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Original Article

Bazedoxifene decreases neuronal apoptosis via transforming growth factor-β-mediated AKT/GSK3β signal pathway in a cerebral hemorrhage rat model

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Abstract: Brain injury is the most common injury in human cerebrovascular disease and frequently leads to ischemic stroke. Bazedoxifene (BZA) is an efficient drug for the treatment of patients with ischemic brain damage. However, the potential mechanisms mediated by BZA remain unclear. The purpose of this study was to investigate neuroprotective effects of BZA and explore potential mechanisms mediated by BZA in a rat model of cerebral hemorrhage induced by ischemia reperfusion. A cerebral hemorrhage rat model was established and treated by BZA or vehicle over a 30-day period. Cerebral infarct volume, neurological functions, hippocampus apoptosis, neuron viability, and transforming growth factor (TGF)-β-mediated AKT/GSK3β signal pathway signaling pathways were analyzed after treatment with BZA. Our results demonstrate that BZA treatment improved cognitive ability and motor functions, and attenuated body weight loss compared to the vehicle group. BZA treatment also markedly decreased cerebral infarct volume, neurological functions, and hippocampus apoptosis compared with vehicle-treated rat after the 30-day treatment. In addition, BZA treatment improved cerebral water content (CWC) and blood brain barrier (BBB) disruption and increased neuronal viability compared to the vehicle group. Furthermore, BZA treatment up-regulated anti-apoptosis protein and down-regulated pro-apoptosis protein expression in neurons of the hippocampus in the cerebral hemorrhage rat model. BZA inhibited neuronal apoptosis through down-regulation of TGF-β-mediated AKT/GSK3β signal pathway. These results indicate that BZA could improve ischemia reperfusion-induced neuronal apoptosis by regulation of TGF-β-mediated AKT/GSK3β signal pathway.

Keywords: Bazedoxifene, cerebral hemorrhage, neuronal apoptosis, TGF-β, AKT/GSK3β

Introduction

Cerebral hemorrhage is a cardiovascular system disease, which often causes cerebrovascular death [1]. Previous reports have indicated that cerebrovascular injury caused by ischemia reperfusion frequently results in cerebral infarct, neurological functions dysfunction, and apoptosis of hippocampus [2-4]. It is widely accepted that neuronal apoptosis may lead to cognitive disorders in cerebrovascular injury induced by ischemia reperfusion [5-7]. Thereby, understanding the pathological processes of neuronal apoptosis is essential for improvement of cognition impairment in cerebral hemorrhage.

Bazedoxifene (BZA) is a compound with multifunctional activity in the treatment of human diseases [8]. BZA has been approved for use in the European Union for the treatment of osteoporosis and may represent a near-term therapeutic option for patients with advanced breast cancer [9]. A study showed that Bazedoxifene ameliorated homocysteine-induced apoptosis and accumulation of advanced glycation end products by reducing oxidative stress in MC-3T3-E1 cells [10]. However, the role of BZA in the cerebral hemorrhage has not been investigated.

Currently, transforming growth factor (TGF)-β is reported to association with brain injury and apoptosis of nerve cells in the hippocampus [11]. A previous study has provided an insight into understanding a novel role for betaig-h3 protein induced by TGF-β in the response of astrocytes to brain injury [12]. A report found that increasing of TGF-β expression may be one of indicators during acute brain injury caused by Toxocara canis in mice [13]. Endo et al.

have shown that the Akt/GSK3beta pathway might be involved in neuronal survival in acute brain injury after subarachnoid hemorrhage [14]. However, relationships between TGF-β and Akt/GSK3beta signal pathway have not clearly elaborated in neuronal apoptosis in rat model of cerebrovascular injury.

In this study, we first investigated the therapeutic effects of BZA in rat model of cerebrovascular injury. The possible mechanism of TGF-β-mediated Akt/GSK3β signal pathway was analyzed in neurons. We also analyzed whether decreased TGF-β expression could lead to reduced neuron apoptosis.

Materials and methods

Ethical statement

This study was approved by the Ethics Committee of Zhejiang Chinese Medical University (Hangzhou, China).

Cerebral hemorrhage rat model

Six-eight male Sprague-Dawley rats (280-320 g) were purchased from Shanghai Slack experimental animals Co., LTD (Shanghai, China). All rats were housed in a temperature-controlled facility at 23±1°C and relative humidity of 50±5% with a 12-hour light/dark cycle. A cerebral hemorrhage rat model was established using the modified ischemia reperfusion method [15]. Rats received right middle cerebral artery occlusion for 90 minutes and reperfusion by withdrawal of the filament at 37.0°C during and after surgery. Immediately, ischemia reperfusion-induced cerebrovascular injury rats were randomly divided into two groups (n=10 in each group) and received intravenous injection of BZA (10 mg/kg/day, Sigma-Aldrich) [16] or the same volume of PBS (Vehicle) [17]. The treatments were continued to 30 days.

Behavioral tests

Behavioral functional tests were performed including neurological deficits score and open-field tests. Neurological deficits score was measured using a modified scoring system [18]. Open-field tests (locomotor activity) were used to analyze the efficacy of BZA on ischemia reperfusion injury performed as described [19].

Analysis of brain water content

On day 30, brain water content was measured after ischemia reperfusion-induced cerebrovascular injury rat model after treatment with BZA as described report [20]. The brains of the rats were isolated as described previously [21]. Two hemispheres were weighed using an electronic analytical balance to obtain wet weights. The brain was dried in an electric oven at 100°C for 24 hours to analyze the water content in the intracerebral hemorrhage rat model. The brain water content was calculated as the following formula: (wet weight-dry weight/wet weight) × 100 (%).

Quantitative analysis of blood-brain barrier permeability

BBB leakage was assessed as previously described with slight modification [22]. The experimental rats were received 100 μl of a 5% solution of Evan's blue in BZA or saline administered intravenously 10 days following ischemia reperfusion-induced injury. Two hours after Evan's blue injection, cardiac perfusion was performed under deep anesthesia with 200 ml of saline to clear the cerebral circulation of Evan's blue. The brain was isolated and sliced. The two hemispheres were homogenized in 750 μl of N,N-dimethylformamide (DMF). Quantitative analysis of blood-brain barrier permeability was analyzed (λ_{ex} 620 nm, λ_{em} 680 nm) using Evan's blue content.

TGF-β overexpression

On day 30, neuron cells were isolated from experimental rats as referenced described [23]. Neuron cells (1×10^5) were cultured in six-well plate until 85% confluence and the media was then removed from the culture plate followed three PBS washes. Neuron cells were transfected by plentivirus-TGF-β (pTGF-βB) or plentivirus-Vector (pvector) using Lipofectamine 2000 (Sigma-Aldrich) according to the manufacturers' instrument. After 48 hours of transfection, TGF-β-overexpressed neuron cells were treated with BZA (1, 1.5, 2 and 2.5 mg/ml, Sigma-Aldrich) for further analysis.

Cells viability assay

Neuron cells (2×10^3 cells/well) were seeded in 96-well plates and cultured at 37°C for 12 hours. treated with 10 μl of MTT (5 mg/ml, Sigma-Aldrich) for 3 hours at 37°C. After incubation, Cells were captured with light microscopy (Bx51, Olympus Corporation, Shinjuku-ku, Japan) and purple formazan crystals were dissolved using isopropanol (15 μl, isopropanol). The absorbance was recorded on a micropla-

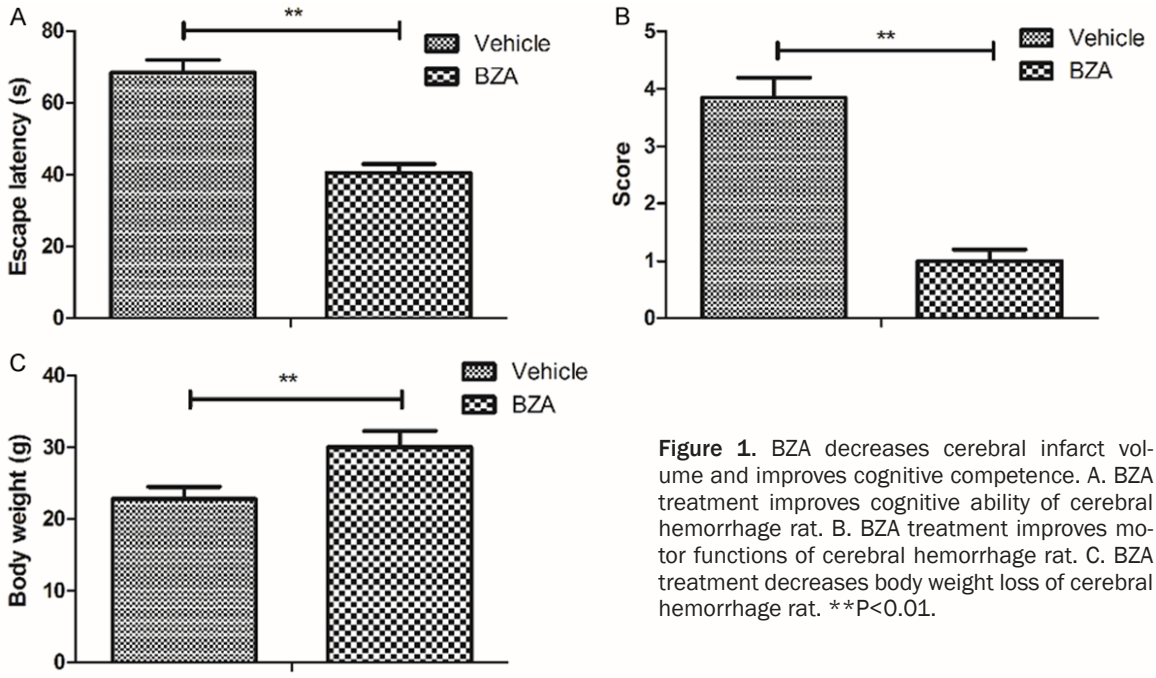


Figure 1. BZA decreases cerebral infarct volume and improves cognitive competence. A. BZA treatment improves cognitive ability of cerebral hemorrhage rat. B. BZA treatment improves motor functions of cerebral hemorrhage rat. C. BZA treatment decreases body weight loss of cerebral hemorrhage rat. **P<0.01.

te reader (Multiskan FC, THERMO SCIENTIFIC) at a wavelength of 570 nm. Neuronal viability was determined by percent of cell viability calculated as the ratio between mean absorbance of three samples and mean absorbance of controls.

Western blot

On day 30, neuron and TGF-β-overexpressing cells were lysed in RIPA buffer (M-PER reagent for the cells and T-PER reagent for the tissues, Thermo Scientific) followed homogenized at 4°C for 10 minutes. Protein concentration was measured by a BCA protein assay kit (Thermo Scientific, Pittsburgh PA, USA). A total of 20 μg protein extracts was electrophoresed on 12.5% polyacrylamide gradient gels and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Massachusetts, USA). The membranes were incubated in blocking buffer (5% milk) prior to incubation with primary antibodies at 4°C overnight. The primary rabbit anti-rat antibodies used in the immunoblotting assays were: TGF-β (1:1200, ab31013, Abcam), Bcl-2 (1:1000, ab692, Abcam), Abcam), Bcl-xl (1: 1200, ab32370, Abcam), caspase-8 (1:1000, ab25901, Abcam), caspase-3 (1:1200, ab2171, Abcam), AKT (1:500, ab151279, Abcam), pAKT (1:500, ab8805, Abcam), GSK3β (1:1000, ab-32391, Abcam) and β-actin (1:2000, ab8226, Abcam). After incubation, the membrane was washed three times in TBST and incubated with HRP-conjugated goat anti-rabbit IgG mAb (PV-6001, ZSGB-BIO, Beijing, China) for 1 hour at 37°C. After three-time washing in TBST, membrane was developed using a chemiluminescence assay system (Roche) and exposed to Kodak exposure film. Densitometric quantification of the immunoblot data was performed by using the software of Quantity-One (Bio-Rad).

TUNEL assay

Tissues in hippocampus or neuronal cells in cerebral hemorrhage rat model were analyzed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (DeadEnd™ Colorimetric Tunel System, Promega) according to the manufacturer's instructions. TGF-β-overexpressed cells were treated with BZA (2 mg/ml, Sigma-Aldrich) or PBS for 12 hours at 37°C. Cells were incubated TUNE (DeadEnd™ Colorimetric Tunel System, Promega). Cells were washed with PBST (Sigma-Aldrich) three times for 5 minutes at 37°C followed by incubated with 5% DPAI (Sigma-Aldrich) for 15 minutes at 37°C. Finally, images were captured with a ZEISS LSM 510 confocal microscope at 488 nm. The infarct volume was calculated by using the software of Developer XD 1.2 (Definiens AG, Munich, Germany).

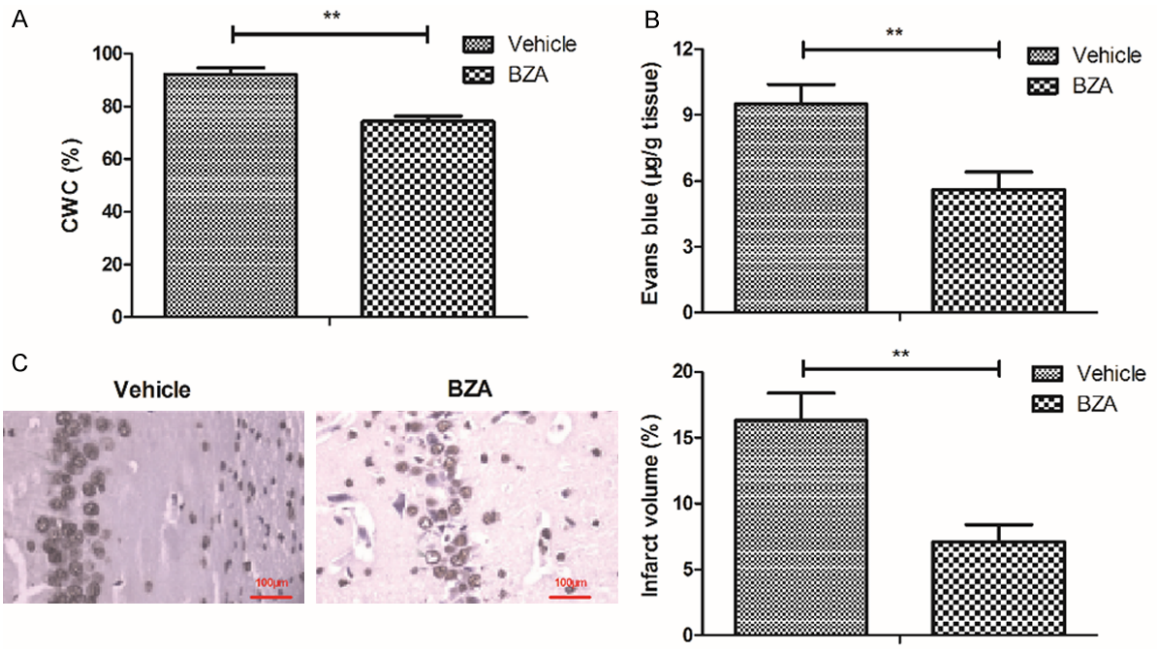


Figure 2. BZA improves CWC and BBB disruption in cerebral hemorrhage rat model. A. BZA treatment decreases the CWC of cerebral hemorrhage rat compared to vehicle group. B. BZA treatment decreases BBB disruption of cerebral hemorrhage rat. C. BZA treatment decreases cerebral infarct volume compared to vehicle group determined by TUNEL assay under ZEISS LSM 510 confocal microscope. **P<0.01.

Statistical analysis

Data are presented as means ± SD of triplicate. All data were analyzed by SPSS 19.0 software (SPSS, Chicago, IL, USA). Significant differences between two groups were analyzed by two-tail unpaired Student's *t*-test. Multiple groups differences were analyzed using one-way analysis of variance (ANOVA) followed Tukey HSD test. A *P*-value of <0.05 and <0.01 was considered to indicate a statistically significant.

Results

BZA decreases cerebral infarct volume and improves cognitive competence

The *in vivo* efficacy of BZA in cerebral hemorrhage rat model was first investigated. As shown in **Figure 1A**, BZA treatment markedly improved cognitive ability compared to vehicle group. BZA treatment improved motor functions, and attenuated body weight loss compared to vehicle group (**Figure 1B** and **1C**). These results indicate that BZA is an efficient drug for the treatment of cerebral hemorrhage rat model induced by reperfusion injury.

BZA improves CWC and BBB disruption in cerebral hemorrhage rat model

The effects of BZA on CWC and BBB disruption, as well as cerebral infarct volume were next investigated. BZA treatment significantly decreased the cerebral water content (CWC) compared to vehicle group (**Figure 2A**). As shown in **Figure 2B**, more Evans blue passed from the circulation through the BBB in the BZA group than vehicle, which suggests that blood brain barrier (BBB) disruption was decreased by BZA treatment in cerebral hemorrhage rat model (**Figure 2B**). BZA treatment also decreased cerebral infarct volume compared to vehicle group (**Figure 2C**). These results suggest that BZA is beneficial for improvement of cerebral hemorrhage-induced symptoms.

BZA treatment decreases neuron cells apoptosis via regulation of apoptosis-related protein

The neuronal viability and apoptosis was investigated in cerebral hemorrhage rat model. As shown in **Figure 3A**, BZA increased neuronal viability in cerebral hemorrhage rat model. TUNEL-positive neurons in hippocampus were markedly decreased by BZA treatment com-

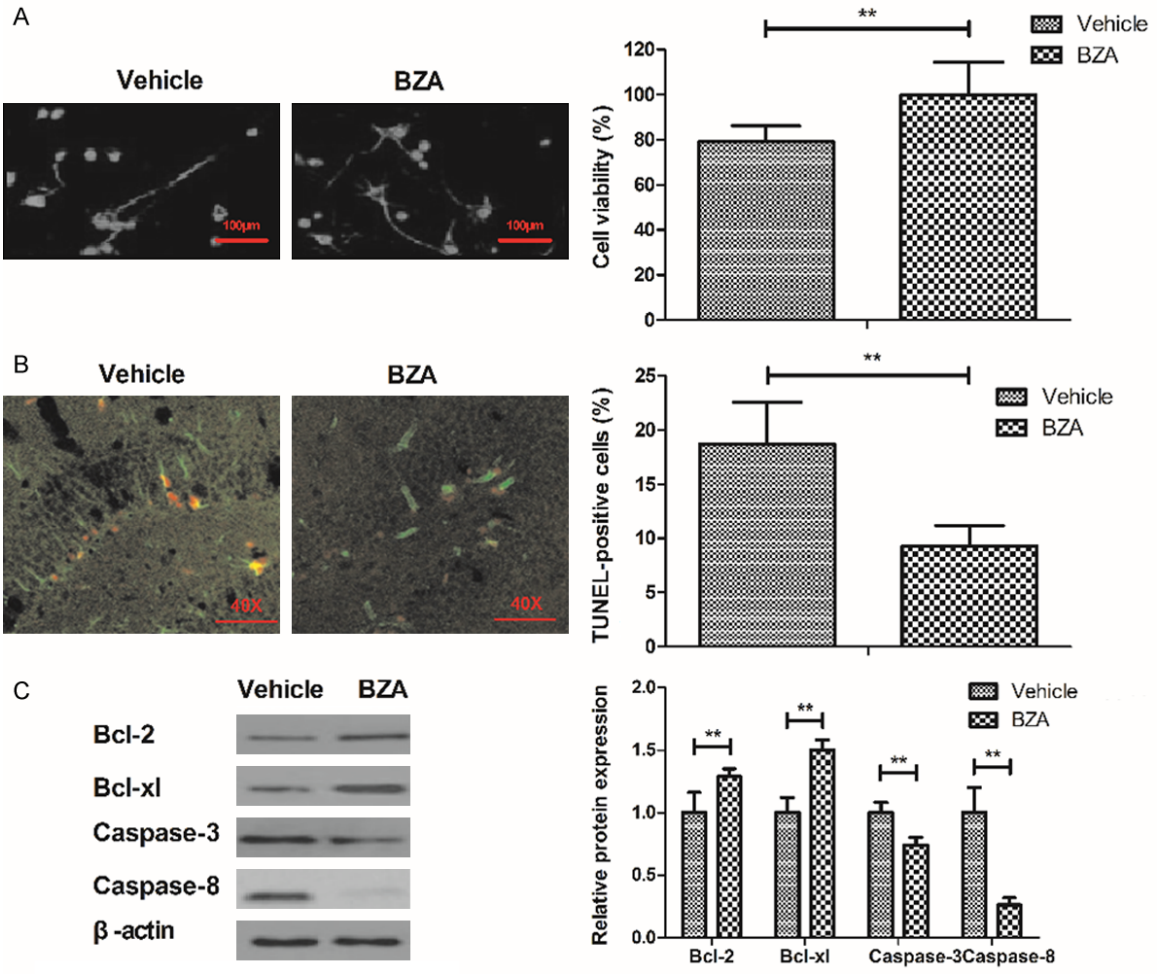


Figure 3. BZA treatment decreases neuron cells apoptosis via regulation of apoptosis-related protein. A. Neuronal viability in BZA and vehicle group under light microscope. B. BZA decreases TUNEL-positive neurons in hippocampus treatment compared to control group. C. BZA regulates apoptosis-related protein of Bcl-2, Bcl-xl, Caspase-3, and Caspase-8 in neuron cells.

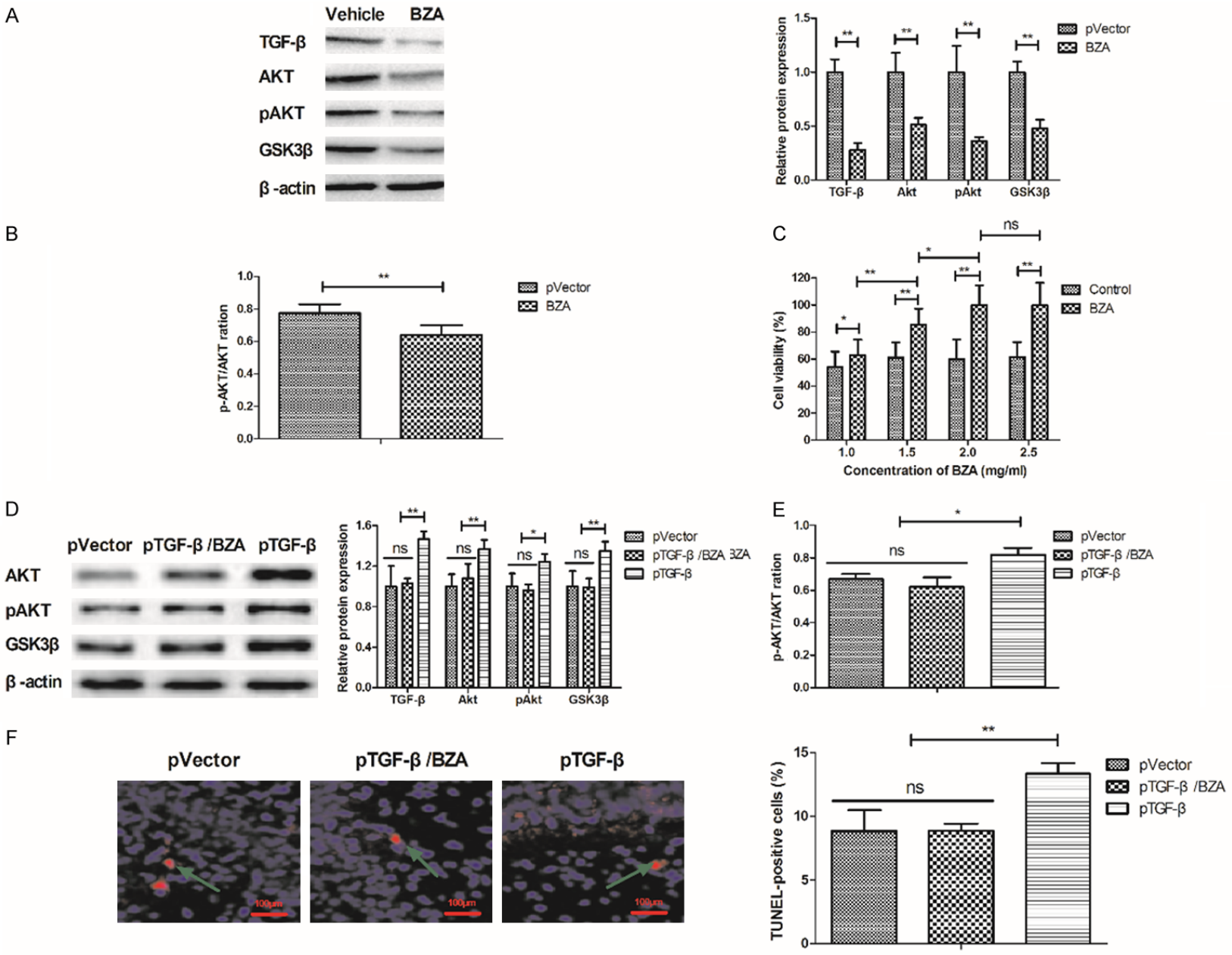
pared to control group (**Figure 3B**). Anti-apoptosis protein expression levels of Bcl-2 and Bcl-xl were up-regulated and pro-apoptosis protein expression levels of Caspase-3 and Caspase-8 were down-regulated by BZA treatment compared to control (**Figure 3C**). These results indicate that BZA could attenuate neuron cells apoptosis via regulation of apoptosis-related protein.

BZA ameliorates neuron cells apoptosis through regulation of TGF-β-mediated Akt/GSK3β signal pathway

The TGF-β-mediated AKT/GSK3β signal pathway was analyzed in neurons. As shown in **Figure 4A**, **4B**, BZA decreased TGF-β, Akt, pAKT, GSK3β expression as well as pAkt/Akt ratio in neurons. Addition of 2 mg/ml BZA could markedly increase viability of neurons, therefore, 2 mg/ml BZA was used for the *in vitro* assays (**Figure 4C**). TGF-β overexpression (pTGF-β) up-regulated both Akt and GSK3β expression levels, and abolished BZA-regulated Akt and GSK3β expression in neurons (**Figure 4D**). TGF-β overexpression abolished BZA-decreased pAkt/Akt ration in neurons (**Figure 4E**). TGF-β overexpression (pTGF-β) also canceled BZA-decreased apoptosis of neurons (**Figure 4F**) thus indicating that BZA can decrease neuron cells apoptosis through down-regulation of TGF-β-mediated Akt/GSK3β signal pathway.

Discussion

Currently, neuronal apoptosis plays essential role in the behavioral function loss in patients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TUNEL-positive cells (%)
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Figure 4. BZA ameliorates neuron cells apoptosis through regulation of TGF-β-mediated Akt/GSK3β signal pathway. (A, B) BZA decreases TGF-β, Akt, GSK3β expression (A) and pAkt/Akt ration (B) in neurons. (C) Effects of BZA (1.0, 1.5, 2.0 and 2.5 mg/ml) on viability of neurons. (D, E) TGF-β overexpression abolishes BZA-regulated Akt, GSK3β expression (D) and pAkt/Akt ration (E) in neurons. (F) TGF-β overexpression cancels BZA-decreased apoptosis of neurons. Arrow indicates the TUNEL-positive cells.

with cerebral hemorrhage [24]. Therefore, increasing anti-apoptosis effects may be contribute to the excitability in the hippocampus in the treatment of cerebral hemorrhage-induced nerve cell injury. In this study, the therapeutic effects of BZA were first analyzed in cerebral hemorrhage rat model induced by reperfusion injury. Findings in this study found that BZA efficiently decreased hippocampus cells apoptosis via down-regulation of TGF-β-mediated Akt/GSK3β signal pathway that is one of the novelties in this study.

A previous report has identified that alleviation of neural apoptosis can improve anti-BBB disruption after subarachnoid hemorrhage [25]. Dong et al. have identified that inhibition of apoptosis signaling attenuated early brain injury induced by subarachnoid hemorrhage [26]. Cerebral hemorrhage in the rat model increased apoptosis of nerve cells in the hippocampus. Evidence has also indicated that BZA ameliorates homocysteine-induced apoptosis and it may be a potent therapeutic drug for preventing Hcy-induced bone fragility [10]. In this work, we observed that BZA attenuated neuron cells apoptosis via increasing anti-apoptosis protein Bcl-2 and Bcl-xl, as well as decreasing pro-apoptosis protein caspase-3 and caspase-8. *In vivo* assays found that BZA improved cerebral hemorrhage-induced symptoms, such as cognitive ability, motor functions, and body weight loss, which may be an efficient drug for the treatment of cerebral hemorrhage rat model induced by reperfusion injury. However, further study should be performed to evaluate the therapeutic effects of BZA in the pathological processes in cerebral hemorrhage.

TGF-β can modulate microglial phenotype and promote recovery after intracerebral hemorrhage, suggesting that TGF-β1 may be a therapeutic target for the treatment of acute brain injury [27]. Here we found that BZA down-regulated TGF-β1 expression in neuron cells in hippocampus in cerebral hemorrhage rat model. Hong et al. have shown that hydrogen-rich saline can attenuate neuronal apoptosis in early brain injury and improve the neurofunctional outcome after subarachnoid hemorrhage via the Akt/GSK3β pathway [28]. Li et al. have found that NGF attenuated high glucose-induced endoplasmic reticulum stress, which could prevent Schwann cells against apoptosis by activating the PI3K/Akt/GSK3β and ERK1/2 pathways [29]. We found that 2 mg/ml of BZA could markedly improve viability of neuron cells in hippocampus *in vitro* assay. In this work, we found that BZA treatment down-regulated Akt/GSK3β signal pathway in neuron cells in hippocampus. Importantly, BZA treatment regulated neuron cells apoptosis in hippocampus via TGF-β1-mediated Akt/GSK3β signal pathway.

In conclusion, down-regulation of TGF-β1-mediated Akt/GSK3β signal pathway can be beneficial for inhibiting neuronal cell apoptosis in cerebral hemorrhage. Administration of BZA to disrupt TGF-β1-mediated Akt/GSK3β signal pathway resulted in neuroprotective for ischemia reperfusion-induced cerebrovascular injury, suggesting BZA may be a potential therapeutic agent for cerebral hemorrhage therapy.

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Disclosure of conflict of interest

None.

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