of Neurology, Affiliated Wenling Hospital of Wenzhou Medical University, 333 Chuan'an South Road, Chengxi Street, Wenling, Zhejiang 317500, P.R. China
E-mail: zhanglindoctor@163.com

*Contributed equally

Key words: Dl-3-n-butylphthalide, Alzheimer's disease, β-amyloid, striatal-enriched protein tyrosine $phosphatase_{61}$, ERK, cAMP-response element-binding protein

basis of learning and memory. Abnormal hippocampal synaptic plasticity has been reported to be a key biological basis for cognitive dysfunction in AD (6). Therefore, the identification of a biological reagent that can improve synaptic remodeling in the hippocampus could serve as a therapeutic strategy for AD.

Dl-3-n-butylphthalide (NBP) is a synthetic compound based on l-3-n-butylphthalide that is isolated from the seeds of *Apium graveolens*. NBP was approved by the Food and Drug Administration (FDA) of China for the treatment of ischemic stroke in 2002 (7). It has also been approved by the US FDA to undergo a phase II trial for the treatment of ischemic stroke (8). Studies have shown that NBP can not only significantly ameliorate the acute symptoms of stroke (9,10) but can also play a strong neuroprotective role in improving the recovery of stroke-related disabilities, as well as alleviating cognitive impairment of vascular dementia and AD (11,12). The neuroprotective effects of NBP may involve multiple mechanisms, including improving microcirculation and ATP metabolism (13), decreasing oxidative damage (14), attenuating inflammatory responses (15) and reducing neuronal apoptosis (16).

Striatal-enriched protein tyrosine phosphatase (STEP) is a central nervous system (CNS)-enriched member of the protein tyrosine phosphatase (PTP) family encoded by the PTP non-receptor type 5 (PTPN5) gene and has two spliced isoforms; $STEP_{61}$ and $STEP_{46}$. $STEP_{61}$ targets the endoplasmic reticulum and postsynaptic density of dendritic spines and is an important regulator of synaptic function (17). Increased $STEP_{61}$ expression and/or activity disrupts synaptic function and is associated with a number of neuropsychiatric disorders, such as AD (17). Studies have shown that the application of Aβ oligomers activates $STEP_{61}$, which subsequently leads to the downregulation of N-methyl-D-aspartic acid or N-methyl-D-aspartate receptor (NMDAR), and that ERK1/2 could be the basis of cognitive deficits in early AD (18). The results of our previous study revealed that $STEP_{61}$ negatively regulates the Aβ protein-mediated ERK/cAMP-response element-binding protein (CREB) signaling pathway, providing a theoretical basis for $STEP_{61}$ as an effective new target for AD (19). It was therefore hypothesized that the $STEP_{61}$/ERK1/2/CREB pathway may be involved in the mechanism of NBP improving the cognitive function of AD mice.

In the present study, using APP/PS1 transgenic mice as the AD model, the efficiency of NBP on the cognitive function of AD mice was investigated and the possible molecular mechanism of NBP regulating the $STEP_{61}$/ERK1/2/CREB pathway was explored in an AD animal model.

Materials and methods

Preparation of NBP. NBP (purity, >98%) was purchased from Shijiazhuang Pharmaceutical Group Co., Ltd. and dissolved in vegetable oil at a working concentration of 5 mg/ml.

Animals and treatment. A total of 30 male APP/PS1 transgenic mice (model mice of AD) aged 12 months and weighing 30.0±0.5 g were purchased from Beijing Huafukang Biotechnology Co., Ltd. A total of 10 age-matched male C57BL/6 mice weighing 30.0±0.5 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (wild-type controls). The mice were housed under a 12-h light/dark cycle at 20-25˚C, 40-45% humidity and provided with free access to food and water. All animal experiments were approved by the Animal Care and Use Committee of Jinzhou Medical University (approval no. 20191110), and in accordance with the Guide for the Care and Use of Laboratory Animals (20). All mice were acclimated to the laboratory environment for 1 week prior to the experiment.

The experimental groups were as follows: C57BL/6 group (WT; n=10), APP/PS1 group (APP/PS1; n=10), NBP 10 mg/kg group (NBP 10 mg/kg; n=10) and NBP 30 mg/kg group (NBP 30 mg/kg; n=10). NBP was dissolved in vegetable oil. In the NBP 10 and 30 mg/kg groups, the APP/PS1 mice underwent intragastric administration of 10 or 30 mg/kg NBP, respectively, once a day. The C57BL/6 and APP/PS1 groups received equal amounts of vegetable oil in the same manner. All mice were monitored daily for general health. The body weight of each mouse was measured weekly. NBP was administered continuously for 16 days, and behavioral experiments were performed on days 10-15. Mice were administered NBP 30 min before the start of the Morris water maze (MWM) training and euthanized on day 16 for histological examination. A schematic overview of the experimental design is presented in Fig. 1.

MWM. Hippocampus-dependent spatial learning and memory function were assessed using a MWM (21). The apparatus included a circular pool (diameter, 120 cm; height, 50 cm) filled with opaque water to a depth of 30 cm, which was maintained at 24±1˚C. The pool was artificially divided into four equal quadrants. An escape platform (diameter, 10 cm) was placed 1 cm below the water surface in the center of one quadrant of the pool. The acquisition phase consisted of 6 consecutive days of testing, with four trials per day. During the first 5 days of training, the mice were given 60 sec per trial to find the hidden platform. The time that the mice spent searching for the platform was recorded as escape latency. If the mice failed to find the platform within 60 sec, the training was terminated, escape latency time was recorded as 60 sec, and the mice were manually guided to the hidden platform. The escape latency, swim paths and distance were recorded with a video camera (TOTA-450SIII) and analyzed using the ANY-maze video tracking system (Stoelting Co.). On day 6, a probe trial was performed with the platform removed, in order to estimate spatial memory retention. The mice were released into the pool directly opposite the platform location and allowed to swim for 60 sec. The time spent in the target quadrant and the number of crossings over the previous position of the platform were recorded.

Sample preparation. Following the MWM training, all mice were anesthetized through an intraperitoneal injection of 50 mg/kg sodium pentobarbital (Sigma-Aldrich; Merck KGaA) and transcardially perfused with 100 ml of 4% paraformaldehyde. The brain was quickly removed and placed on an ice-cold glass dish and sagittally bisected. The left hemisphere was routinely fixed in 4% paraformaldehyde at 4˚C for 24 h, dehydrated with ethanol and embedded in paraffin. The coronal sections were serially cut at a thickness of 5 μm. The

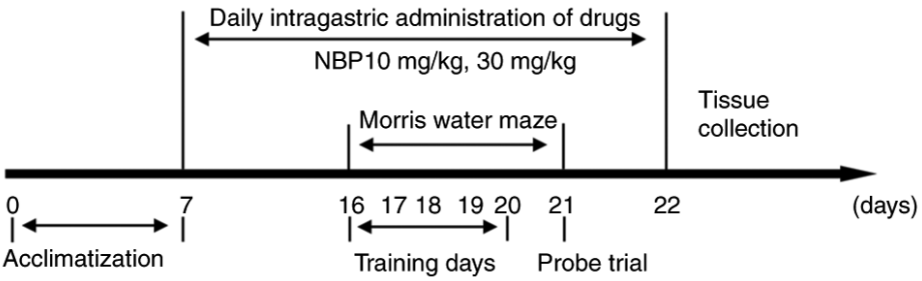

Figure 1. Schematic overview of the experimental design of the study. NBP, Dl-3-n-butylphthalide.

right cortex and hippocampus were separated and stored at -80˚C until analysis.

Immunohistochemistry. Following dewaxing and rehydration of the paraffin sections, antigen retrieval was carried out with citric acid buffer (pH=6.0). The sections were incubated at room temperature with 3% hydrogen peroxide solution for 30 min to block endogenous peroxidase activity. Following rinsing with PBS, the slides were incubated with 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) blocking solution at a room temperature of 30 min to remove the non-specific reaction. Following incubation with the primary antibodies $STEP_{61}$ (1:400; cat. no. ER1917-25; Hangzhou Huaan Biotechnology Co., Ltd.), phosphorylated (p)-p44/42 MAPK (ERK1/2) (Tht202/Tyr204) (1:300; product no. 4370; Cell Signaling Technology, Inc.) and p-CREB (Ser133) (1:400; product no. 9198; Cell Signaling Technology, Inc.) at 4˚C overnight, the sections were incubated with a biotinylated secondary antibody (1:200; product no. ZB2010; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) at 37˚C for 30 min. Peroxidase activity was evaluated using 3,3'-diaminobenzidine and counterstained with hematoxylin at room temperature for 10 min. Immunopositive expression in the cortex and hippocampus was analyzed using an Olympus BX53 light microscope (Olympus Corporation).

Immunofluorescence. Following dewaxing and rehydration of the paraffin sections, antigen retrieval was carried out with citric acid buffer (pH=6.0). The sections were incubated at room temperature with 3% hydrogen peroxide solution for 30 min to block endogenous peroxidase activity. Following rinsing with PBS, the slides were incubated with 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) blocking solution at room temperature for 1 h to remove the non-specific binding. Following incubation with the primary antibodies, NeuN (1:200; product code ab177487; Abcam), $STEP_{61}$ (1:400), p-ERK1/2 (1:300) and p-CREB (1:400), at 4˚C overnight, the sections were incubated with goat anti-mouse secondary antibody conjugated with FITC (1:200; product no. ZF0312) and goat anti-rabbit secondary antibody conjugated with tetramethylrhodamine (1:200; product no. ZF0316; both from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) at 37˚C for 30 min. Cell nuclei were counterstained with DAPI (0.005 mg/ml) for 5 min at 37˚C. The sections were mounted using anti-fluorescence quenching sealing tablets, and observed, and images were captured under a PerkinElmer Mantra™ Quantitative Pathology Imaging system (PerkinElmer, Inc.). The mean number of positively stained cells was counted from three visual fields in the cortex and hippocampus of each section, and three discrete sections of each sample were manually selected for statistical analysis.

Western blot analysis. The frozen brain samples were weighed and then homogenized in RIPA lysis buffer (cat. no. R0020; Beijing Solarbio Science & Technology Co., Ltd.) containing protease and phosphatase inhibitor cocktails. Subsequently, the homogenate was centrifuged at 12,000 x g for 30 min at 4˚C to collect the supernatant. The protein concentrations were quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of total protein (30 μg/lane) were separated on 10% SDS-PAGE gel electrophoresis and transferred to PVDF membranes. The membranes were blocked against non-specific binding for 1 h with 5% non-fat dry milk in TBS containing 0.1% Tween-20 (TBST) at room temperature and incubated with the primary antibodies $STEP_{61}$ (1:2,000), p-ERK1/2 (1:2,000), p-CREB (dilution, 1:2,000) and GAPDH (1:2,000; cat. no. AM1020b-400; Abgent Biotech Co., Ltd.) at 4˚C overnight. They were then rinsed with TBST three times for 10 min, followed by incubation with HRP-labeled secondary antibody (1:5,000; product no. 7074; Cell Signaling Technology, Inc.) at room temperature for 1 h. Subsequently, the target protein expression was visualized by SuperSignal West Pico PLUS chemiluminescence tsubstrate (Thermo Fisher Scientific, Inc.) and analyzed using ImageJ software version 1.52a (National Institutes for Health). GAPDH was used as the loading control. All experimental procedures were repeated at least three times.

Statistical analysis. GraphPad Prism 8 software (GraphPad software, Inc.) was used to carry out the statistical analysis of the experimental data, which are all presented as the mean ± SEM. One- or two-way ANOVA was performed. LSD and Tukey's post hoc tests were used for comparisons between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

NBP improves spatial learning and memory in APP/PS1 transgenic mice with AD. To investigate the effects of NBP on spatial learning and memory function in APP/PS1 transgenic mice, their behavior was assessed using the MWM. Following

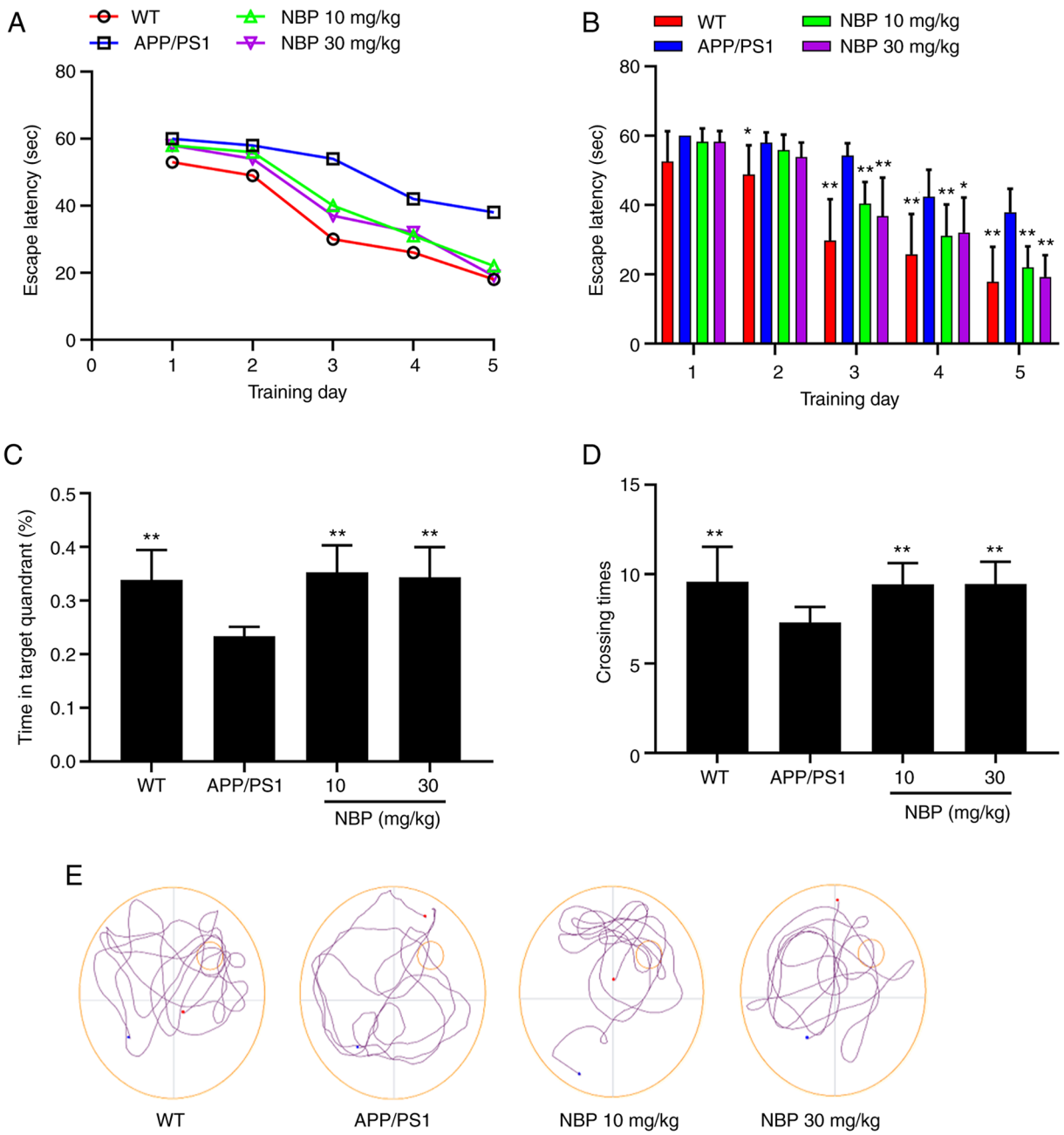

Figure 2. NBP improves spatial learning and memory in APP/PS1 transgenic mice. (A and B) Escape latency in 5 training days. (C) Percentage of swimming time in target quadrant in the probe trial. (D) Number of times crossing the target platform in the probe trial. (E) Representative swim paths of mice during the probe trial. The data are expressed as the mean ± SEM (n=10). *P<0.05 and **P<0.01 vs. the APP/PS1 group (A-D, one-way ANOVA; B, two-way ANOVA). NBP, Dl-3-n-butylphthalide; APP/PS1, amyloid precursor protein/presenilin 1; WT, C57BL/6 group.

5 training days in the MWM, the escape latency to find the platform was significantly increased in the APP/PS1 group compared with the C57BL/6 group. The results revealed that the spatial learning of APP/PS1 transgenic mice was clearly impaired in the water maze. In addition, compared with the APP/PS1 group, the escape latency in the NBP 10 and 30 mg/kg groups had significantly declined (P<0.01; Fig. 2A and B), indicating that NBP alleviated the spatial learning impairment in APP/PS1 transgenic mice. In the probe trial of the MWM, mice from the APP/PS1 group spent significantly less time in the target quadrant and had a lower number of crossings, which was significantly different from the C57BL/6 group. However, an obvious improvement was observed following NBP treatment. As compared with the APP/PS1 group, mice from the NBP 10 and 30 mg/kg groups spent considerably more time in the target quadrant and had a higher number of crossings (P<0.01; Fig. 2C and D). Representative swim paths from all groups during the probe trial are presented in Fig. 2E. There was no significant difference in the average swimming speed among all groups, indicating that the APP/PS1 transgenic mice exhibited no impairment in motor ability (P>0.05).

These results indicated that the spatial learning and memory impairment in the APP/PS1 mice could be effectively ameliorated following NBP treatment, but no significant difference was observed between the NBP 10 and 30 mg/kg groups.

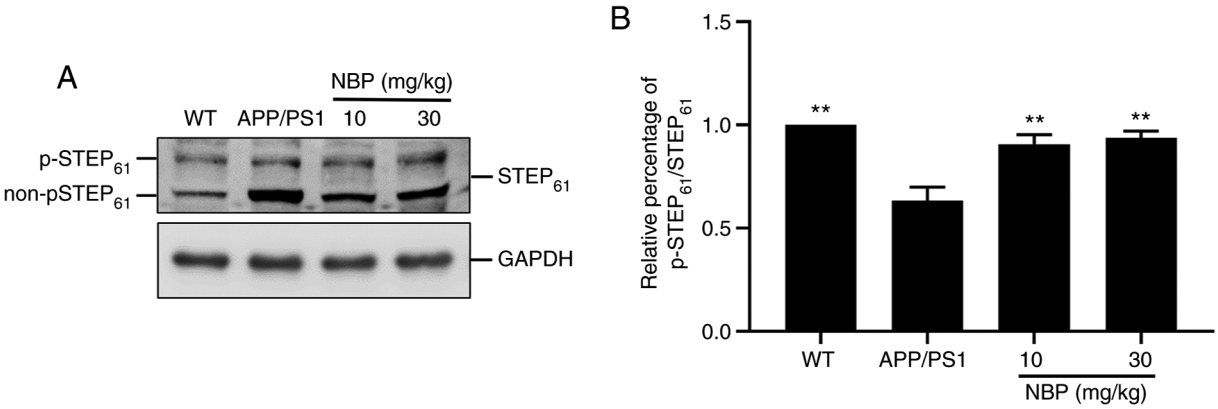

Figure 3. NBP increases $STEP_{61}$ phosphorylation in APP/PS1 transgenic mice. (A) Representative western blot analysis images of $STEP_{61}$. Equal amounts of protein from each group samples were analyzed with western blot analysis using an anti-$STEP_{61}$ antibody. GAPDH was used as an internal reference. (B) Quantification of p-$STEP_{61}$. All data are presented as the mean ± SEM (n=3). **P<0.01 vs. p-$STEP_{61}$ from the APP/PS1 group. NBP, Dl-3-n-butylphthalide; STEP, striatal-enriched protein tyrosine phosphatase; p-, phosphorylated; APP/PS1, amyloid precursor protein/presenilin 1; WT, C57BL/6 group.

NBP increases $STEP_{61}$ phosphorylation in APP/PS1 transgenic mice. $STEP_{61}$ is expressed in neurons of the hippocampus, cerebral cortex and striatum and its activity is modulated by the phosphorylation/dephosphorylation of a serine residue in the kinase-interacting motif domain, which affects the affinity of $STEP_{61}$ towards its substrate. The dephosphorylation of the serine residue, referred to as the 'active form', causes $STEP_{61}$ to bind to its substrates, thus inhibiting their activities, while the phosphorylation of the serine residue prevents the binding of $STEP_{61}$ to its substrates (22). To evaluate whether NBP impacted $STEP_{61}$ activity in APP/PS1 mice, cell lysates were analyzed by western blot analysis using an anti-$STEP_{61}$ antibody (Fig. 3A). The predominant isoform of $STEP_{61}$ expressed in neurons was mainly exhibited as a doublet, with the top band representing the $STEP_{61}$ phosphorylated form and the bottom band representing the $STEP_{61}$ non-phosphorylated (or active) form (22). The intensity of p-$STEP_{61}$ is presented as a percentage of the total intensity of p-$STEP_{61}$ and non-p-$STEP_{61}$. Western blot analysis demonstrated a marked decrease in the level of $STEP_{61}$ phosphorylation in the APP/PS1 group compared with that in the C57BL/6 group. By contrast, the level of $STEP_{61}$ phosphorylation was significantly increased following NBP treatment compared with that in the APP/PS1 group (P<0.01; Fig. 3A and B), particularly in the NBP 30 mg/kg group.

$STEP_{61}$ is a brain-specific phosphatase that modulates key signaling molecules involved in synaptic plasticity and neuronal function (23). An increase in $STEP_{61}$ activity is considered to interfere with synaptic strengthening, resulting in cognitive and behavioral deficits in AD (24). Western blot analysis suggested that the decrease in $STEP_{61}$ activity in both the hippocampus and the cortex of APP/PS1 mice was restored by NBP treatment.

NBP increases the phosphorylation level of ERK1/2 and CREB. Among the downstream targets of $STEP_{61}$, ERK1/2, which regulates memory and synaptic plasticity, was of particular interest in the present study. In addition, p-ERK1/2 activates the transcription factor CREB, which regulates LTP induction and maintenance, and synaptic plasticity (25). Thus, the levels of p-ERK1/2 and p-CREB were evaluated. Western blot analysis demonstrated a marked decrease in the levels of p-ERK1/2 and p-CREB in both the hippocampus and cortex of the APP/PS1 group compared with that in the C57BL/6 group. Conversely, the levels of p-ERK1/2 and p-CREB were significantly increased following NBP treatment compared with those in the APP/PS1 group (P<0.01, Fig. 4A and B; P<0.05, Fig. 4C and D), particularly in the NBP 30 mg/kg group, suggesting that NBP can increase the levels of p-ERK1/2 and p-CREB in APP/PS1 transgenic mice.

Localization, distribution and expression changes of $STEP_{61}$, p-ERK1/2 and p-CREB in the brain of APP/PS1 transgenic mice. To investigate the effects of NBP on the localization, distribution, and expression of $STEP_{61}$, p-ERK1/2 and p-CREB in APP/PS1 transgenic mouse brains, mouse brain sections from four experimental groups were analyzed using immunohistochemistry and immunofluorescence.

Immunofluorescence staining for $STEP_{61}$, p-ERK1/2 and p-CREB was performed using a PerkinElmer Mantra™ Quantitative Pathology Imaging system. Sections were co-stained with the neuronal marker NeuN (green) and anti-$STEP_{61}$, anti-p-ERK1/2 and anti-p-CREB antibodies (red), respectively (Fig. 5). $STEP_{61}$ (Fig. 5A), p-ERK1/2 (Fig. 5B) and p-CREB (Fig. 5C) staining was observed within hippocampal neurons. The results showed that compared with the C57BL/6 group, the fluorescence intensity of $STEP_{61}$ in the APP/PS1 group was significantly increased, while the fluorescence intensity of p-ERK1/2 and p-CREB in the APP/PS1 group was significantly decreased. After treatment with NBP, the fluorescence intensity of $STEP_{61}$ decreased, which was close to the level of the C57BL/6 group, and particularly in the NBP 30 mg/kg group, the fluorescence intensity of p-ERK1/2 and p-CREB was significantly increased.

Immunohistochemistry revealed a positive distribution of $STEP_{61}$ in the cerebral cortex and hippocampal CA1 region of mice in the APP/PS1 group, and the expression of $STEP_{61}$ was markedly increased in both the cortex and hippocampus of the APP/PS1 group compared with the C57BL/6 group, but not in the NBP 10 and 30 mg/kg groups (Fig. 6A). p-ERK1/2 and p-CREB expression was similar (Fig. 6B and C). Immunohistochemistry indicated that p-ERK1/2 and p-CREB

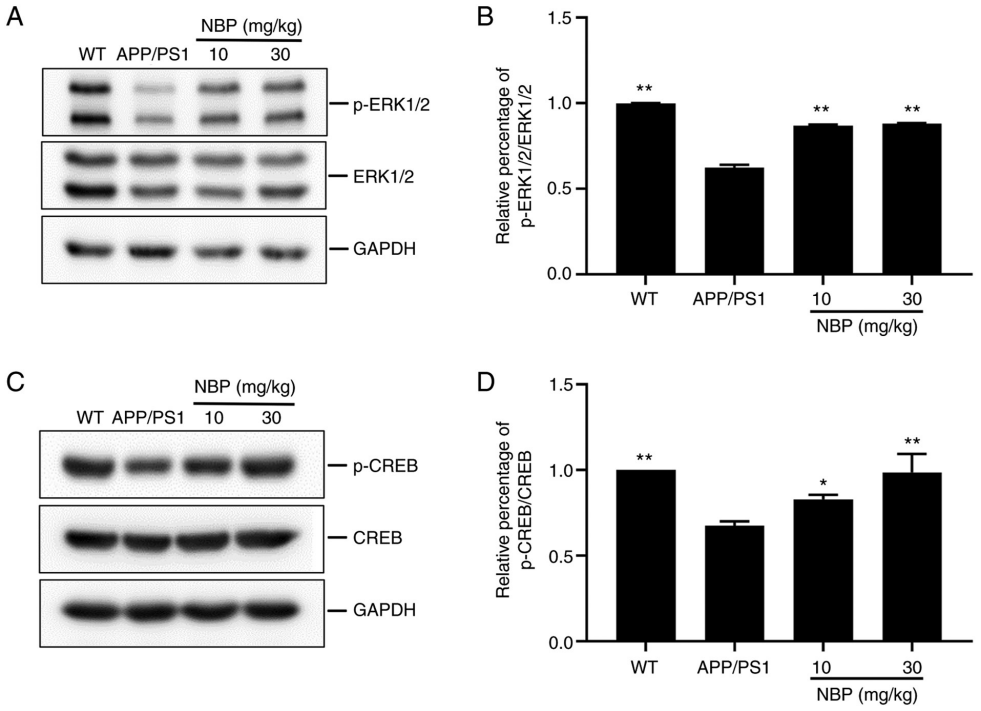

Figure 4. NBP increases the level of p-ERK1/2 and p-CREB. (A and C) Representative western blot analysis images of p-ERK1/2 and p-CREB. The level of p-ERK1/2 and p-CREB were analyzed using western blot analysis with anti-p-ERK1/2 and anti-p-CREB antibodies, respectively. Total ERK1/2 and CREB were analyzed with western blot analysis using anti-ERK1/2 and anti-CREB antibodies, respectively. GAPDH was used as an internal reference control. (B and D) Quantification of p-ERK1/2 and p-CREB. All data are presented as the mean ± SEM (n=3). *P<0.05 and **P<0.01 vs. APP/PS1 group. NBP, Dl-3-n-butylphthalide; p-, phosphorylated; CREB, cAMP-response element-binding protein; APP/PS1, amyloid precursor protein/presenilin 1; WT, C57BL/6 group.

immunoproducts were decreased in the cerebral cortex and hippocampus of APP/PS1 mice compared with those of WT mice. In addition, the decrease in p-ERK1/2 and p-CREB was restored following NBP treatment, particularly in the NBP 30 mg/kg group. It was further demonstrated that NBP reduced amyloid-induced $STEP_{61}$ levels, while increasing p-ERK1/2 and p-CREB levels.

Discussion

AD is one of the most common age-linked neurodegenerative diseases causing dementia. The aggregation of Aβ peptides in the brain is the predominant pathological hallmark of AD (26). APP/PS1 AD model mice were double-transfected with the human APP695swe and the human PS1dE9 mutation, both of which are associated with early-onset AD (27). The mouse/human APP695swe and PS1dE9 double transgene allowed mice to secrete human-amyloid oligopeptides. In the early stages of AD physiopathology, soluble Aβ oligomers interfere with synaptic function by controlling the synaptic levels of the NMDAR (28). STEP, encoded by the PTPN5 gene, is a CNS-enriched member of the PTP family that regulates tyrosine residues on NMDARs through dephosphorylation, resulting in the internalization of these receptor complexes (29). The protective effect of NBP on synaptic function has been previously reported (12,30). In the present study, the mechanism through which NBP protects synaptic function by inhibiting $STEP_{61}$ activity, thus improving cognitive function in the AD mouse model, was investigated.

The activity of $STEP_{61}$ was modulated due to the altered $STEP_{61}$ phosphorylation/dephosphorylation level, leading to a correlative change in the phosphorylation level of its substrate. The MAPK family member ERK1/2 is the substrate of $STEP_{61}$. The ERK1/2 cascade plays a key role in maintaining and inducing synaptic plasticity. p-ERK1/2 initiates transcription by activating the transcription factor CREB in the nucleus, while the $STEP_{61}$-mediated dephosphorylation of ERK1/2 at TYR204/187 inactivates ERK1/2, thereby limiting the duration of ERK1/2 activity following NMDAR stimulation (23). CREB is a transcription factor and a downstream target of ERK1/2. Our previous study has shown that $STEP_{61}$ affects the ERK/CREB signaling pathway in animal and cell models of AD (19).

In the present study, APP/PS1 transgenic mice were used to study the effects of NBP on cognitive impairment. Cognitive function was evaluated using a MWM. Furthermore, the mechanism of NBP therapy in APP/PS1 transgenic mice was studied by detecting the levels of p-$STEP_{61}$, p-ERK1/2 and p-CREB in the cortex and hippocampus using western blot analysis and immunostaining. In the MWM, the APP/PS1 group revealed a clear impairment in spatial learning and memory compared with the C57BL/6 group, which manifested as prolonged escape latency, shorter swimming time spent in the target quadrant and a lower number of platform crossings in the probe trial. NBP administration markedly decreased escape latency, and increased the percentage of target quadrant search time and number of platform location crossings in APP/PS1 transgenic mice. The results revealed that NBP clearly alleviated learning and memory functions in APP/PS1 transgenic mice. In the present study, it was also

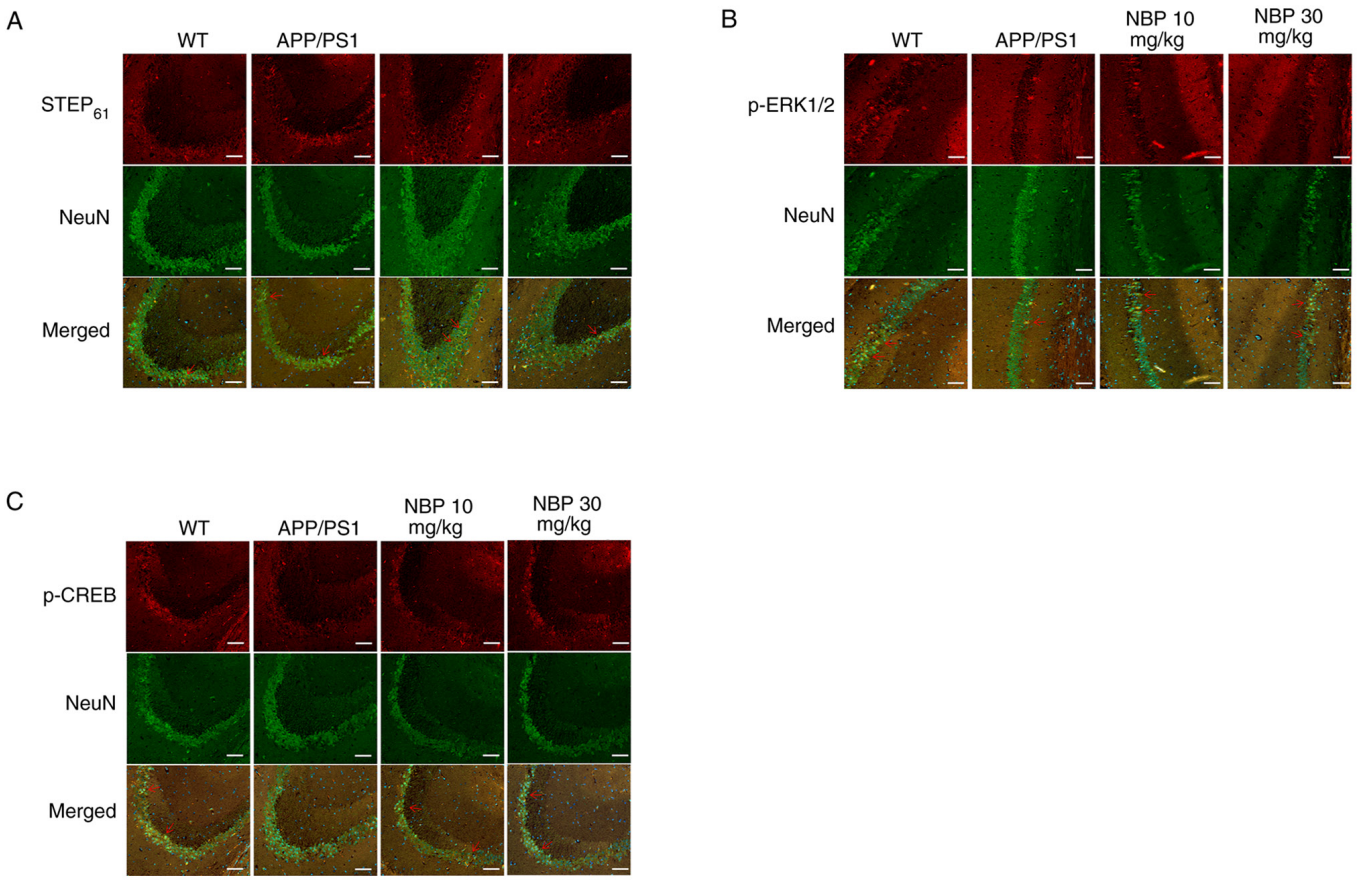

Figure 5. Colocalization of NeuN with $STEP_{61}$, p-ERK1/2 and p-CREB, as revealed using confocal microscopy. Red stains indicate positive staining for (A) $STEP_{61}$, (B) p-ERK1/2 and (C) p-CREB (top panels). The green stains indicate positive staining of cells for NeuN (A-C; middle panels) in the hippocampus. Arrows represent merged images (A-C; bottom panels). Scale bars, 50 μm. STEP, striatal-enriched protein tyrosine phosphatase; p-, phosphorylated; CREB, cAMP-response element-binding protein; NBP, Dl-3-n-butylphthalide; WT, C57BL/6 group.

revealed that the inhibition of ERK1/2 and CREB phosphorylation in the cortex and hippocampus of APP/PS1 transgenic mice was accompanied by an increase in $STEP_{61}$ dephosphorylation. However, the continuous administration of 10 or 30 mg/kg NBP significantly reduced the levels of activated $STEP_{61}$ and increased those of p-ERK1/2 and p-CREB, but no significant differences were observed between the NBP groups (10 vs. 30 mg/kg; $P>0.05$). NBP concentrations were set to 10 and 30 mg/kg according to the published literature (7,31). In conclusion, NBP alleviated amyloid-induced learning and memory impairment, and decreased Aβ-induced activated $STEP_{61}$ levels and promote ERK1/2 and CREB phosphorylation. Therefore, it was hypothesized that NBP improved learning and cognitive defects in APP/PS1 transgenic mice, likely through the regulation of the STEP/ERK/CREB pathway. The results showed that, as a multi-target drug, NBP may exert a neuroprotective effect. NBP may serve as an effective treatment for AD. These effects will help elucidate and possibly underlie the development of effective drugs to improve cognitive function in patients with AD. Further study is required to determine the mechanism underlying the effect of NBP on phosphorylation level of $STEP_{61}$.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Science Foundation of Liaoning Province, China (grant no. 2019-ZD-0603) and the Taizhou Science and Technology Support Plan of Jiangsu Province (Social Development; grant no. TS202014).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ, JY and HRZ designed the study. WQY and LY performed experiments and collected all experimental data. YZ and JY drafted the manuscript and contributed substantially to its revision. YZ and JY performed the statistical analysis. JY contributed reagents and materials. YZ and LZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of Jinzhou Medical University

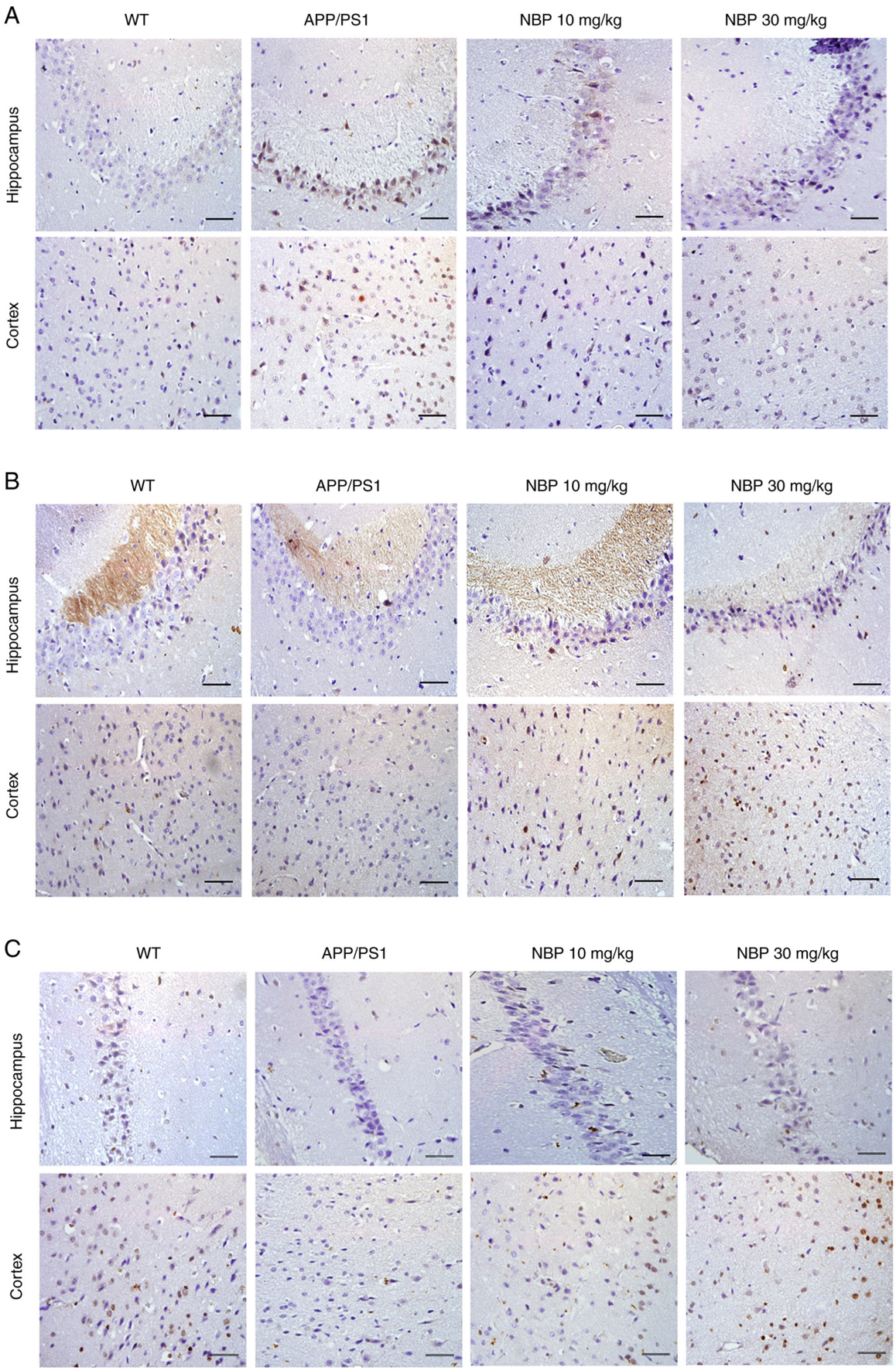

Figure 6. Effects of NBP on the expression of $STEP_{61}$, p-ERK1/2 and p-CREB in APP/PS1 transgenic mouse brains. (A) Representative images of immunohistochemical staining of the $STEP_{61}$ in the cortex and hippocampus. (B) Representative images of immunohistochemical staining of the p-ERK1/2 in the cortex and hippocampus. (C) Representative images of immunohistochemical staining of the p-CREB in the cortex and hippocampus. Brown indicates positive staining. Scale bars, 50 μm. NBP, Dl-3-n-butylphthalide; STEP, striatal-enriched protein tyrosine phosphatase; p-, phosphorylated; CREB, cAMP-response element-binding protein; WT, C57BL/6 group.

(approval no. 20191110), and in accordance with 'The detailed rules for the implementation of the management of medical laboratory animals' (Ministry of Health of China, 1998).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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