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Original Research

Jatrorrhizine Hydrochloride alleviates tert-butyl hydroperoxide-induced endothelial cell injury through its anti-inflammatory activity and PPAR-γ activation

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Abstract: The aim of this study was to investigate whether Jatrorrhizine hydrochloride (JAH) can attenuate oxidative damage of endothelial cells by regulating mitochondrial function and inflammatory response. It was found that JAH inhibited tert-butyl hydroperoxide (t-BHP)-induced oxidative damage in mouse brain endothelial cells (MBECs) by increasing cell viability and inhibiting cell apoptosis. Moreover, JAH significantly inhibited the production of reactive oxygen species (ROS) and lipid peroxidation. It enhanced mitochondrial membrane potential (MMP) and maintained ATP synthesis. In addition, JAH regulated the expressions of inflammatory cytokines and increased the activation of endothelial nitric oxide synthase (eNOS). The protective effect of JAH was related to the protein expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) gene. In conclusion, these results suggest that JAH may have therapeutic potential for ischemic stroke associated with endothelial dysfunction through its antioxidant and anti-inflammatory properties.

Key words: Jatrorrhizine hydrochloride; Endothelial cells; Ischemic stroke; Endothelial dysfunction.

Introduction

Ischemic stroke, also known as cerebrovascular accident, which is a worldwide health problem, induces cerebrovascular and neuronal damage (1, 2). Although the exact molecular mechanism of apoplexy-induced neuronal damage has not been fully elucidated, oxidative stress and endothelial dysfunction are considered to play important roles in its pathogenesis (3, 4). Brain endothelial cells (BECs) are the basis for the formation of blood brain barrier (BBB) which can prevent uncontrolled entry of ions, amino acids and peptides into the brain. In ischemic conditions, endothelial dysfunction leads to the formation of brain edema and the penetration of inflammatory cells into parenchyma tissue, which may be related to the acceleration of nerve injury (5).

The renin-angiotensin system (RAS) plays an important role in regulating fluid volume and blood pressure (6). In the brain, angiotensin II (Ang II) exerts different effects on fluid balance through two subtypes of G protein-coupled receptors: Ang II 1 and Ang II 2 (AT1-R and AT2-R) (7). The AT1-R blockers (ARBs) have been used as antihypertensive drugs for many years (8). Several clinical trials have shown the effectiveness of ARBs in primary and secondary prevention of stroke in patients with hypertension and/or elevated cardiovascular risk (9). Natural products used as medicines, are characterized by small side effects, multiple targets, and strong efficacy, and are well-patronized by the people (10). Therefore, efforts are on to identify a natural product that can offer protection from ischemic stroke, and also treat same.

Jatrorrhizine hydrochloride (JAH) is a tetrahydroisoquinoline alkaloid and the main component of coptidis rhizome (the rhizome of *Coptis chinensis* Franch, Ranunculaceae). It has antagonistic effects against arrhythmias induced by myocardial ischemia and reperfusion in rats. It has been found that jatrorrhizine plays an important neuroprotective role in apoptosis of cortical neurons induced by hydrogen peroxide (11). However, the role of JAH in ischemic stroke needs further studies. In this study, the effect of JAH on t-BHP-induced endothelial cell damage in mouse endometrial cells (MBECs) was investigated. The results showed that the mechanisms of JAH-induced protection involved oxidative stress, mitochondrial dysfunction, endothelial nitric oxide synthase (eNOS) and peroxisome proliferator-activated receptor-γ (PPAR-γ) pathways.

Materials and Methods

Primary MBECs were obtained in 2-month-old C57BL/6 mice, as previously reported (12). The brain was removed from the mice, and the meninges were stripped and cut. The tissue was dissociated in MEM-HEPES solution containing 20 U/ml papain and 250 U/ml DNA type I IV. The dissected brain tissue was

ground and added to a 10-ml tube containing 22% bovine serum albumin. It was then centrifuged at 1000g for 20 minutes at 4 °C . The separated cells were then washed and suspended in an endothelial cell growth medium composed of Hams F12 supplemented with 10% fetal bovine serum, heparin, ascorbic acid, L-glutamine and endothelial cell growth supplements. The MBECs were cultured in a CO_2 incubator at a humidity of 5% at 37°C, and half of the culture medium was replaced every other day.

Cell viability assay

Cell viability was determined using WST-1 assay kit (Beyotime, Nanjing) according to standard procedures. The MBECs were cultured in a micro-medium at a density of 3 x 10^5 cells/100 µl. After treatment with t-BHP and JAH, 10 µl WST-1 was added to each well and incubated at 37°C for 4 hours. Then, in the absence of cells, 100 ml medium and 10ml WST-1 were added to each well, and the absorbance was used as blank value. The cells were shaken thoroughly on a shaker for 1 minute, and the absorbance of the sample was measured with an ELISA reader.

Flow cytometry

After 24 hours of t-BHP and JAH treatment, the MBECs were collected, washed with cold Ca^{2+} PBS, and suspended in conjugated buffer. The cell suspension was transferred to a tube and stained with Annexin V (AV) and propylene iodide (PI) for 15 minutes in the dark at room temperature. The stained cells were analyzed using flow cytometry (BD, USA) after adding 400 µl buffer. The fluorescence emission wavelength was 530 nm and > 575 nm. FlowJo analysis results were used.

Measurement of intracellular ROS production

Intracellular ROS levels were quantified with H2DCF-DA probe. The MBEC cells were incubated with H2DCF-DA (10 µM) at 37°C for 1 hour in the dark, and then suspended in PBS. The fluorescence intensity of H2DCF-DA was measured under Olympus BX60 microscope to quantify intracellular ROS (at excitation wavelength of 480 nm, and emission wavelength of 530 nm).

Measurement of lipid peroxidation

Malondialdehyde (MDA) and 4-hydroxy nonanenal (4-HNE) are two indicators of lipid peroxidation. Cell Bio labs kit was used to determine the contents of these two indicators according to the manufacturer's instructions. The absorbance of the sample was measured in an ELISA reader. The results were expressed as multiples of the control group value.

Measurement of mitochondrial membrane potential (MMP)

Mitochondria membrane potential (MMP) depolarization results in loss of Rh123 in mitochondria and decrease in intracellular fluorescence. After treatment and washing with PBS, Rh123 was added to MBECs to a final concentration of 10 mM at 37°C for 30 minutes. The fluorescence was measured using Olympus BX60 microscope with appropriate fluorescent filter, at excitation wavelength of 480 nm, and emission wavelength of 530 nm.

Measurement of mitochondrial ATP generation

ATP synthesis in isolated mitochondria was determined based on luciferase/fluorescein system. Mitochondrial-rich precipitation of 30 µg was suspended in 100 µl buffer A (150 mM KCl, 25 mM Tris-HCl, 2 mM potassium phosphate, 0.1 mM $MgCl_2$, pH 7.4) containing 0.1% bovine serum albumin (BSA), 1 mM malate, 1 mM glutamate and buffer B (0.8 mM luciferase, 0.5 M triacetate and 20 mg/ml luciferase, pH 7.75,). The reaction was initiated by adding 0.1 mM ADP, and it was monitored for 5 minutes using enzyme labeling.

Total RNA was prepared from MBECs (13). The expressions of eNOS and PPAR-γ mRNA were assayed using real-time reverse transcription-polymerase chain reaction (RT-PCR) on iQ5 polychromatic real-time PCR detection system (Bio-Rad, Hertfordshire, UK). The specific primers used were:

eNOS: forward: 5'-ACC CTC ACC GCT ACA ACA TC-3', reverse, 5'-GCC TTC TGC TCA TTC TCA TCC TCC AG-3';

PPAR-gamma: forward: 5'ACC - CAC AAT GCC ATC AGG TTT GG-3', reverse, 5'-GTG ATT CCG TTG TCT TTC CC-3'.

In the reaction, according to the manufacturer's instructions, 1µl cDNA of each sample was mixed with SYBR Green JumpStart Taq ReadyMix (Fisher Science, Atlanta, GA, USA). All samples had three parallel wells and the experiment was repeated at least three times. The amplification conditions were as follows: 5 minutes at 94°C, 45 seconds at 94°C, 35 cycles, 56°C for 1 minute, 72°C for 1 minute, and then 10 minutes at 72°C. The expressions were calculated relative to that of GAPDH.

Western blot analysis

Total protein (40mg per lane) was loaded and separated with 10% SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was sealed with 5% skimmed milk containing 0.1% Triton X-100 (TBST) in Tris buffer for 1 hour, and then incubated at 4°C with p-eNOS primary antibody (1:500, Cell Signaling), eNOS primary antibody (1:1000, Cell Signaling), PPAR-γ primary antibody (1:400, Cell Signaling) or β-actin (1:800, Cell Signaling) diluted with TBST overnight incubation. The membranes were then incubated with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology) at room temperature. Enhanced chemiluminescence (ECL) was used for immunoblotting. Image J software was used to determine the amount of protein which was normalized to the control value.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0. Statistical evaluation of the data was performed with one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons or unpaired t test (two groups). A value of $p < 0.05$ was considered statistically significant.

Results

JAH protected against t-BHP-induced injury in MBECs

The MBECs were treated with 50 μMt-BHP to induce injury *in vitro*. Cell viability (Figure 1A) showed that JAH significantly attenuated t-BHP-induced cytotoxicity in a dose-dependent manner, although 0.5 and 2 μM JAH were ineffective. Treatment with JAH alone had no significant effect on cell viability. As shown in Fig. 1B, apoptosis was detected flow cytometrically. The results showed that t-BHP significantly increased the degree of apoptosis but JAH reversed the apoptotic effect to a certain extent.

JAH inhibited t-BHP-induced oxidative stress

Compared with t-BHP treatment group, JAH significantly reduced ROS production in cells by about 50% within 24 hours after injury (Figure 2A). Lipid peroxidation was estimated through determination of the levels of 4-HNE and MDA. The results showed that JAH significantly reduced the levels of 4-HNE and MDA (Figure 2B and Figure 2C) in MBECs treated with t-BHP. These results suggest that JAH inhibits t-BHP-induced oxidative stress.

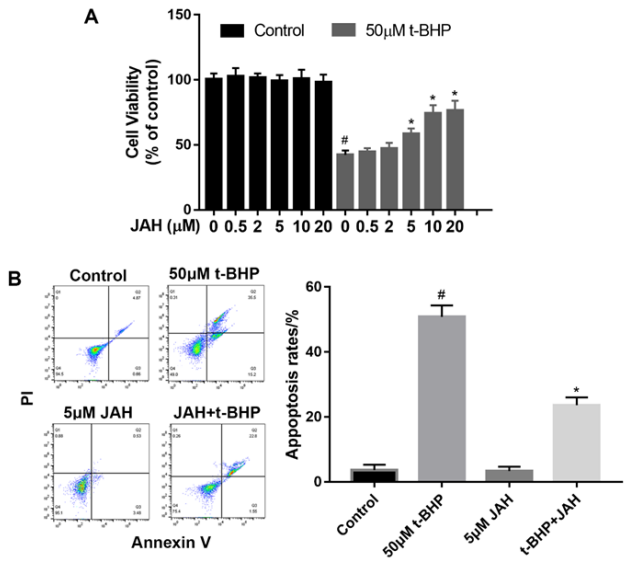

Figure 1. Effect of JAH on t-BHP-induced apoptosis of MBECs. (A) MBECs were separately treated with JAH at different concentrations (0.5, 2, 5, 10 and 20 μM). Cell viability (B) was measured 24 hours later. Apoptosis induced with 50 μM t-BHP was mitigated by 10 μM JAH. Data are shown as mean ± SD of three experiments. $^{\#}p < 0.05$ vs. control; $^{*}p < 0.05$ vs t-BHP.

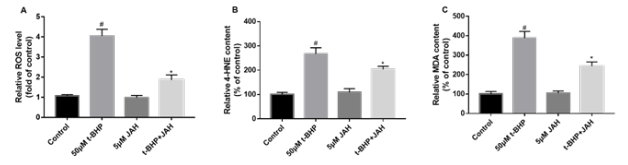

Figure 2. Effect of JAH on t-BHP-induced oxidative stress in MBECs. (A) Intracellular ROS production; (B) 4-HNE and (C) MDA levels. Data are shown as mean ± SD of three experiments. # $p < 0.05$ vs. control; * $p < 0.05$ vs. t-BHP. n.s: not statistically significant.

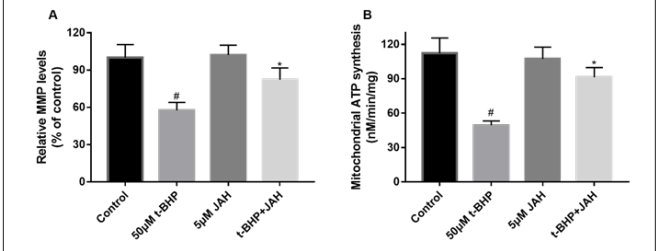

Figure 3. Effect of JAH on t-BHP-induced mitochondrial dysfunction. (A) Relative MMP levels of different experimental groups; (B) ATP levels in the different experimental groups. Data are shown as mean ± SD of three experiments. # $p < 0.05$ vs. control; * $p < 0.05$ vs. t-BHP.

JAH attenuated t-BHP-induced mitochondrial dysfunction

An attempt was made to find out whether the protective effect of JAH was related to preservation of mitochondrial function. Fluorescent dye Rh123 was used to measure changes in MMP. The results showed that JAH treatment partially prevented t-BHP-induced MMP loss (Figure 3A). In addition, JAH alleviated the reduction in ATP levels in mitochondria of MBECs treated with t-BHP (Figure 3B).

JAH exerted anti-inflammatory effect through eNOS activation

ELISA results showed that t-BHP-induced cytotoxicity was mediated through inflammation in MBECs. As shown in Figure 4A, Figure 4B and Figure 4C, JAH partially prevented the t-BHP-induced expressions of IL-1β, TNF-α and IL-6, respectively. In order to study the potential mechanism of JAH-induced protection, eNOS expression was assayed using RT-PCR and Western blotting. Even in the presence of t-BHP, JAH significantly increased the expression of eNOS (Figure 4D). As shown in Fig.4E, JAH increased the protein expressions of p-eNOS and eNOS, suggesting that the phosphorylation of eNOS increased after JAH treatment. Thus, JAH exerted anti-inflammatory effect.

PPAR-γ activation contributed to JAH-induced protective effects

The likelihood of involvement of PPAR-γ agonist

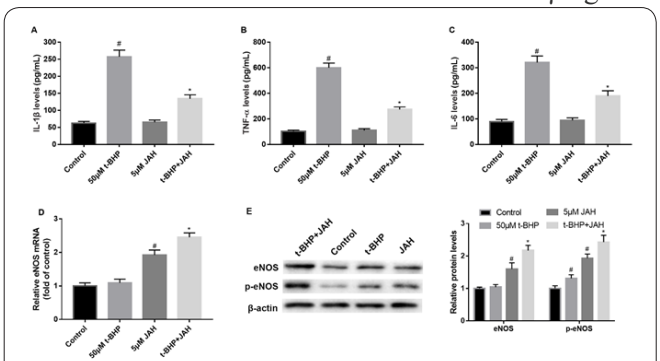

Figure 4. Involvement of eNOS activation in the anti-inflammatory role ofJAH. The expression levels of IL-1β (A), TNF-α (B) and IL-6 (C) in different treatment groups were measured using ELISA kit. The expression of eNOS gene was assayed with RT-PCR (D), while the protein expressions of p-eNOS and eNOS were measured with Western blotting (E). Data are shown as mean ± SD of three experiments. $^{\#}p<0.05$ vs. control; $^{*}p<0.05$ vs. t-BHP.

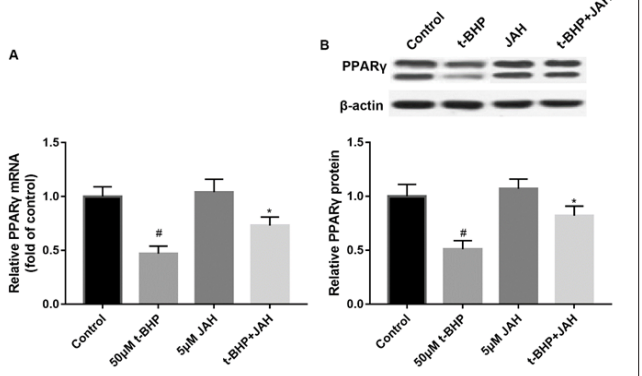

Figure 5. Involvement of PPAR-γ activation in the protective effect of JAH. MBECs were treated with 50 μM t-BHP. The expression of PPAR-γ mRNA (A) was determined with RT-PCR in different experimental groups, and the expression of PPAR-γ protein (B) was determined with Western blotting. Data are shown as mean ± SD of three experiments. $^{\#}p < 0.05$ vs control; $^{*}p < 0.05$ vs t-BHP.

potential in the protective effect of JAH was investigated. When MBECs treated with t-BHP were compared with cells treated with JAH at the same time, the expression of PPAR-γ was significantly decreased (Figure 5A). In addition, JAH prevented decreases in PPAR-γ protein expression after t-BHP exposure (Figure 5B).

Discussion

For many years, people have been looking for effective neuroprotective agents for the treatment of nervous system diseases. This study investigated the protective effect of JAH on t-BHP-induced endothelial cell injury. It was found that t-BHP induced apoptosis in MBECs, but JAH treatment reduced the apoptosis, oxidative stress, mitochondrial dysfunction and inflammatory response. It was also shown that the beneficial effects of JAH may be due to the activation of p-eNOS and PPAR-γ. Therefore, JAH may have potential therapeutic value in the management of neurological disorders, because it plays a multifaceted role in the cascade of endothelial injury in MBECs.

Initially, ARB was identified as a treatment for hypertension, diabetic nephropathy and congestive heart failure. Since RAS exists in the vascular system and the central nervous system, ARB affects the vascular and nervous system. Recently, ARBs have been shown to reduce encephalitis, prevent stroke and exert neuroprotective effects, all of which are thought to be associated with increased cerebral blood flow (14). In the present study, the protective effect of JAH was extended to the direct protection of brain endothelial cell injury, because it is well known that brain injury is related to several neurological diseases.

Mitochondria play an important role in inducing apoptotic cell death after oxidative stress (15). Tert-butyl hydroperoxide (t-BHP) is widely used to induce oxidative cell damage and ROS production (16). *In vitro* and *in vivo* studies have demonstrated that mitochondrial energy metabolism is extremely sensitive to ROS damage. Excessive synthesis of ROS during oxidative stress may aggravate mitochondrial dysfunction and lead to mitochondrial swelling, which is the most common ultrastructural change and damage of cells (17). Therefore, the effect of JAH on mitochondrial function in MBECs was also studied. The results showed that JAH significantly retained MMP levels and increased mitochondrial ATP synthesis.

Nitric oxide (NO) production by endothelial cells is essential for the regulation of vascular responses, and relative lack of biologically available NO in blood vessels leads to endothelial dysfunction (18, 19). The ENOS, also known as NOS III, is a constitutive low-output enzyme expressed in endothelial cells. The production of NO derived from eNOS is an endogenous protective principle under neurological conditions. Previous studies have shown that elevated eNOS activity and subsequent NO availability are compensatory mechanisms for ineffectiveness arising from traumatic or ischemic brain injury (20). Increased phosphorylation of eNOS protein was also observed in the *in vitro* model in this study. The expressions of eNOS mRNA, eNOS protein and p-eNOS were increased in mBECs after JAH treatment, indicating that JAH plays a role in regulating the transcriptional mechanism or protein stability of eNOS. In recent years, more and more evidence support the important role of inflammation in neuronal cell death and dysfunction. Therefore, the role of cytokines in JAH protection was investigated in the present study. The results showed that even in the presence of t-BHP, JAH significantly reduced the expressions of IL-1β, TNF-α and IL-6. These data strongly suggest that JAH-induced protection may be mediated by eNOS-dependent anti-inflammatory potential.

It is well known that PPAR-γ activation induces anti-inflammatory and anti-oxidative effects in different organs (including the brain) (21). It has been reported that PPAR-γ precursor cells regulated inflammatory response to bacterial endotoxin in the brain and also prevented neuronal death induced by NMDA excitotoxicity *in vivo* and *in vitro* (22). In this study, RT-PCR results showed that JAH increased the expression of PPAR-γ after t-BHP exposure. Although the protective effect of JAH on t-BHP-induced endothelial cell injury was clearly demonstrated, these results still have limitations. Under *in vitro* conditions, the cell microenvironment is absent, and the neurons and glial cells associated with signal cascade cannot be simulated completely.

In conclusion, JAH protects MBECs from oxidative stress and cell apoptosis induced by t-BHP. These protective effects are related to the preservation of mitochondrial function, anti-inflammatory effects mediated by p-eNOS, and activation of PPAR-γ pathway. These findings provide a new clue for the treatment of ischemic stroke.

Acknowledgements
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Xiaolin Chen; Guanji

Wu, Ting Mu, Lihong Zhang, Xiaolin Chen collected and analysed the data; Guanji Wu wrote the text and all authors have read and approved the text prior to publication.

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