Am J Transl Res 2019;11(3):1780-1788
www.ajtr.org /ISSN:1943-8141/AJTR0090866

Original Article

miR-9 stimulation enhances the differentiation of neural stem cells with zoanthamine by regulating Notch signaling

Fang Li[1], Anbao Chen[1], Jie Zhang[2]

[1]*Department of Emergencyinternal Medicine, The Second Affiliated Hospital of Kunming Medical University, Kunming 650221, China;* [2]*Department of Hepatology, The First Affiliated Hospital of Kunming Medical University, Kunming 650032, China*

Received January 5, 2019; Accepted January 19, 2019; Epub March 15, 2019; Published March 30, 2019

Abstract: The protective effect of zoanthamine on Alzheimer's disease by enhancing differentiation of neural stem cells (NSCs) was evaluated. NSCs were isolated from C57BL/6 mice and assessed by cell viability and apoptosis assays. The cells were separated into five different groups: green fluorescent protein (GFP; transfected with GFP), amyloid precursor protein (APP; transfected with APP), APP + zoanthamine, APP + miR-9 inhibitor, and APP + miR-9 inhibitor + zoanthamine. The effects of zoanthamine on the differentiation of NSCs was determined. Moreover, the effects of zoanthamine on the expression of miR-9 and Notch signalling pathway members was assessed by western blot analysis and reverse-transcription polymerase chain reaction. There was a significant increase in cell viability and a decrease in apoptosis of NSCs in the APP + zoanthamine group compared with the APP group. Treatment with zoanthamine attenuated miR-9 expression and neuronal cell differentiation in APP-treated NSCs. Moreover, in the APP + miR-9 inhibitor group, neuronal cell differentiation and miR-9 expression were significantly reduced, and treatment with zoanthamine reduced the number of differentiated cells and miR-9 expression compared with the APP + miR-9 inhibitor group. There was a significant reduction in the expression of Hes1 and NICD proteins in the APP + zoanthamine group relative to the APP group. In addition, the levels of Hes1 and NICD were enhanced by inhibition of miR-9 but zoanthamine prevented these increases. In conclusion, these results suggest that treatment with zoanthamine enhances the differentiation of NSCs by regulating Notch signalling via elevated miR-9 expression.

Keywords: Zoanthamine, amyloid precursor protein, Notch signalling, neural stem cells, Alzheimer's disease

Introduction

Neurodegenerative disorders, such as Alzheimer's disease (AD), have recently become a major issue worldwide [1]. AD commonly develops at an older age and causes dementia. Several pathogenic factors contribute to the development of AD, such as decreases in the levels of acetylcholine, β-amyloid (Aβ) and tau protein, which result in the loss of neurons [2]. Neural stem cells (NSCs) are found in several regions of the brain, including the subventricular zone (SVZ) and hippocampus, and transplantation of these cells improves memory and learning deficits in neurodegenerative disorders [3]. It was reported that differentiation and death of NSCs are regulated by the Notch signalling pathway, and that Notch 1 signalling plays a role in the development of AD [4]. Cleavage of the Notch 1 intracellular domain by gamma secretase leads to the production of Aβ and, subsequently, the development of neurodegeneration [5]. Conventional drugs used for the management of AD have several limitations, and new drugs and/or therapies are therefore needed.

In the past few years, many drugs isolated from marine sources have been confirmed for their therapeutic effects against several chronic disorders. Many alkaloids have been isolated from Zoanthus species, including zoanthamine, which is an alkaloid derived from these marine zoantharians [6]. Alkaloids isolated from

Zoanthus species are reported to possess several pharmacological activities, such as inhibition of platelet aggregation and antibacterial, anti-inflammatory and antiosteoporotic activities [7]. Zoanthamine shows strong anti-inflammatory activity and has a proven role in preventing neuroinflammation [8]. Thus, the present investigation evaluated the effect of zoanthamine on the differentiation of NSCs in AD.

Material and methods

Chemicals

The human amyloid precursor protein (APP) 695swe sequence was procured from DNA-SYN Biotechnology Co. Ltd. (Beijing, China) and the green fluorescent protein (GFP) lentiviral vector from System Biosciences (Palo Alto, CA, USA). The primary antibodies targeting Hes1, Notch intracellular domain (NICD), Aβ, APP and β-actin, and the horse radish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Top Green qPCR SuperMix was purchased from TransGen Biotech (Beijing, China) and cDNA Synthesis Kit from Thermo Scientific (Waltham, MA, USA). The miR-9 oligonucleotide was purchased from GenePharma (Shanghai, China).

Generation of NSCs

The SVZ region was obtained from new-born (0-2 days old) C57BL/6 mice for isolation of NSCs. The SVZ regions from freshly collected brains were cut into 1 mm^3 sections and suspended in 3 mL trypsin-EDTA (0.25%) for 15 min at 37°C. The collected cells (1 × 10^6/mL) were seeded into 24-well plates coated with poly-L-lysine and maintained at 37°C under humidified conditions. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg/mL streptomycin, 100 IU/mL penicillin, 20 ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor and 2% B27. Neurospheres developed after day 5 of culture. NSCs from passage 3-8 were used in the following experiments.

Construction of a lentiviral vector encoding APP and its transfection into NSCs

To generate an APP expression construct, human APP695swe was subcloned into a GFP lentiviral vector, pCDHCMV-MCS-EF1-copGFP, via the XbaI and NotI restriction sites. The pLP/VSV-G, pLP1 and pLP2 plasmids and APP were isolated from bacteria using an endotoxin-free plasmid kit (Qiagen, Hilden, Germany). A plasmid DNA solution containing 3.5 μg pLP/VSV-G, 6.5 μg pLP1, 2.5 μg pLP2 and 15 μg GFP or APP was transfected into 293T human embryonic kidney cells using Lipofectamine 2000. The 293T cells were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% foetal bovine serum (FBS). Fresh culture medium was added after 6 h of transfection, and the culture medium was then collected and filtered through a 0.45 μm membrane. GFP lentiviral particles encoding APP, or GFP only, were transfected into NSCs. After 3 days, the stably transfected cells were either assessed by immunocytochemical staining or cultured for future use.

Cell viability assay

Cell viability was determined using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) per the manufacturer's instructions. In a 96-well plate, 100 μL of the cell suspension were added to 10 μL CCK-8 solution. The plate was incubated for 4 h at 37°C, and a microplate reader was used to determine the absorbance at 450 nm.

Apoptosis assay

Cells (2 × 10^6) were seeded into six-well plates and incubated for 2 days at 37°C. The cells were harvested, washed with phosphate-buffered saline (PBS) and resuspended in 500 μL binding buffer. Then, 5 μL propidium iodide and 5 μL Annexin V-Light 650 were added to the cells, followed by a 15 min incubation in the dark. The number of apoptotic cells was determined using a flow cytometer.

Determination of cell differentiation

APP- or GFP-expressing NSCs were divided into five groups: GFP (transfected with the GFP-expressing plasmid), APP (transfected with the APP-expressing plasmid), APP + zoanthamine, APP + miR-9 inhibitor, and APP + miR-9 inhibitor + zoanthamine. For miR-9 inhibitor treatment, the cells were transfected with an antisense miR-9 oligonucleotide using Lipofectamine 2000 reagent per the manufacturer's instruc-

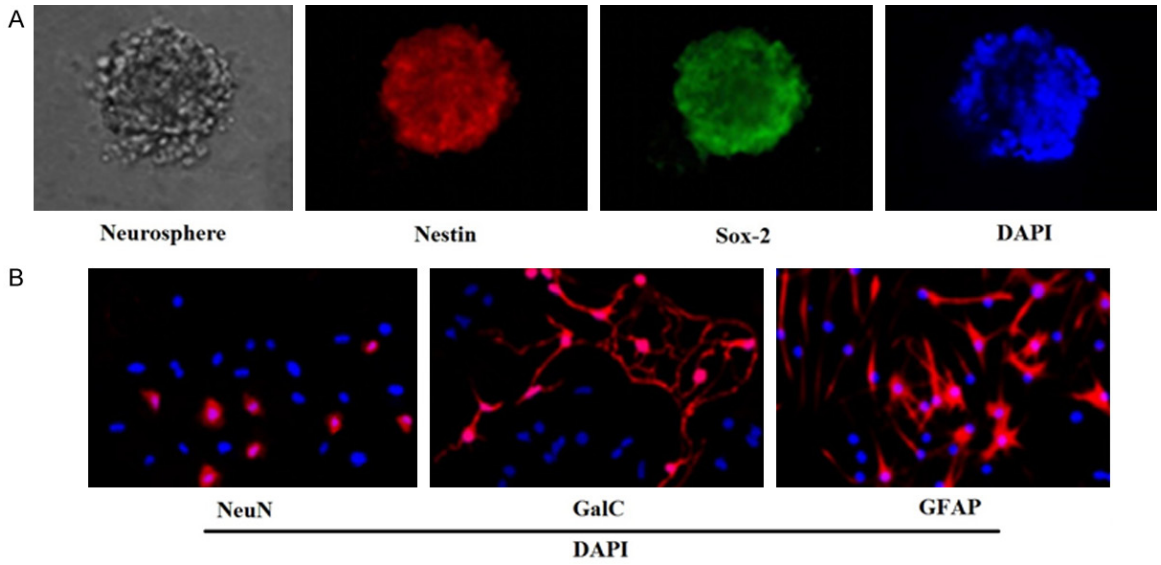

Figure 1. Neural stem cell (NSC) characterization by immunocytochemistry. A. Immunohistochemical staining of neurospheres for nestin (red) and Sox2 (green) and DAPI staining (blue) of the nucleus. B. Immunohistochemical staining for NeuN, GalC and GFAP.

tions (Invitrogen, Carlsbad, CA, USA). After 72 h of transfection, the NSCs were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS for another 10-14 days.

Western blot analysis

Proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane. The proteins on the membrane were blocked with blocking solution for 60 min. The membrane was then incubated with primary antibodies against Hes1 (1:1,000), NICD (1:1,000), Aβ (1:1,500), APP (1:1,000) and β-actin (1:2,000) overnight at 4°C, followed by HRP-conjugated secondary antibodies for 1 h at room temperature. Electrochemiluminescence (ECL) western blotting detection reagents were used to visualize chemiluminescence, and the protein band intensities were quantitated using Image J software (NIH, Bethesda, MD, USA).

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from tissues using TRIzol reagent and quantitated using a spectrophotometer. The Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) was used to reverse-transcribe cDNA from RNA. PCR was conducted using 2 μL cDNA and TransStart Top Green qPCR SuperMix under the following conditions: 94°C for 30 s, followed by 35 cycles of 94°C for 5 s, 60°C for 15 s and 72°C for 10 s. The primers used are listed below. U6 small nucleolar RNA expression was measured as a control for normalization. The data are expressed as Cq values.

Primer	
APP	Forward: GACTGACCACTCGACCAGCAGGTTCTG
	Reverse: CTTGTAAGTTGGATTCTCATATCCG
Notch 1	Forward: TCGTGTGTCAAGCTGATGAGGA
	Reverse: GTTCGGCAGCTACAGGTCACAA
Hes1	Forward: GCAGACATTCTGGAAATGACTGTGA
	Reverse: GAGTGCGCACCTCGGTGTTTA
β-actin	Forward: GGGAAATCGTGCGTGACCT
	Reverse: TCAGGAGGAGCAATGATCCTG

Results

Transfection of GFP and APP into NSCs

NSCs isolated from the SVZ region of new-born C57BL/6 mice were characterized by immunohistochemical staining of Sox2 and nestin markers. Sox2- and nestin-positive NSCs are shown in **Figure 1A**. **Figure 1B** shows the differentiation of NSCs into astrocytes (GFAP+), oligodendrocytes (GalC+) and neurons (NeuN+)

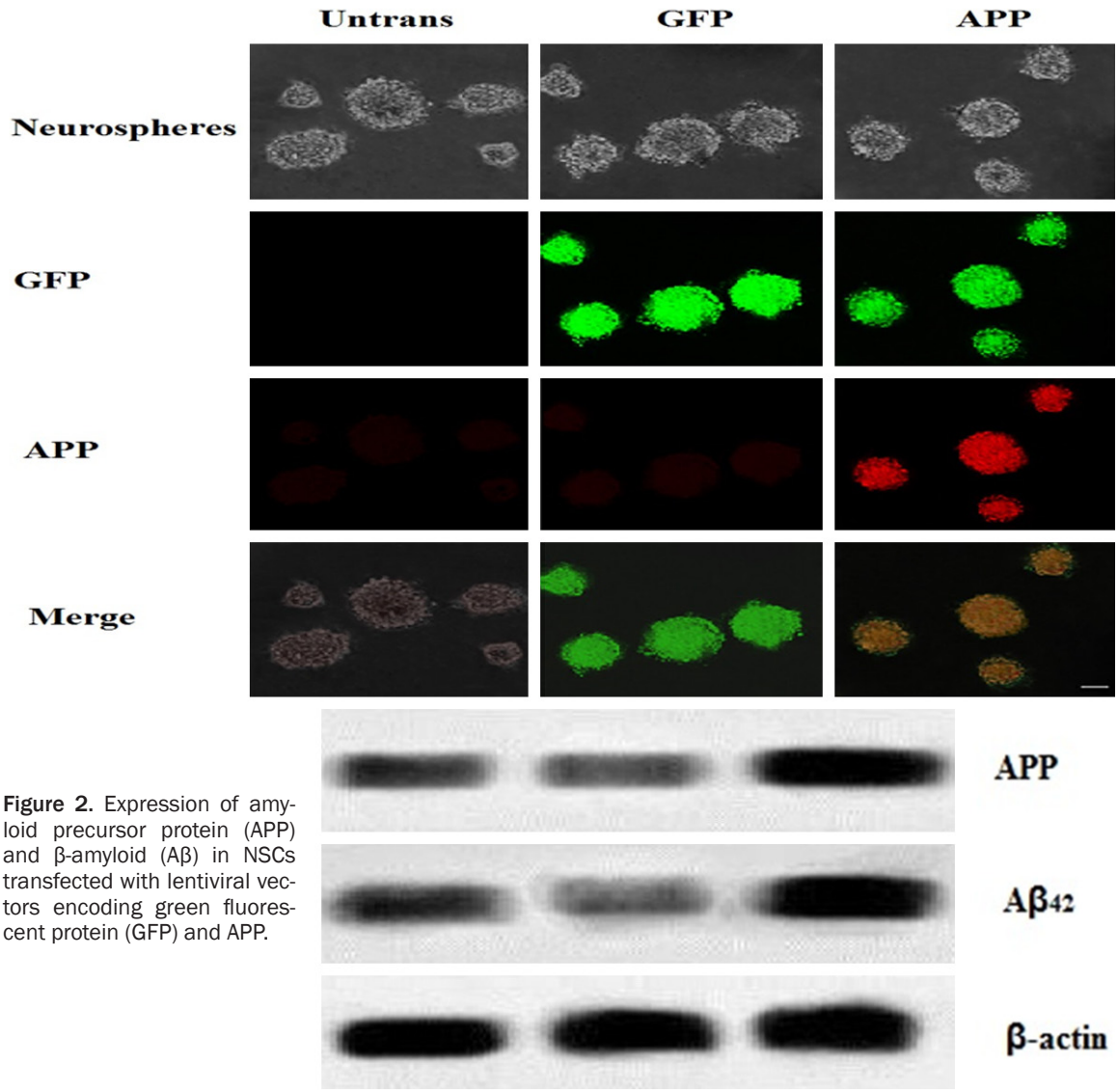

Figure 2. Expression of amyloid precursor protein (APP) and β-amyloid (Aβ) in NSCs transfected with lentiviral vectors encoding green fluorescent protein (GFP) and APP.

after 14 days of culture in differentiation medium. GFP lentiviral vectors encoding APP, or GFP alone, were transfected into the cells. APP and GFP expression was seen in the cells transfected with both vectors (APP group) on day 3 after transfection. Western blot analysis and RT-PCR demonstrated enhanced expression of APP and the Aβ peptide in the APP group. Moreover, there was no difference in the expression of APP or Aβ in the GFP group compared with untransfected cells (**Figure 2**).

Effect of zoanthamine on apoptosis

The protective effect of zoanthamine was assessed by treating APP-transfected NSCs with 100 μmol/L zoanthamine for 1 day followed by the CCK-8 assay. Cell viability was significantly decreased in the APP + zoanthamine group compared with the APP group. However, there was no difference in cell viability between the GFP group and GFP + zoanthamine group (**Figure 3A**).

The number of apoptotic cells, determined by flow cytometry, was higher in the APP group compared with the GFP group. Treatment with zoanthamine decreased the number of apoptotic cells compared with the APP group (**Figure 3B**). These results suggested that our cellular AD model was developed successfully, and that 100 μmol/L zoanthamine had no cytotoxic effect.

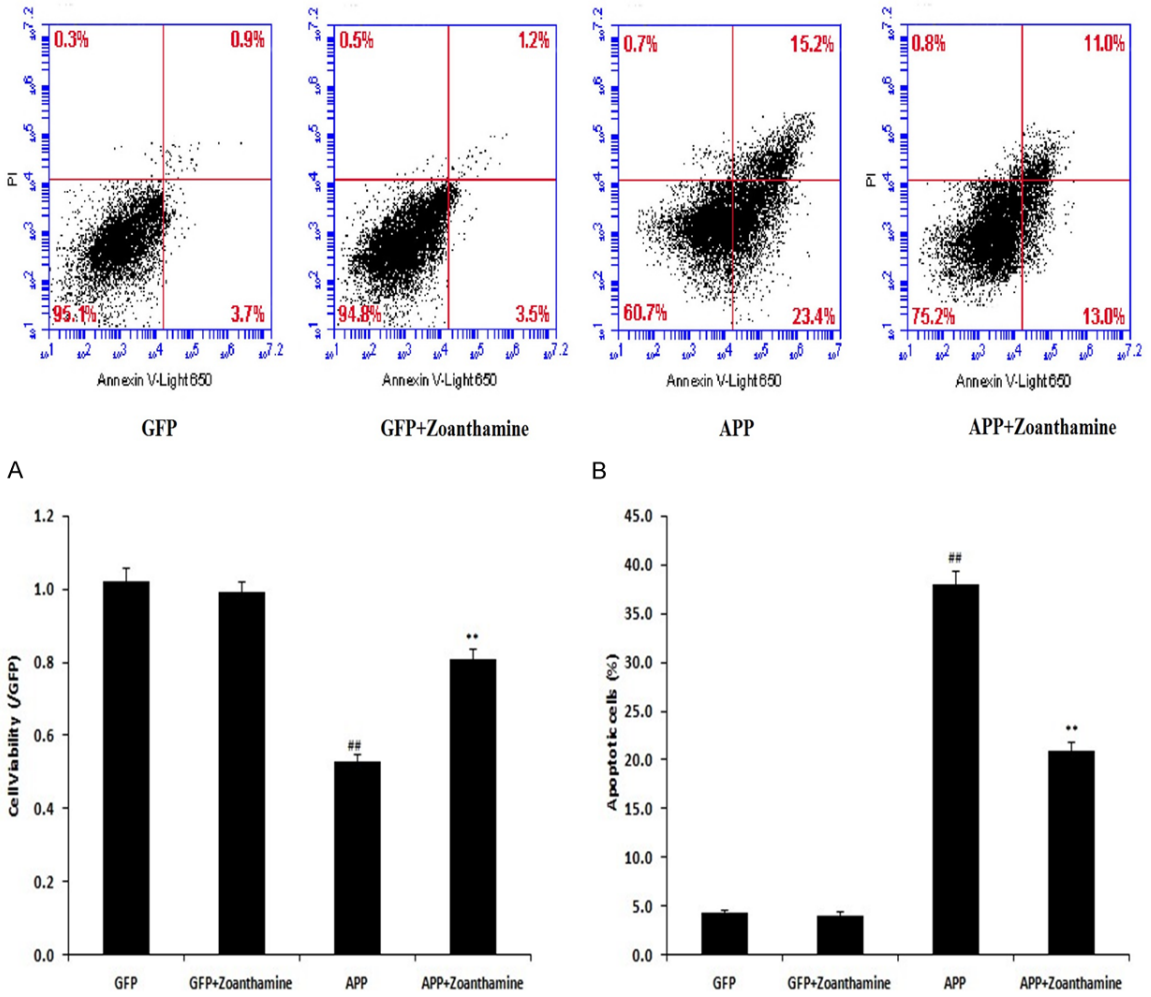

Figure 3. Effects of zoanthamine on apoptosis and viability of APP-expressing NSCs. NSC viability (A) was assessed by CCK-8 assay and apoptosis (B) by flow cytometry with quantitative determination. Mean ± SEM (n = 6); ##P < 0.01 compared with the GFP group; **P < 0.01 compared with the APP group.

Effect of zoanthamine on NSC differentiation

The effect of zoanthamine on the differentiation of NSCs into neuronal cells was evaluated (**Figure 4**). Immunostaining for NF-M indicated fewer NF-M-positive cells in the APP group compared with the GFP group. Zoanthamine attenuated the difference in the proportion of NF-M-positive NSCs between the APP and GFP groups, with a significant increase in the zoanthamine + APP group compared with the APP group. RT-PCR revealed lower miR-9 expression in the APP group compared with the GFP group, and zoanthamine treatment prevented this APP-induced decrease in miR-9 expression. Moreover, in the APP + miR-9 inhibitor group, neuronal cell differentiation and miR-9 expression were significantly reduced compared with the GFP group, and additional treatment with zoanthamine reduced the proportion of differentiated cells and miR-9 expression compared with the APP + miR-9 inhibitor group.

Effect of zoanthamine on the Notch signalling pathway

The effects of zoanthamine and the miR-9 inhibitor on the expression of Notch signalling pathway members were assessed by western blot analysis and RT-PCR (**Figure 5**). RT-PCR showed a significant increase in the expression of Hes1, but not Notch-1, in the APP group compared with the GFP group. However, treatment with zoanthamine attenuated the APP-induced increase in Hes1 expression, with a significant decrease in the APP + zoanthamine group com-

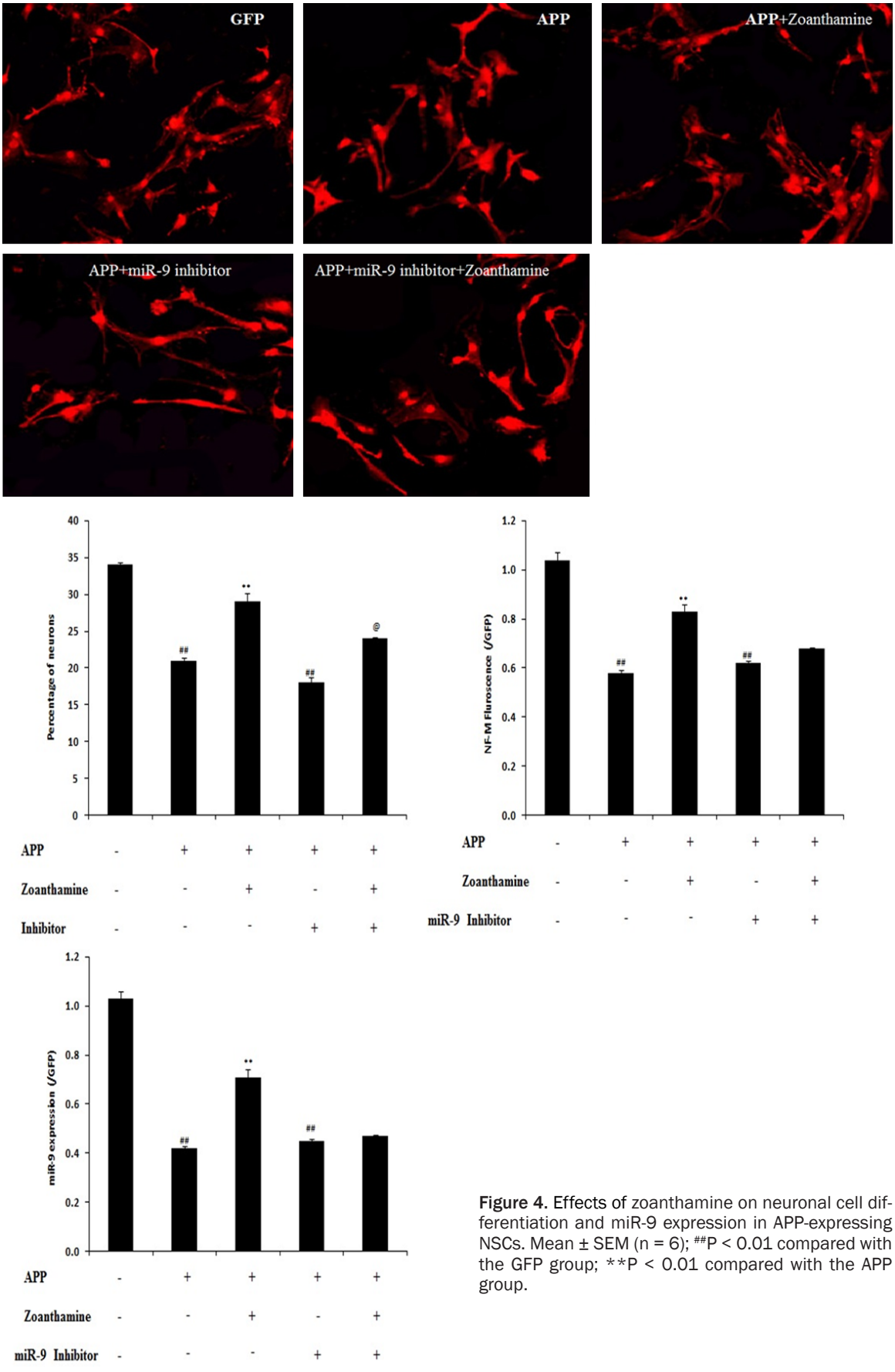

Figure 4. Effects of zoanthamine on neuronal cell differentiation and miR-9 expression in APP-expressing NSCs. Mean ± SEM (n = 6); ##P < 0.01 compared with the GFP group; **P < 0.01 compared with the APP group.

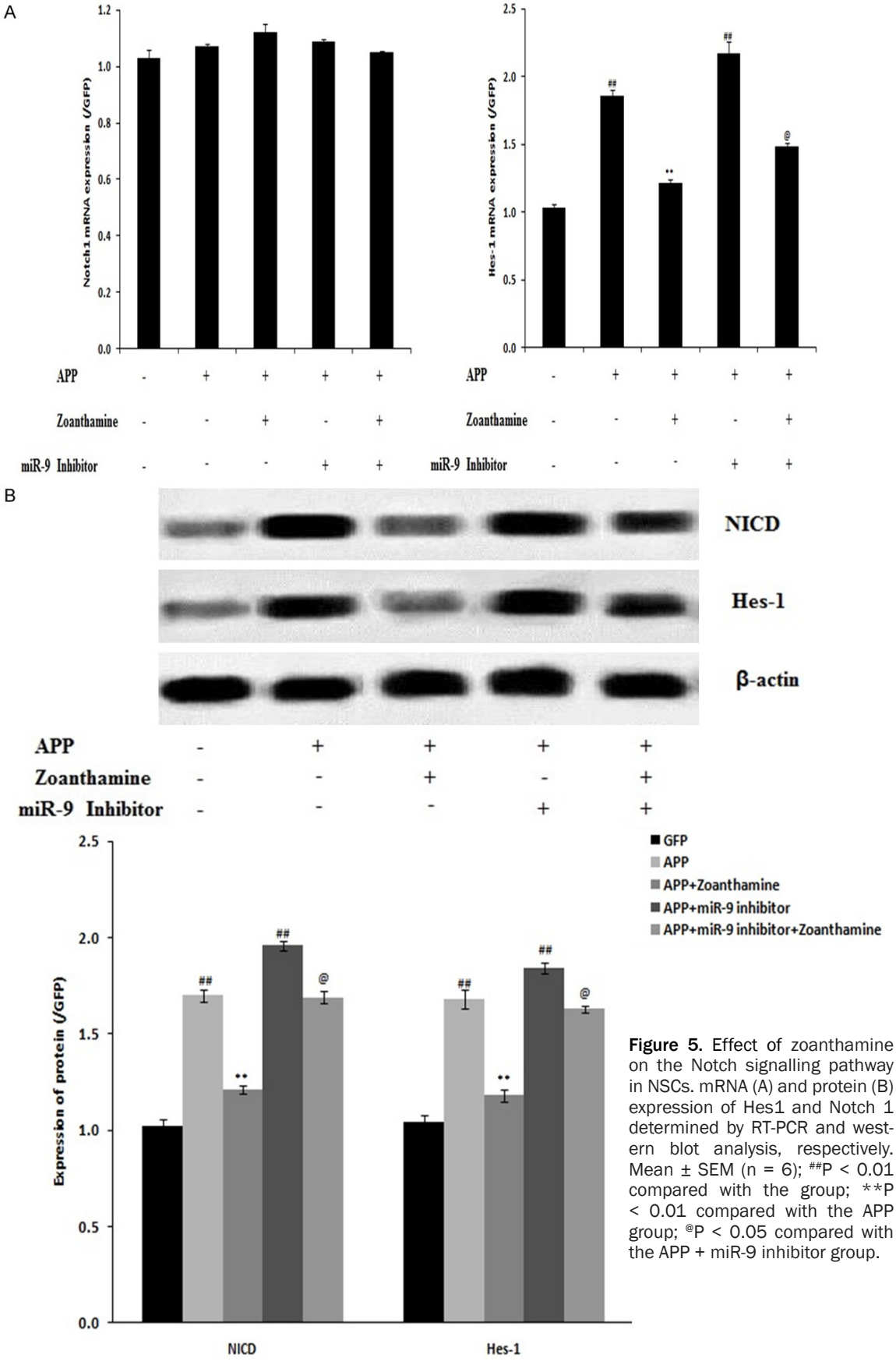

Figure 5. Effect of zoanthamine on the Notch signalling pathway in NSCs. mRNA (A) and protein (B) expression of Hes1 and Notch 1 determined by RT-PCR and western blot analysis, respectively. Mean ± SEM (n = 6); $^{\#\#}P < 0.01$ compared with the group; $^{**}P < 0.01$ compared with the APP group; $^{@}P < 0.05$ compared with the APP + miR-9 inhibitor group.

pared with the APP group (**Figure 5A**). According to western blot analysis, Hes1 and NICD protein levels were significantly increased in the APP group compared with the GFP group and significantly reduced in the APP + zoanthamine group compared with the APP group (**Figure 5B**). Moreover, miR-9 inhibition increased the protein levels of Hes1 and NICD compared with the GFP group, whereas additional treatment with zoanthamine resulted in lower levels of these proteins compared with the APP + miR-9 inhibitor group.

Discussion

The pathogenesis of AD involves several factors, including formation of Aβ from APP cleavage by gamma secretase [9]. Aβ formation results in neurotoxicity and contributes to the development of AD via loss of cognitive function [10]. Previous studies using different cellular models of AD have revealed that APP plays an important role in the development of AD [11]. The present study evaluated the molecular mechanisms underlying the AD-preventative effects of zoanthamine in a novel cellular AD model using western blot analysis and RT-PCR.

It was reported that zoanthamine attenuates neuroinflammation by reducing the production of reactive oxygen species [6] and possesses strong antioxidant and anti-inflammatory activities [6]. miR-9 enhances the differentiation of neurons and promotes neurogenesis by acting on various signalling molecules, including FoxG, Zfp521, STAT-3 and Hes1 [12]. In AD patients, expression of miR-9 was found to be reduced, and thus miR-9 may be a target for the management of AD [13]. Another study revealed that overexpression of miR-9 protected against neuronal injury in Aβ-induced neurotoxicity [14]. Our study suggests that zoanthamine attenuates apoptosis and enhances the viability of APP-treated NSCs.

The biological activities of stem cells and development of the nervous system are regulated by the Notch signalling pathway. Differentiation of NSCs was found to be enhanced by Notch signalling via increased miR-9 expression [15]. APP reduces the differentiation of neuronal cells by activating the Notch signalling pathway [16]. We found that treatment with zoanthamine in the presence of APP enhanced miR-9 expression and reduced Hes1 and NICD protein levels compared with APP alone. Moreover, Hes1 and NICD protein levels were increased by inhibition of miR-9, but zoanthamine prevented these increases.

Conclusion

In conclusion, treatment with zoanthamine promoted the differentiation of NSCs by regulating Notch signalling via increased miR-9 expression.

Acknowledgements

All authors of this manuscript thank the Applied Basic Research Foundation of Yunnan province (no. 2017FE468(-251)) and the Talent Development Program of The Second Affiliated Hospital of Kunming Medical University (no. RCPYXM2017-3-04) for providing the funding to conduct the present study.

Disclosure of conflict of interest

None.

Address correspondence to: Jie Zhang, Department of Hepatology, The First Affiliated Hospital of Kunming Medical University, Kunming 650032, China. Tel: 0086-0871-65324888; Fax: 0086-0871-65324888; E-mail: jgonzalez907@yahoo.com

References

[1] Metcalfe SM, Bickerton S, Fahmy T. Neurodegenerative disease: a perspective on cell-based therapy in the new era of cell-free nanotherapy. Curr Pharm Des 2017; 23: 776-783.

[2] Singh SK, Srivastav S, Yadav AK, Srikrishna S, Perry G. Overview of Alzheimer's disease and some therapeutic approaches targeting Aβ by using several synthetic and herbal compounds. Oxid Med Cell Longev 2015; 2016: 7361613.

[3] Galvan V, Jin K. Neurogenesis in the aging brain. Clin Interv Aging 2007; 2: 605-10.

[4] Lathia JD, Mattson MP, Cheng A. Notch: from neural development to neurological disorders. J Neurochem 2008; 107: 1471-81.

[5] Chow VW, Mattson MP, Wong PC, Gleichmann M. An overview of APP processing enzymes and products. Neuromolecular Med 2010; 12: 1-12.

[6] Guillen PO, Gegunde S, Jaramillo KB, Alfonso A, Calabro K, Alonso E, Rodriguez J, Botana LM, Thomas OP. Zoanthamine alkaloids from the zoantharian Zoanthus cf. pulchellus and their effects in neuroinflammation. Mar Drugs 2018; 16.

[7] Hsu YM, Chang FR, Lo IW, Lai KH, El-Shazly M, Wu TY, Du YC, Hwang TL, Cheng YB, Wu YC. Zoanthamine-type alkaloids from the zoanthid Zoanthus kuroshio collected in Taiwan and their effects on inflammation. J Nat Prod 2016; 79: 2674-2680.

[8] Yoshimura F, Tanino K, Miyashita M. Recent developments in the synthesis of zoanthamine alkaloids. Tetrahedron Lett 2014; 55: 2895-2903.

[9] O'Brien RJ, Wong PC. Amyloid precursor protein processing and Alzheimer's disease. Annu Rev Neurosci 2011; 34: 185-204.

[10] Crews L, Masliah E. Molecular mechanisms of neurodegeneration in Alzheimer's disease. Hum Mol Genet 2010; 19: R12-20.

[11] Singh VK, Kalsan M, Kumar N, Saini A, Chandra R. Induced pluripotent stem cells: applications in regenerative medicine, disease modeling, and drug discovery. Front Cell Dev Biol 2015; 3: 2.

[12] Coolen M, Katz S, Bally-Cuif L. miR-9: a versatile regulator of neurogenesis. Front Cell Neurosci 2013; 7: 220.

[13] Yuva-Aydemir Y, Simkin A, Gascon E, Gao FB. MicroRNA-9: functional evolution of a conserved small regulatory RNA. RNA Biol 2011; 8: 557-64.

[14] Ji Q, Ji Y, Peng J, Zhou X, Chen X, Zhao H, Xu T, Chen L, Xu Y. Increased brain-specific miR-9 and miR-124 in the serum exosomes of acute ischemic stroke patients. PLoS One 2016; 11: e0163645.

[15] Li N, Long B, Han W, Yuan S, Wang K. microRNAs: important regulators of stem cells. Stem Cell Res Ther 2017; 8: 110.

[16] Kwak YD, Marutle A, Dantuma E, Merchant S, Bushnev S, Sugaya K. Involvement of Notch signaling pathway in amyloid precursor protein induced glial differentiation. Eur J Pharmacol 2010; 650: 18-27.