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ORIGINAL PAPER

Tribulusterine Containing *Tribulus terrestris* Extract Exhibited Neuroprotection Through Attenuating Stress Kinases Mediated Inflammatory Mechanism: In Vitro and In Vivo Studies

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Abstract

The present study has been aimed to explore the different secondary messengers of the inflammatory pathway NF-κB, kinases (JNK, P38MAPK, GSK3β/βcatenin), apoptosis pathway (Caspase-3 and AIF), and neuronal survival pathway (BDNF) in order to understand the neuroprotective mechanism of aqueous extract of *Tribulus terrestris* (AQTT). In primary cortical neurons, the ischemic condition was induced through oxygen–glucose deprivation (OGD). Anti-inflammatory activity of AQTT was evaluated in formalin induced inflammation model and carrageenan-induced paw edema test. The bilateral common carotid artery occlusion model was employed for whole animal studies. Treatment of AQTT (100 mg/kg) significantly reduced the inflammation induced by formalin and carrageenan. The neuroprotective mechanism of AQTT (50 and 100 mg/kg) was assessed by pre- and post-administration. The results indicate down regulation of kinases and NFkB, suggesting possible anti-inflammatory activity of AQTT. Additionally, AQTT down regulated both caspase dependent and independent apoptotic pathways suggesting its possible anti-apoptotic activity. The treatment of AQTT also reduced GSK3β levels and increased p-Ser9 GSK3β levels; stabilizing the unphosphorylated form of β-catenin and its translocation into the nucleus suggesting role of AQTT in neuronal survival and GSK3β mediated anti-inflammatory property. In comparison to pretreatment, post treatment of AQTT had lesser effects indicating tribulusterine standardized AQTT may have prophylactic effect. This study can be concluded with the thesis that AQTT has neuroprotective effect through alternating neuroinflammation, apoptosis, and promoting neuron survival. Being that it produced better effect with pretreatment, exploring this with thrombolytic drugs will be beneficial. For the first time AQTT has been reported for this indication.

Keywords *Tribulus* · Nerunjil · Apoptosis · Stress kinases · Neuroprotection · Ischemia

Introduction

Tribulus terrestris (*T. terrestris*) Linn, (Family: Zygophyllaceae) commonly known as Nerunjil, is one of the most widely used traditional medicine in India and China [1]. Our previous study has shown that standardized aqueous *T. terristris* extract attenuated hyperalgesia in diabetic neuropathic pain [2] through down regulation of oxidative stress and inflammatory mediators. Active role of *T. terrestris* in ameliorating mitochondrial dysfunction in H9c2 cells during ischemia has been reported [3]. In a recently reported study, the in silico study, inhibition of c-Jun terminal-NH2 kinase (JNK) pathway with alkaloids of *T. terristris* indicated possible anti-inflammatory activity of the herb [4]. In-vitro studies have shown that *N-trans*-ρ-caffeoyl tyramine isolated from *T.terrestris* exerted anti-inflammatory effects by down regulating COX2 and JNK pathway

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in lipopolysaccharide-stimulated RAW 264.7 cells [5]. Further, tribulosin, a saponin from *T. territris*, was found to be active against hypoxia/re-oxygenation induced injury in cardiac myocytes [6], in middle cerebral artery occlusion (MCAO) rat model [7] and in vincristine induced neuropathic pain model [8].

Ischemic stroke involves many molecular pathways with respect to neurodegeneration and neuroprotection such as cellular calcium homeostasis, neuronal excitotoxicity, free radicals, inflammatory cascade, and apoptosis [9].Hypoxic stress induced cellular injury involves activation of kinases pathways and NF-κB. Activation of this pathway stimulates the release of pro-inflammatory cytokines.They are the major central functioning molecules contributing neurodegenerative diseases.The inflammatory cascades are induced by molecules such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF-α), transforming growth factor (TGF-β), chemokines (CXC), chemokine cytokine-induced neutrophil chemo AQTTractant (CINC), monocyte chemo tactic protein-1 (MCP-1), inducible nitric oxide synthase (iNOS) produced by endothelial cells, activated astrocytes, microglial cells, and leukocytes [10, 11]. These processes are mediated by the mitogen-activated protein kinases (MAPKs) signal transduction pathway [12, 13] and the NFκB transcription factor, which exacerbates the immune response, lead to the most common form of ischemia/reperfusion-induced delayed neuronal cell death [14, 15].

Enhanced pro-inflammatory mediator release has been observed through the activation of microglia with GSK-3β stimulation [16, 17]. GSK-3β is constitutively active and ubiquitously expressed in various cell types especially neurons. It phosphorylates at ser9 and stabilizes β-catenin, which accumulates in cytosol and then translocates into the nucleus and complexes with T-cell factor/lymphoid enhancer factor (Tcf/Lef) reporter to initiate inflammatory response, neuronal survival, and synaptic plasticity [18, 19]. Further, GSK-3β, through caspase dependent (caspase-3) and independent (p53) mechanisms, promotes apoptosis. Therefore, inhibition of GSK-3β reversed the neuroinflammation through down regulation of TNFα through NF-κb, PI3K/Akt/JNK signaling pathway [20]. Wingless integration (Wnt), a canonical signaling pathway for neuronal survival, involves AXIN, adenomatous polyposis coli (APC), glycogen synthase kinase-3 (GSK-3β), and β-catenin complex [21, 22].

The apoptotic pathway in ischemia/reperfusion involves activation of cytochrome C/Caspase-3 or apoptosis inducing factor (AIF).This stimulus will lead to the cleavage of hundreds of potential substrates, resulting in programmed cell death [23, 24]. Despite inflammation and apoptosis induced cell death in neurons, neuronal survival pathway is the key factor that helps in recovery of cells from ischemic injury. The role of brain derived neurotrophic factor (BDNF) has been emphasized in neuronal survival, proliferation, and in differentiation and synaptic formation [25].

From the plethora of information available in literature, it has been observed that, *T. terrestris* possess anti-inflammatory activity. In ischemic states, the hypoxic and energy deprived induced stress will enhance the release of inflammatory mediators precipitating neuro-inflammation. Hence, in the present study, different secondary messengers of the inflammatory pathway NFκB, stress kinase (JNK, P38MAPK, GSK3β/β-catenin), apoptosis pathway (Caspase-3 and AIF), and neuronal survival pathway (BDNF) were investigated to explore the possible neuroprotective mechanism of *T. Terrestris* under hypoxic stress induced conditions; both in vitro and in vivo methods.

Materials and Methods

Chemicals and Reagents

Cytosine β-D-arabinofuranoside, all-trans retinoic acid, Eagle's minimal essential medium (MEM) with nonessential amino acids (NEAAs), Ham's F12, RPMI medium, fetal bovine serum (FBS), and sodium pyruvate were purchased from Sigma-Aldrich, MO, USA. Caspase-3 enzyme assay kit was obtained from BioVision, Milpitas, CA, USA. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Amersham™ Hybond™-enhanced chemiluminescent (ECL) Western blot nitrocellulose membrane was purchased from GE Healthcare Life Science, Chalfont, and Buckinghamshire, UK. Chemiluminescent HRP substrate (Pierce ECL) was obtained from Thermo Scientific, Vernom Hills, IL, USA.

Aqueous Extract of *Tribulus terrestris* (AQTT) Preparation and HPLC Quantification

Aqueous extract of *Tribulus terrestris* (AQTT), prepared as per Ayurvedic principles, and was provided by N. Rama Varier Ayurveda foundation as a gift sample. The active alkaloid tribulusterine or perlolyrine [26], was quantified by HPLC technique.

In Vitro Studies

Primary Cortical Neuronal Cultures and Stress Induction

Mouse primary cultures of cortical neurons were extracted according to a protocol mentioned previously, with minor modifications [27, 28]. Brain cortices from fetal brains of 14-day pregnant female C57BL/6 mice were used in

the study. **C**old Hanks' balanced salt solution (HBSS) medium (Fisher Scientific, Hanover Park, IL, USA) was used. Approximately, 2×10^5 neurons/well were seeded in a 24-well plate coated with poly-L-lysine (0.1 mg/ml) for cell viability studies and 2×10^6 neurons/60 mm dish were seeded for Western blot analysis. All treatment experiments were performed on day 5 after plating cells.

The cells were dissociated and resuspended in neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing penicillin and streptomycin (50 U/ml of medium), glutamine (0.5 mM), and 2% B27 serum-free supplement (Invitrogen, Carlsbad, CA, USA). Approximately, 2×10^5 neurons/well were seeded in a 24-well plate coated with poly-L-lysine (0.1 mg/ml) for cell viability studies and 2×10^6 neurons/60 mm dish were seeded for western blot analysis. The cell cultures were maintained at 37 °C in 95% air and 5% CO_2. Cortical neurons were treated with cytosine β-D-arabinofuranoside (Sigma-Aldrich, St. Louis, MO, USA) at a 5-µM concentration on day 2 in vitro (DIV 2) for 24 h to curb glial cell growth. To induce stress, cortical neuronal cells growth medium were deprived of oxygen and replaced with glucose free HBSS (OGD model). This was initiated by AnaeroGen (OXOID, Germany sachet containing ascorbic acid) in hypoxic chamber [29]. In the OGD/reperfusion (OGD/R) model, cells were subjected to OGD for 1 h, followed by reperfusion for 24 h. AQTT was dissolved in neuronal cells growth medium and was incubated with cells after they were subjected to OGD for 1 h.

Cell Viability

Briefly, primary cultures of cortical neurons were incubated with MTT reagent for an additional duration of 4 h. The medium was discarded and the formazan blue insoluble crystals that formed in the cells were dissolved with 150 µl of DMSO. The percentage toxicity was calculated as per our previous study [30].

In Vivo Studies

Animals

Male C57BL/6 mice (20–25 g) were used in the ischemic study. All animals were housed at 22 ± 1 °C with a 12 h light/dark cycle and fed a standard pellet diet with tap water ad libitum. The experimental protocols were approved by the Institutional Animal Ethical Committee and experiments were performed in accordance to the CPCSEA guidelines for ethical use of animals proposal number 162/2014/IAEC dated 07.10.2014.

Bilateral Common Carotid Artery Occlusion Model (BCCAO)

Bilateral common carotid artery occlusion model was performed as per Tulsulkar and Shah, 2013, with minor modifications [31]. C57BL/6 mice (25–30 g) were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (20 mg/kg). A midline incision in the neck region was made and a dissection was made between the sternocleido mastoid and the sternohyoid muscles parallel to the trachea and both the common carotid arteries were exposed. Then arteries were isolated and occluded with aneurysm clips for 15 min. Ischemic duration was measured from the application of the last clip to the left common carotid artery. After 15 min, both clips were removed, followed by reperfusion of blood flow and this was confirmed in every case by direct inspection of each artery under a microscope. After suturing, antibiotics were administered to the mice and returned to their home cage.

Animal Groupings and Drug Treatment

Animals were divided into six groups: group 1 sham operated (naïve group) and remaining five groups were vehicle (0.3% CMC) treated BCCAO mice, AQTT pretreated (50 and 100 mg/kg) mice and, AQTT post treated (50 and 100 mg/kg) mice [2, 7, 8]. The study plan was to understand the mechanism of action of AQTT in inflammatory pathways. Hence, AQTT effect has not been compared with a standard drug. Further, the dose dependent effect of AQTT was tested by administering two doses (50 and 100 mg/kg).

In pretreatment, the mice were treated with test drugs orally for 7 days prior to BCCAO (N = 9). Similarly, in post treatment, test drugs were administered orally for 7 days after BCCAO (N = 9). In our earlier studies, we have shown time dependent changes of inflammatory marker in ischemic condition. Hence, tissues were harvested (from 3 mice) on the 3rd day for second messenger measurements in order to analyze the pathways [32]. The experimental design regarding the treatment schedule is depicted in Fig. 1.

Behavioral Assessment

Functional recovery studies such as locomotor activity and grip strength assessment were performed as per protocol on 7th day after BCCAO surgery for all the treatment groups. The cortical section of the mouse brain was isolated 72 h after surgery in both pre and post-treatment group for Western blot analysis.

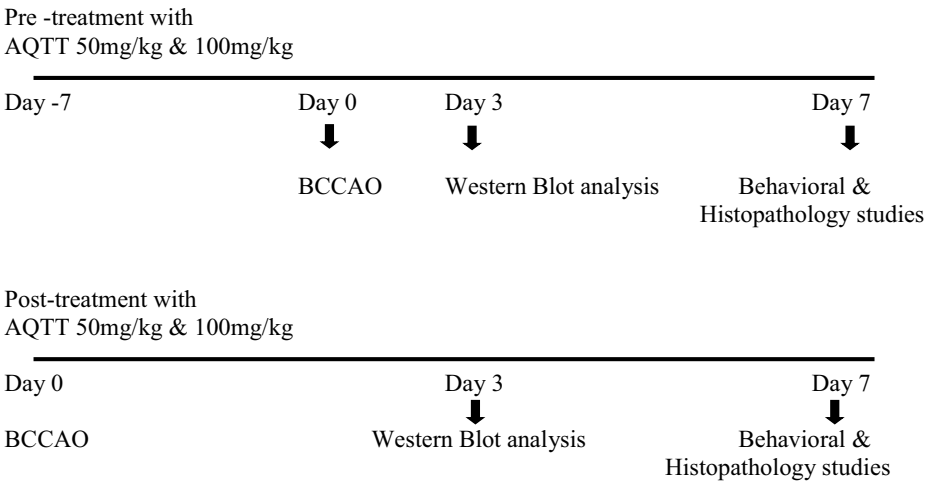


Fig. 1 Experimental design with treatment schedule

Sub Cellular Fractionation

Briefly, to obtain sub cellular fractions, brain cortices were dissected, weighed, and homogenized using ice cold lysis buffer [250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 10 mMKCl, 20 mM HEPES (pH 7.5), 1mMdithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride, 10 mM sodium orthovanadate, 20 mM sodium pyrophosphate and protease inhibitor cocktail]. Tissue homogenates were kept on ice for 15 min and then centrifuged at 10,000×*g* for 15 min to sediment nuclear pellets. The supernatant obtained was the cytosolic fraction. Nuclear pellets were re suspended in NET buffer [20 mM HEPES pH 7.9, 20% glycerol, 0.5M NaCl, 1.5 mM MgCl2, 1% Triton-X-100, 1 mM DTT, 1 mM PMSF, 50 mM sodium fluoride, sodium ortho vanadate, 20 mM sodium pyrophosphate, and protease inhibitor cocktail (Sigma Aldrich,USA) to yield nuclear fraction [27, 33].

Western Blot Analysis of Protein

Protein concentration was determined by Bio-Rad Bradford reagent (Bio-Rad Laboratories, Hercules, CA), and the protein samples were analyzed by loading equivalent amount of total cytoplasmic and nuclear proteins (40 µg) onto 10% SDS-polyacrylamide gels. The proteins were transferred from the gel to PVDF membrane (Biorad, USA) and blocked with 5% dry nonfat milk and/or 3% BSA for 1 h at RT, followed by overnight incubation at 4 °C with the respective antibodies : Rabbit anti-JNK (1:500), Rabbit anti-p38MAPK (1:500), and Rabbit anti-NFkB (1:1000), Rabbit anti-Cytochrome C, Rabbit anti-caspase-3, and Rabbit anti-AIF antibodies were used in following dilutions (1:1000), Rabbit anti-GSK-3β (1:1000), Mouse anti-S9-phospho GSK-3β (1:1000), Rabbit β-catenin (1:2000), and Rabbit anti-BDNF (1:1000). Following this step, goat anti-rabbit IgG-HRP and goat anti-mouse IgG1-HRP secondary antibodies were added to the membrane in a separate procedure according to the primary antibody used in the previous step and incubated for 2 h at room temperature. The membrane was developed and detected by Gel Doc system, G: Box (Syngene, Frederick, MD, USA). The cytosolic proteins were normalized with sham and β actin as internal control and nuclear proteins were normalized with sham and Histone as internal control.

Nissl Staining

The mouse brain was carefully removed after 7 days and post fixed in 10% neutral-buffered formalin for 3 days. Coronal sections of paraffin embedded tissue were de-paraffinized in xylene and in graded series of ethanol; incubated with 20 µg/ml of proteinase. Using a rotating microtome, 4 µm paraffin-embedded coronal sections were made and after standard dehydration and diaphanization procedures, slides that contained sections were immersed in distilled water and submerged in 0.2% cresyl violet solution (Nissl staining) for 5 min. The slides were then rinsed in distilled water and cleared in xylene, and then cover slipped with xylene using Permount and visualized using TS100 eclipse microscope (Nikon microscope Inc, NY, USA). The degree of damage in the hippocampal CA1 region (four samples from each group) that was evident in the Nissl staining on the 7th day after ischemia was semi-quantitatively scored from 0 to 3 based on the percentage of neurons damaged irreversibly [34]:

0: No morphological signs of damage and very few dark stained cells.

1: 0–30% (meager eosinophillic or dark neurons).
2: 30–60% (dark stained cell bodies with shrunken cell).
3: 60–100% (severe lesions with dark stained degenerative neurons and shrunken cell bodies).

Statistical Analysis

The results were expressed as arithmetic mean and their standard deviations. The collected data was subjected to one-way analysis of variance ANOVA followed by post hoc Tukey's multiple comparison test for all experiments. Nissl staining the semi quantitative scores were (medial value for each samples) analyzed by ANOVA. Probability values of less than 0.05 were considered to be significant. The analysis was carried out using Graph pad prism software (version 4.03).

Results

Active Constituents

The AQTT extract was standardized using HPTLC and contained not less than 0.4 g%w/w of total alkaloids. HPLC techniques were employed to quantify tribulusterine/perlolyrine. A standard plot was made at various concentrations (1-100 µg/ml) to determine the unknown concentration in the extract. The AQTT extract was found to contain 0.54 mg%w/w of tribulusterine (data are shown in Supplementary).

Effect of AQTT on Cell Viability and Different Biomolecules of Primary Neuronal Culture Exposed to OGD

Cell Viability Growing of primary neuronal cells in oxygen glucose deprivation medium followed by reperfusion (OGD/R) resulted in cell loss as indicated by decreased percentage cell viability. Treatment of AQTT (25 µg/ml) significantly protected the primary neuronal cells exposed to hypoxic and glucose deprivation [$F(4,40) = 6.26$ $p < 0.001$]. At 50 µg/ml AQTT did not show significant protection though a trend was observed (Fig. 2a).

OGD Effect Mimicking the ischemic condition of primary neuronal culture to OGD/R for 1 h resulted in significant increase in inflammatory markers JNK [$F(2,6) = 12.69$ ($p < 0.05$)], GSK3β [$F(2,6) = 103.0$ ($p < 0.001$)], and p38MAPK [$F(2,6) = 110.5$ ($p < 0.01$] protein levels after 24 h in comparison to DMSO treated neuronal cells. Similarly, after 1 h of OGD, the apoptotic protein levels after 24 h have shown significant increase in cytochrome C [$F(2,6) = 162.5$ ($p < 0.01$)] and caspase 3 [$F(2,6) = 531.7$ ($p < 0.001$)] levels (Fig. 2b).

AQTT Treatment Treatment of AQTT (25 µg/ml) after OGD exposure in primary neuronal culture Attenuated the elevated inflammatory markers as observed by decreased kinases JNK ($p < 0.01$), GSK3β ($p < 0.001$), and p38MAPK ($p < 0.001$) in cytosolic fraction in comparison to OGD exposed cells. Further, AQTT (25 µg/ml) significantly reduced the cytochrome C ($p < 0.001$) and caspase 3 ($p < 0.001$) levels in comparison to OGD challenged neuronal cells. NFκB could not be detected in nuclear fraction of primary neuronal cells in both control and AQTT treatment. Likewise, caspase independent pathway protein AIF was not detected in primary neuronal culture (Fig. 2b).

Effect of AQTT on Locomotor Activity and Grip Strength

Locomotor activity in BCCAO mice was found to be significantly reduced ($p < 0.01$) in comparison to sham operated mice. Pre- and post-treatment of AQTT (100 mg/kg) significantly increased [$F(5,30) = 6.794$; $p < 0.05$] the locomotor activity of BCCAO mice in comparison to vehicle treated BCCAO mice. AQTT (50 mg/kg) did not alter the locomotor activity of BCCAO mice (Fig. 3a).

The muscle tone assessment of BCCAO mice using grip strength parameter is represented in Fig. 5b. No significant effect [$F(5,30) = 1.76$] of ischemic induction on muscle tone alterations was observed in BCCAO mice. All the treatments remain unaltered (Fig. 3b).

Effect of AQTT on Different Biomolecules in Nuclear Fraction of Ischemic Brain Tissue

Inducement of global ischemia is mice elevated the NFκB ($p < 0.001$), AIF [$F(3,8) = 12.12$ ($p < 0.01$)], and β-catenin ($p < 0.05$) levels in nuclear fraction of BCCAO mice brain samples in comparison to sham operated mice.

Pretreatment of AQTT (50 & 100 mg/kg) significantly increased [$F(3,8) = 89.26$; $p < 0.01$] and decreased ($p < 0.05$) the NFκB levels respectively in comparison to vehicle treated BCCAO mice. The β-catenin level was found to be significantly elevated [$F(3,8) = 22.17$;

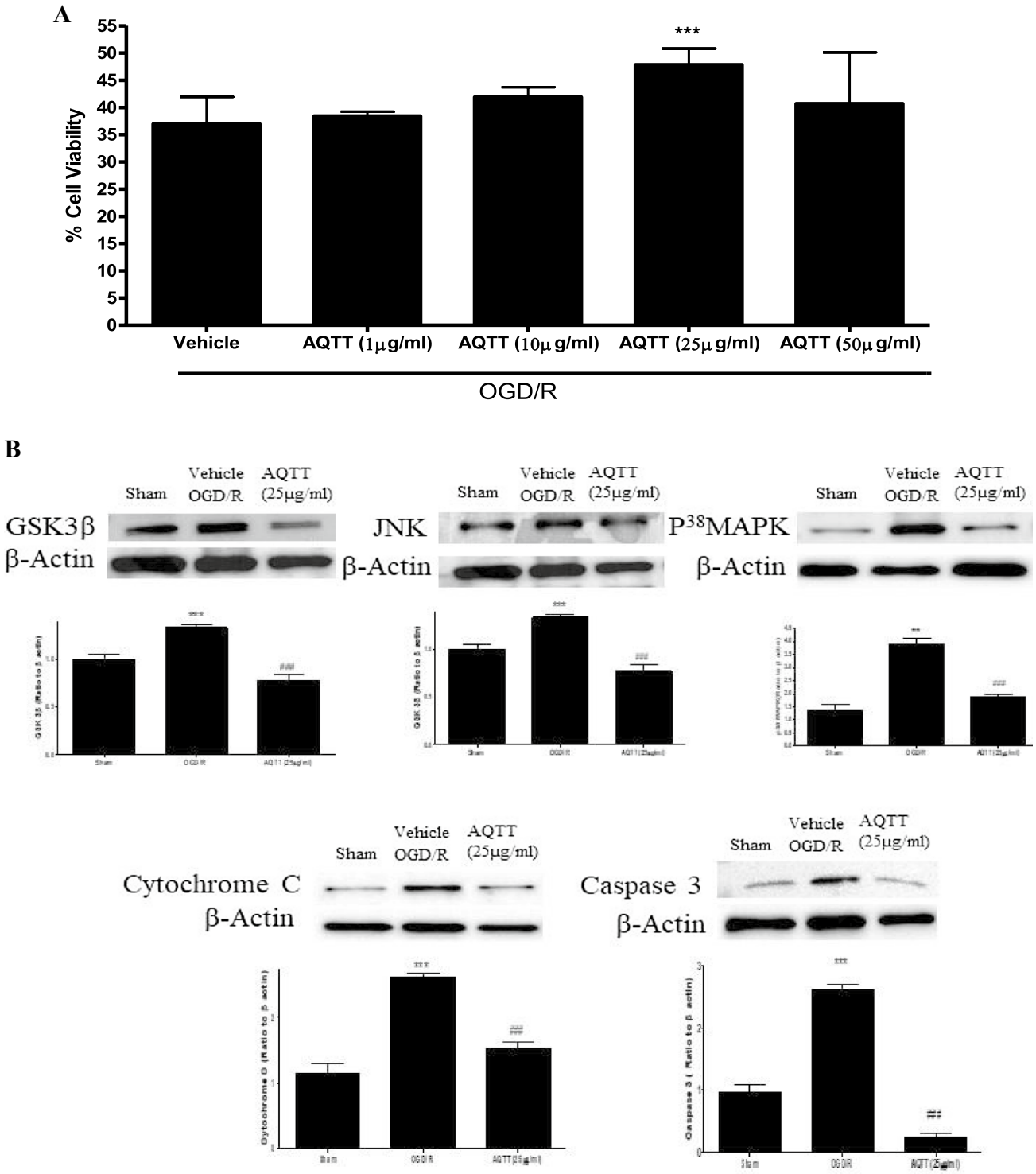


Fig. 2 Effect of AQTT on primary cortical neurons after OGD/R: **a** Phase contrast images of primary cortical neurons at DIV2 and cell viability assay. Statistical significance ***$p < 0.001$ as compared to OGD/R. **b** Represents protein levels of GSK3β, JNK, p38MAPK, Cytchrome C, Caspase 3 and β-actin serves as internal control. All data are expressed as mean ± SD and *,**,***$p < 0.05$, $p < 0.01$, $p < 0.001$ as compared to DMSO treated cells. ##, ###$p < 0.01$, $p < 0.001$ as compared to OGD/R

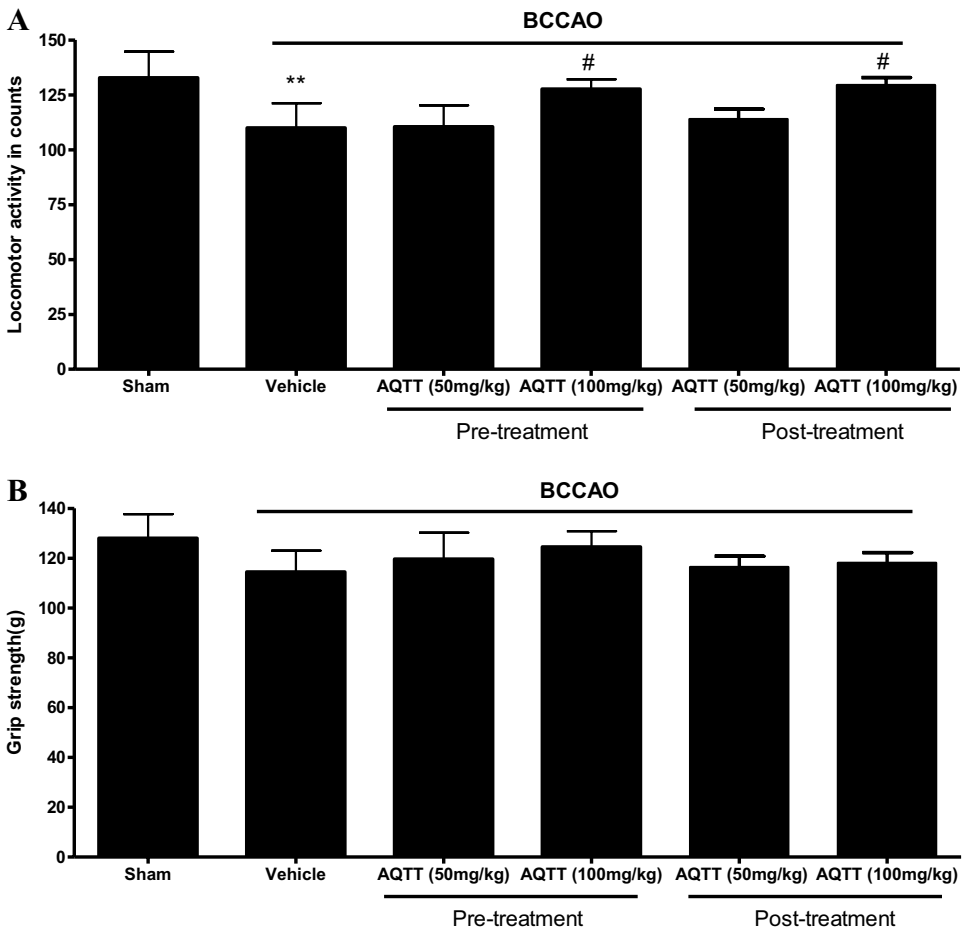


Fig. 3 Effect of AQTT on functional recovery, **a** locomotor activity and **b** grip strength post BCCAO evaluation on 7th day. All data are expressed as mean ± SD and statistical significance *$p < 0.05$ compared to sham operated mice. $^{\#}p < 0.05$ compared to vehicle treated BCCAO mice

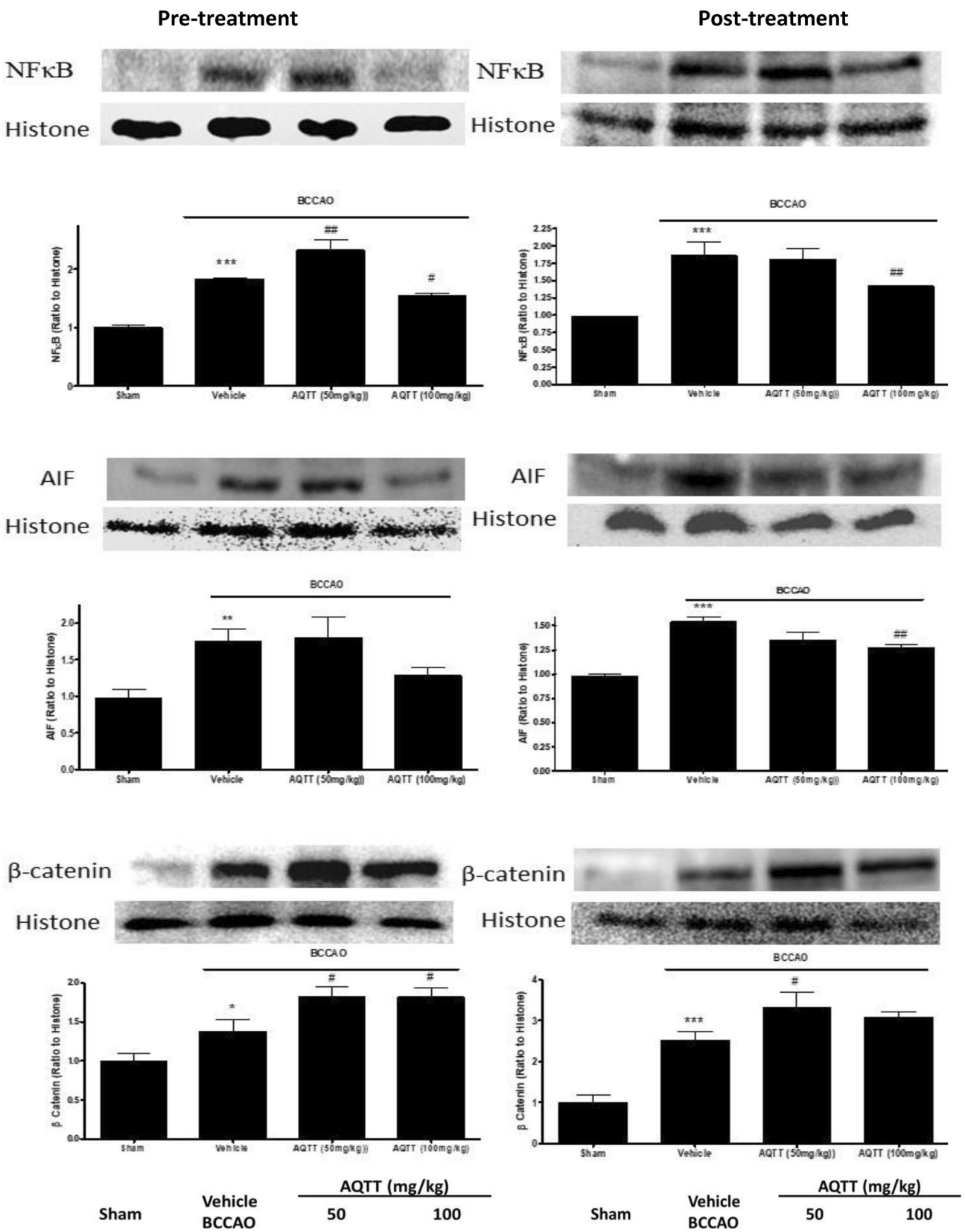
$p < 0.05$] with AQTT (50 and 100 mg/kg) treatment in comparison to BCCAO mice. The AIF level remains unaltered with AQTT treatment (Fig. 4a).

In post treatment AQTT (100 mg/kg) significantly reduced [$F(3,8) = 33.38$; $p < 0.01$] the nuclear NFκB and AIF activity [$F(3,8) = 46.51 (p < 0.01)$] induced due to ischemia. The β-catenin activity was found to be elevated with AQTT 50 mg/kg treatment [$F(3,8) = 50.19 (p < 0.05)$] in comparison to BCCAO vehicle treated mice (Fig. 4b).

Effect of Pretreatment of AQTT on Different Biomolecules in Cytosolic Fraction of Ischemic Brain Tissue (Fig. 5)

Cytosolic fraction of ischemia brain samples have shown significantly increased JNK[$F(3,8) = 69.13 (P < 0.01)$], p38MAPK [$F(3,8) = 139.7 (p < 0.01)$], cytochrome c[$F(3,8) = 13.38 (p < 0.05)$], and caspase 3 [$F(3,8) = 15.88 (p < 0.001)$] levels in comparison to sham operated mice. Further, the levels of BDNF [$F(3,8) = 144.1 (p < 0.001)$], β-catenin [$F(3,8) = 27.12 (p < 0.05)$], and pGSK3β:GSK3β ratio[$F(3,8) = 35.03 (p < 0.05)$] were found to be significantly decreased in the cytosolic fraction of ischemic mice.

Pretreatment of AQTT (100 mg/kg) significantly decreased the JNK level ($p < 0.001$) in cytosol in comparison to BCCAO vehicle treated mice. The p38 MAPK level ($p < 0.001$) and cytochrome c level ($p < 0.01$) were found to be decreased with AQTT (50 and 100 mg/kg) treatment in BCCAO mice. The effect was found to be dose dependent. The β-catenin level was found to be elevated with AQTT [50 ($p < 0.05$) and 100 mg/kg ($p < 0.001$)] treatment in comparison to BCCAO vehicle treated mice. In this parameter, AQTT exhibited a dose dependent effect. The cytosolic BDNF parameters exhibited biphasic response with AQTT (50 and 100 mg/kg) treatment. At 50 mg/kg, BDNF level was found to be decreased ($p < 0.001$), whereas with 100 mg/kg, it was found to be significantly increased ($p < 0.01$) in comparison to vehicle treated BCCAO mice. Caspase 3


Pre-treatment
Post-treatment
NFκB
Histone
BCCAO
NFκB (Ratio to Histone)
Sham
Vehicle
AQTT (50mg/kg))
AQTT (100mg/kg)
AIF
AIF (Ratio to Histone)
β-catenin
β Catenin (Ratio to Histone)
AQTT (mg/kg)
50
100

◂**Fig. 4** Effect of AQTT on protein levels of NFκB, AIF, β-catenin in nuclear fraction (pre and post treatment). **a** Pre-treatment, **b** post-treatment. Histone serves as internal control for nuclear fraction. All data are expressed as mean ± SD and statistical significance *,**,***$p < 0.05$, $p < 0.01$, $p < 0.001$ in comparison to sham operated mice. #,##$p < 0.05$, $p < 0.01$, $p < 0.001$ as compared to vehicle treated BCCAO mice

activity and pGSK3β:GSK3β ratio were found to be significantly decreased ($p < 0.01$) and increased ($p < 0.001$) respectively with AQTT 100 mg/kg treatment in comparison to BCCAO mice.

Effect of Post Treatment of AQTT on Different Biomolecules in Cytosolic Fraction of Ischemic Brain Tissue (Fig. 6)

Global ischemia in mice significantly increased the cytosolic JNK [$F(3,8) = 10.27$ ($p < 0.01$)], p38MAPK [$F(3,8) = 5.69$ ($p < 0.05$)], cytochrome c [$F(3,8) = 16.2$ ($p < 0.05$)], caspase [$F(3,8) = 6.65$ ($p < 0.05$)] activities in comparison to sham operated mice. The levels of β catenin [$F(3,8) = 31.22$ ($p < 0.05$)] and pGSK3β:GSK3β ratio [$F(3,8) = 34.16$ ($p < 0.001$)] were found to be significantly decreased in BCCAO mice in comparison to vehicle treated sham operated mice.

Post treatment of AQTT (100 mg/kg) significantly decreased the JNK ($p < 0.01$) and caspase levels ($p < 0.05$) in ischemic mice in comparison to BCCAO vehicle treated mice. AQTT (50 mg/kg) did not alter the JNK and caspase levels. AQTT [50 ($p < 0.01$) and 100 mg/kg ($p < 0.001$)] treatment for 7 days after global ischemia in mice significantly elevated the cytosolic β-catenin and pGSK3β:GSK3β ratio in comparison to vehicle treated BCCAO mice and this effect of AQTT was found to be dose dependent. Cytochrome c level was found to be significantly decreased with AQTT [50 ($p < 0.05$) and 100 mg/kg ($p < 0.01$)] treatment. AQTT has shown dose dependent effect in the reduction of cytochrome C levels. The p38MAPK and BDNF cytosolic levels remain unaltered with AQTT treatment.

Effect of AQTT on BCCAO-Induced Delayed Neuronal Death in Hippocampal CA1 Region

Nissl staining ($p < 0.001$) histopathological analysis of hippocampal CA1 region after 7 days of ischemia induction has shown neuronal loss in vehicle treated BCCAO mice in comparison to sham operated mice. Severe lesions with dark stained degenerative neurons and shrunken cell bodies of neuron in vehicle treated BCCAO indicates neurodegeneration.

Pretreatment of AQTT (100 mg/kg) has shown significant improvement in neurodegeneration. The post treatment of AQTT (100 mg/kg) ($p < 0.05$) in BCCAO mice exhibited significant [$F(5, 18) = 12.98$; ($p < 0.05$)] reduction in severity of neuronal damage as evident by very few dark stained cells with more number of normal viable cells in hippocampal CA1 region (Fig. 7).

Discussion

AQTT Protects Primary Cortical Neurons Controls

It is well known that, the activated JNK and p38MAPK mainly function as inflammatory mediators of cellular stress in cerebral I/R injury by phosphorylation of intracellular enzymes, cytosolic proteins, transcription factors, and leads to production of inflammatory mediator and apoptosis [35]. In our study, OGD exposure for a period of 1 h followed by reperfusion for 24 h, up regulated the stress kinases JNK, p38MAPK, and apoptotic pathway proteins. Previous study has shown up-regulation of nitric oxide, TNF-α, IL-1β, ERK1/2, JNK1/2, and p38MAPK in primary cortical neurons exposed to 2 h of OGD followed by 24 h of reperfusion [36]. AQTT has shown increase in cell viability in primary cortical neurons exposed to OGD/R injury. Treatment of AQTT down regulated these stress kinase proteins, signifying the anti-inflammatory property of AQTT and its' possible anti-inflammatory pathway mechanism in exhibiting neuroprotective activity. Further, AQTT down regulated apoptosis pathway protein (cytochrome C dose dependently and caspase 3), suggesting the anti-apoptotic role through attenuating the caspase dependent pathway.

AQTT Ameliorated Inflammation and Global Cerebral Ischemia Induced Damage

In our study we have observed anti-inflammatory activity of tribulosterine containing AQTT in both formalin, carrageenan model (refer Supplementry Data) as well as LPS treated primary neuronal cells. AQTT down regulated inflammatory pathway protein NFκB. Earlier studies have shown that topical application of TT extract in atopic dermatitis mice model controlled the skin inflammation and this effect was found to be mediated through modulation of calicum channel and mass cell stabilization [37]. TT protected the endothelial function in hypertension

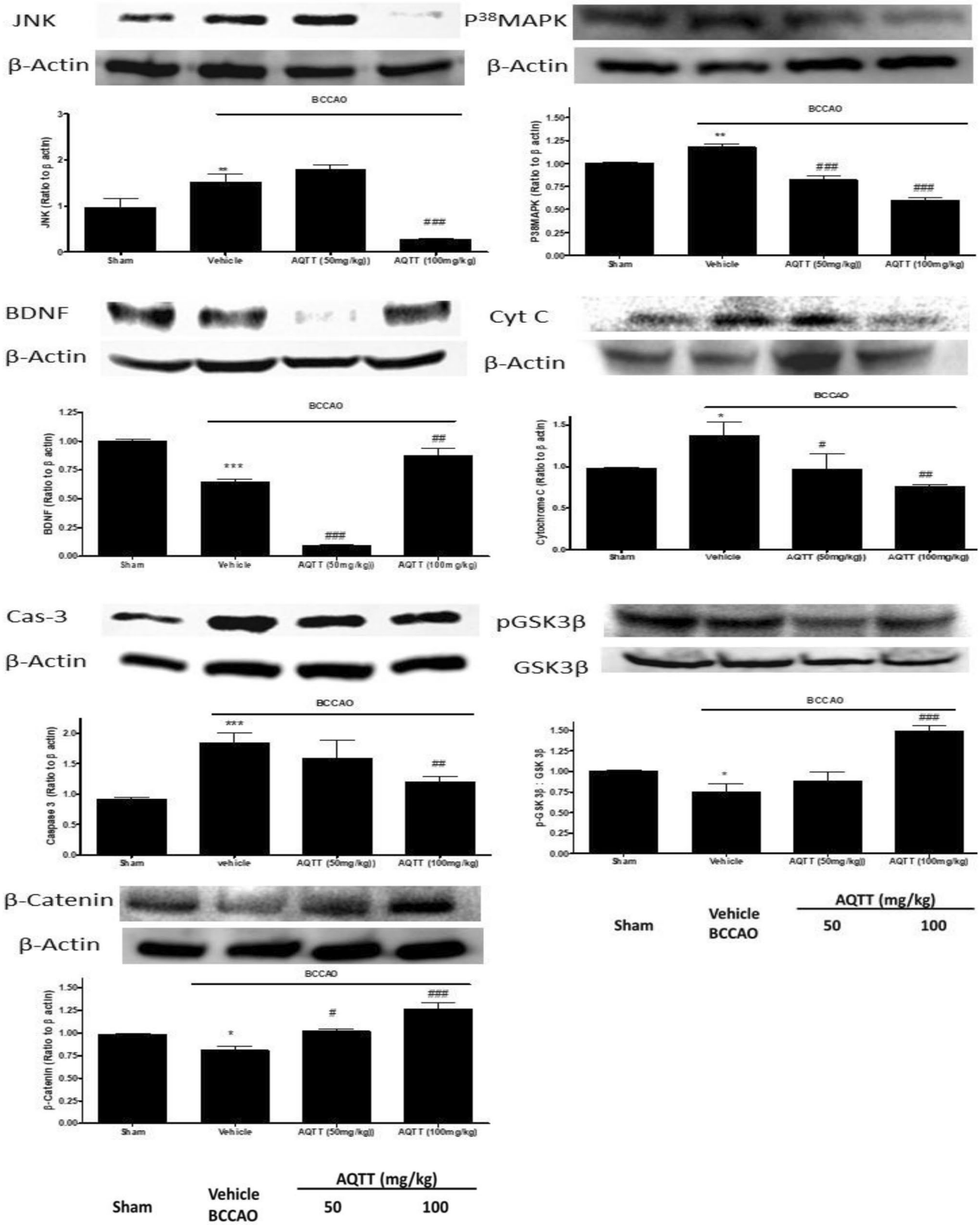


Fig. 5 Pre-treatment effect of AQTT on protein levels of JNK, p38MAPK, Cytochrome C, Caspase 3, pGSK3β:GSK3β, β-catenin and BDNF in BCCAO mice. β-actin serves as internal control for cytosolic fraction. All data are expressed as mean ± SD and statistical significance *,**,***$p < 0.05$, $p < 0.01$, $p < 0.001$ as compared to sham operated mice. #,##,###$p < 0.05$, $p < 0.01$, $p < 0.001$ as compared to vehicle treated BCCAO mice. Western blot

related endothelial injury through decreased Erk2, FAK and NFkB expression [38]. Tribulusamide D an active principle of TT produced anti-inflammatory acitivity by attenenuating inflammatory mediators expression [39] these studies support our present findings. Further, TT have altered the production of nitric oxide, and the expression of proinflammatory cytokines and COX-2 in LPS stimulated RAW 264.7 cells [5, 40]. Recently, in obesity related glomerulopathy model treamtent of TT for 8 weeks protected the kidney function and improved the acute phase biochemical and immunity [41]. Ozawa et al. [42] reported that ischemia–reperfusion produced a strong activation of the JNK, ERK, and p38 enzymes in the CA1 region after transient global ischemia. Present study results have shown 15 min of transient global ischemia produced a significant increase in levels of JNK and p38MAPK. AQTT reduced stress kinases such as JNK and p38MAPK level. Systemic administration of SP600125, a small molecule JNK-specific inhibitor, diminished JNK activity after ischemia and attenuated ischemia induced mitochondrial translocation of Bax and Bim, release of cytochrome c and Smac, and activation of caspase-9 and caspase-3 [43]. JAK2/ STAT3 and or PI3K/ AKT pathways mediated endothelial functional protection [44] and attenaution of chemical induced pancreatitis has been reported with TT extract [45]. These observations clearly suggest that AQTT possesses anti-inflammatory property by down regulating stress kinases and nuclear translocation of NFκB.

AQTT Promote Neuronal Survival by GSK-3β/β-Catenin Pathway and Confirms Its Anti-inflammatory Property

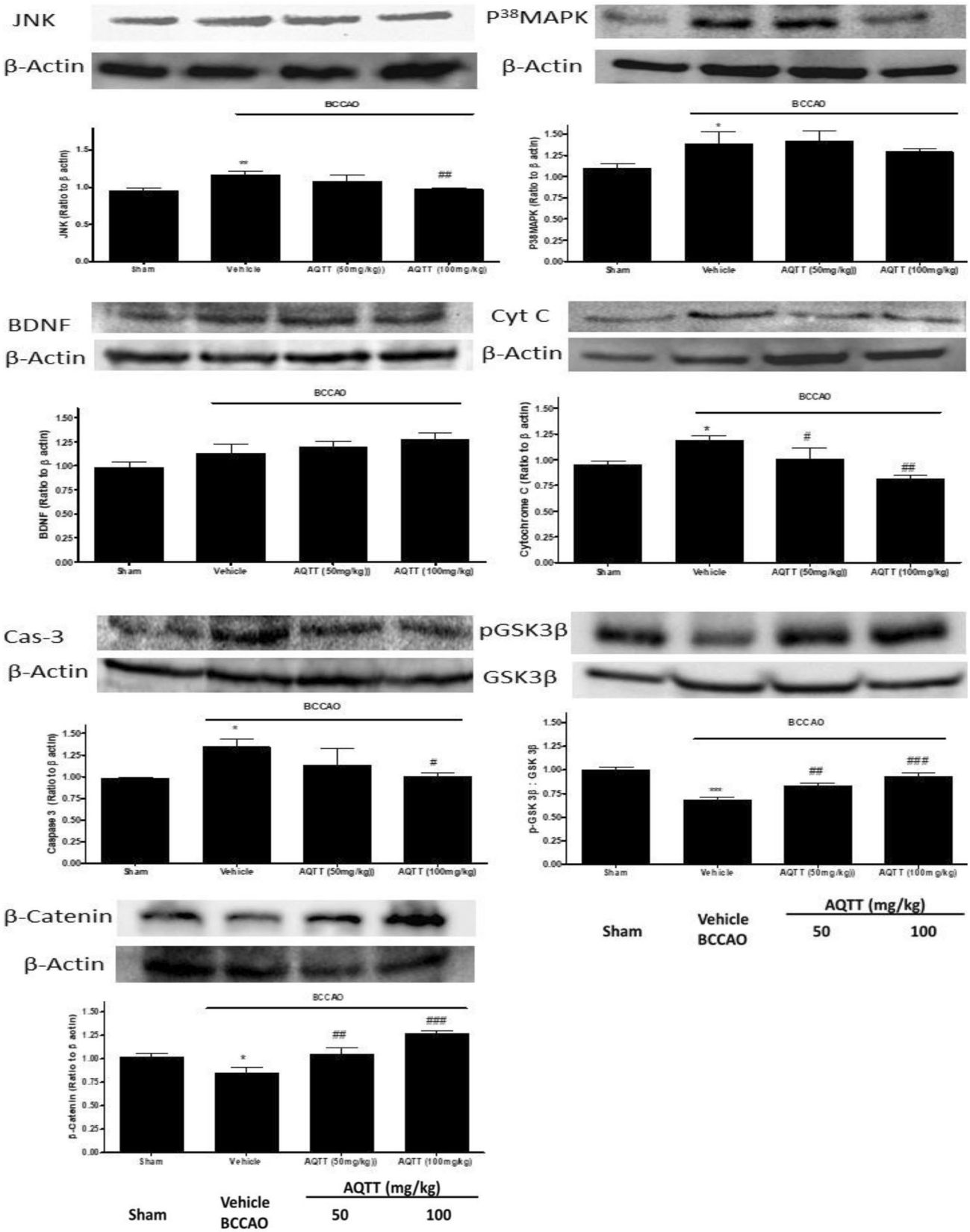
GSK-3β acts as a center bridge in a wide spectrum of signaling mechanism [46]. Many studies have proven that GSK3β promotes inflammation, partly by regulating key transcription factors in the inflammation signaling pathway such as NLRP3/IL-1β, NFκB [47]. Further studies have shown inhibitors of GSK3 act on both arms of the inflammatory response, reducing inflammatory cytokines, and increasing anti-inflammatory cytokines such as IL10 [48]. Ischemic condition leads to up-regulation of GSK-3β, post translational phosphorylation, and downstream signaling proteins in propagating neurodegeneration [49]. A recent study has shown that GSK-3β and pser9-GSK-3β (promotes cell survival) were significantly changed in pyramidal cells or astrocytes in the gerbil hippocampal CA1 area following 5 min of transient cerebral ischemia [50]. In our study, AQTT reduced the GSK3β levels and increased the pSer9-GSK3β level dose dependently. pSer9-GSK3β is known to stabilize the unphosphorylated form of β-catenin, which translocates into the nucleus and activates genes that regulate neuronal homeostasis and promote cell survival [51]. In the present study, ischemic insult has shown to reduce transcriptional activity of β-catenin by cytosolic degradation. Also, AQTT stabilized β-catenin in cytosolic fraction. Further, it also increased the nuclear translocation of β-catenin in both doses of post treatment of AQTT in dose dependent manner. β-catenin translocates into the nucleus, where it acts as a transcription factor and forms a complex with TCF and LEF-1 for transcription of multiple target genes [52].

AQTT Attenuates Apoptosis in Global Cerebral Ischemia Induced Damage

AQTT down regulated both caspase dependent and independent pathway. It is evident that cytochrome c is translocated from the mitochondria to the cytosolic compartment. In cytosol, cytochrome c activates caspase 3, which is a key regulator of apoptosis after transient or permanent focal cerebral ischemia [53]. AQTT reduced both levels of cytochrome *c* and caspase 3 in cytosol. Apoptosis-inducing factor (AIF) is a major caspase-independent protein involved in neuronal cell death after global cerebral ischemia (GCI). Thal et al. investigated the role of AIF in C57/Bl6 or low AIF expressing Harlequin mutant mice (AIF_{low}) and their wild-types. Their report shows that hippocampal CA1 neurons cell death, following GCI, was associated with nuclear translocation of AIF [54]. In our study, the nuclear levels of AIF were reduced by AQTT, which suggest that they also attenuate apoptosis through caspase independent pathway.

AQTT Pre-treatment Versus Post-treatment

In this report, we demonstrated that most of the signaling molecules required for neurogenesis were up-regulated in pre-treatment paradigms at day 7. Although both treatments were able to protect mice brain from ischemic stroke, which is evident from Nissl staining, the difference in expression levels may be attributed to duration of AQTT doses administered in both pre and post treatment. We conclude that pretreatment of AQTT promoted up regulation of neuronal survival better as compared to post treatment; therefore it


JNK
β-Actin
BCCAO
JNK (Ratio to β actin)
Sham
Vehicle
AQTT (50mg/kg))
AQTT (100mg/kg)
P38MAPK
β-Actin
BCCAO
P38MAPK (Ratio to β actin)
Sham
Vehicle
AQTT (50mg/kg))
AQTT (100mg/kg)
BDNF
β-Actin
BCCAO
BDNF (Ratio to β actin)
Sham
Vehicle
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AQTT (100mg/kg)
Cyt C
β-Actin
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Cytochrome C (Ratio to β actin)
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β-Actin
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Caspase 3 (Ratio to β actin)
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◂**Fig. 6** Post-treatment effect of AQTT on protein levels of JNK, p38MAPK, Cytochrome C, Caspase 3, pGSK3β:GSK3β, β-catenin and BDNF in BCCAO mice. β-actin serves as internal control for cytosolic nuclear fraction. All data are expressed as mean ± SD and statistical significance *,***$p < 0.05$, $p < 0.001$ as compared to sham operated mice. #,##,###$p < 0.05$, $p < 0.01$, $p < 0.001$ as compared to vehicle treated BCCAO mice

can be stated that it acted like a neuroprotective agent by controlling inflammation.

Conclusion

In conclusion, AQTT down-regulated the stress kinases and also inhibited the nuclear level of NFkB, suggesting that it possesses anti-inflammatory properties. Furthermore, it was evident that AQTT mediated neuronal survival through down regulation of apoptotic pathway and promotion of GSK3β/*β*-catenin. AQTT may serve as a promising therapeutic strategy for ischemic stroke by promoting regenerative repair.

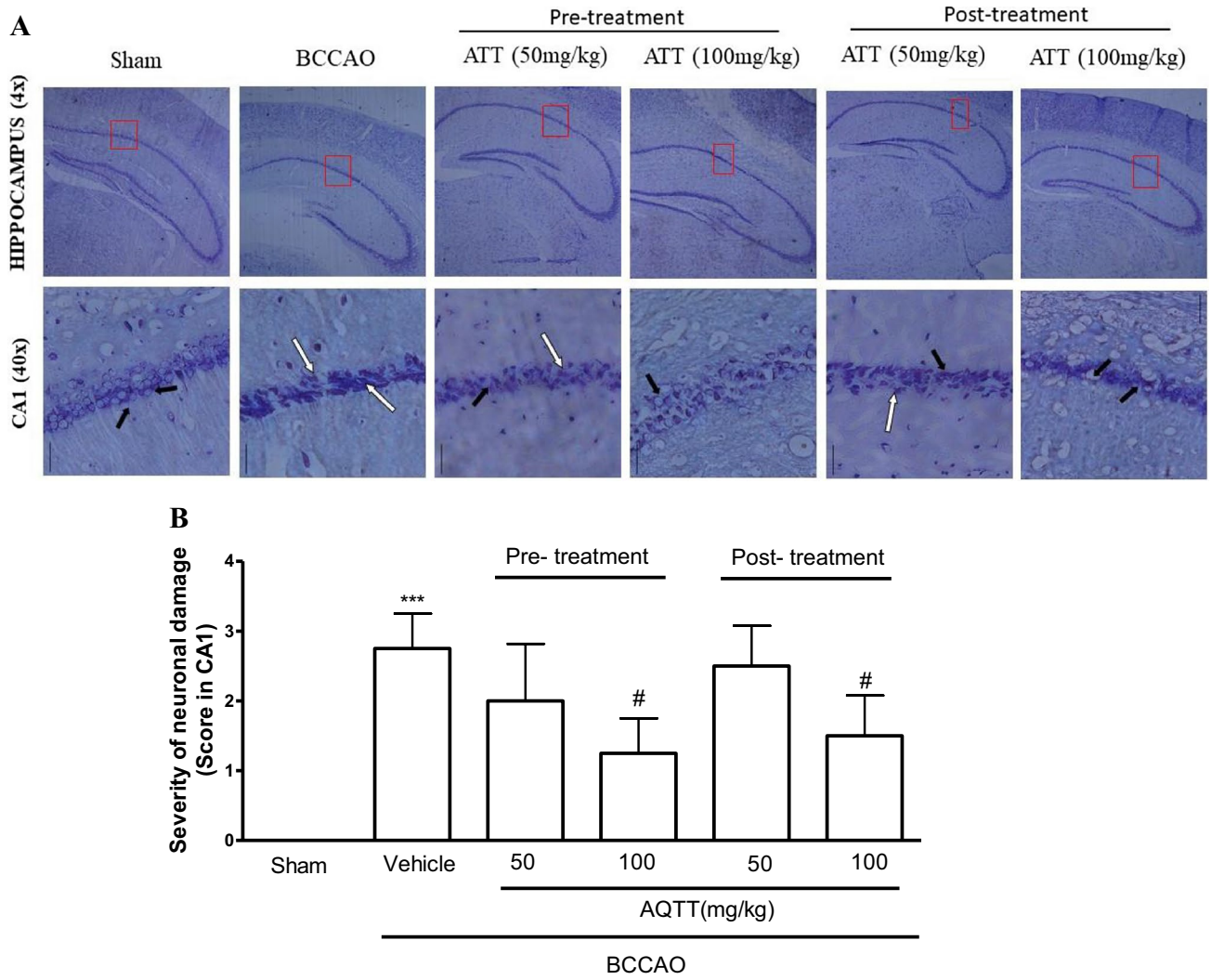

Fig. 7 **a** Representative microphotographs of Nissl staining in hippocampal region after 7th day of global cerebral ischemia in mice. Each column below is magnified view of the rectangular region of the above column. White arrow heads indicate degenerative cells with a dark shrunken nucleus and black arrow heads indicate normal viable cells. Bar = 500 µm. **b** All data expressed as mean ± SD (n = 4) and statistical significance ***$p < 0.001$ compared to sham operated mice. #$p < 0.05$ compared to vehicle treated BCCAO mice

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All protocols were approved by the Institutional Animal Ethical Committee, PSG IMS&R, and experiments were performed in accordance to the CPCSEA guidelines for ethical use of animals.

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