

Original Research Article

Evaluation of anisodamine-mediated amelioration of hypoxic injury in brain microvascular endothelial cells

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Abstract

Purpose: To investigate the therapeutic effect of anisodamine on hypoxic injury of brain microvascular endothelial cells, and the underlying mechanism of action.

Methods: A hypoxic injury model of rat primary brain microvascular endothelial cells was established. Cell viability, proliferation, percent survival and apoptosis were assessed using MTT, EdU staining, trypan blue staining and TUNEL staining assays, respectively. The activities of lactate dehydrogenase (LDH) and matrix metalloproteinase-9 (MMP-9) were measured using colorimetry and enzyme-linked immunosorbent assay (ELISA), respectively. Expression levels of key proteins in the PI3K/Akt signal route were determined by Western blotting.

Results: Anisodamine treatment significantly reduced apoptosis and LDH leakage in the rat cerebral vascular endothelial cell hypoxia injury model group ($p < 0.001$). At doses of 3 and 10 mM, anisodamine markedly reduced the cellular level of MMP-9 in rat cerebral vascular endothelial cell hypoxia injury model group ($p < 0.001$). Treatment of rat brain microvascular endothelial cells with anisodamine significantly reduced levels of caspase-3 and Bax, but up-regulated the expression levels of Bcl2, p-Akt and PI3K ($p < 0.001$).

Conclusion: Anisodamine exerts significant protective effect against hypoxic injury in rat brain microvascular endothelial cells by modulating PI3K/Akt signaling pathway. This finding provides a scientific basis for the clinical application of anisodamine in the treatment and prevention of ischemic cerebrovascular disease.

Keywords: Anisodamine, Stroke, Hypoxic injury, Brain microvascular endothelial cells

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INTRODUCTION

Stroke is a neurodegenerative disease. Two types of stroke are known: ischemic stroke and hemorrhagic stroke [1,2]. Ischemic stroke has high incidence, mortality and disability.

Atherosclerosis and thrombosis are among the leading causes of ischemic stroke.

When a patient develops ischemic stroke, the cerebral vascular endothelium at the end of the embolism site becomes hypoxic, leading to necrosis and shedding of the cerebral vascular

endothelium, abnormal apoptosis and differentiation in brain microvascular endothelial cells (BMECs), as well as dysregulation of angiogenesis and proliferation [3,4]. Vascular endothelial cells injured after ischemic stroke release inflammatory cytokines and adhesion molecules which adhere platelets to vascular endothelial cells. The blood-brain barrier transport becomes dysregulated, platelets are activated, and thrombosis is accelerated [5]. Therefore, the reversal of hypoxic injury to brain microvascular endothelial cells has important clinical significance for the treatment of ischemic stroke.

Anisodamine (Ani) is a naturally occurring tropane alkaloid [6]. Its pharmacological effects are similar to those of atropine: both are M choline blockers. Anisodamine exerts pharmacological effects such as anticholinergic relaxation, smooth muscle relaxation, and analgesia [7-9].

Studies [10-12] have found that anisodamine protects the brain from myocardial ischemia/reperfusion injury by inhibiting oxidative stress, inflammation and apoptosis. However, its role in ischemic stroke, and the mechanism involved have not been reported. This study found that anisodamine increased the viability, survival, and proliferation of cerebral microvascular endothelial cells in hypoxic rats by regulating the PI3K/Akt signaling pathway, inhibiting apoptosis and reducing levels of LDH and MMP-9. This study was aimed at providing an important reference for the treatment of ischemic stroke using anisodamine.

EXPERIMENTAL

Culture and verification of brain microvascular endothelial cells

Sprague Dawley suckling rats were sacrificed with cervical dislocation after 1 to 3 days of birth. The cerebral cortex was harvested under aseptic conditions. After washing three times in pre-chilled D-Hank's solution (Thermo, USA), the cortex was sliced into 1 mm³ sections using a pair of ophthalmic scissors. The tissue blocks were alternately digested at 37 °C with 0.25 % trypsin (Beyotime, China) and type II collagenase (Sigma, USA) for 30 min. To stop the digestion, high glucose (HG) DMEM containing FBS (10 %) and streptomycin-penicillin (1%) mixture was added, and the mixture was centrifuged at 1000 rpm for 10 min. After the cell pellet was homogenized with high-sugar DMEM, the cells were subjected to incubation at 37 °C in a 5 % CO₂ incubator (Thermo, USA). The culture

medium was changed every three days. The cells were digested and passaged when 85% confluency was reached. Approval for this investigation was given by the Ethics Committee of our institution.

After the cells were cultured for 48 h, they were examined under an inverted fluorescence microscope to observe and take photos of any changes in cell morphology.

Establishment of hypoxic injury model of rat brain microvascular endothelial cells

This study was approved by the Animal Ethics Committee of the Affiliated Hospital of Hebei University of Engineering (no. 2020 (K) 1221), and performed according to international guidelines [13]. Rat brain microvascular endothelial cells at passage 3 were used in this study. Cells in the control group were maintained in HG DMEM containing FBS (10 %) and antibiotics mixture at 37 °C in an incubator containing 5 % CO₂ for 24 h. A cell model of hypoxic injury was established by incubating the cells in sugar-free Earle's balanced salt solution at 37 °C for 6 h in an incubator containing 95 % N₂ and 5 % CO₂. Then, oxygen and glucose were restored by replacing the medium with high-glucose DMEM medium supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin mixture, and incubation at 37 °C in an incubator containing 5 % CO₂ for 24 h [14,15].

Anisodamine treatment

Hypoxia-damaged rat cerebral vascular endothelial cells were seeded in a well plate or petri dish, and when the cell confluence reached 50 - 60 %, injury intervention and different concentrations of anisodamine were administered. The cell groups used were: control group, oxygen injury group, and hypoxia injury + anisodamine treatment group (with different anisodamine concentration i.e., 300 μM, 1 mM, 3 mM, 10 mM and 30 mM). After treatment, the cells were incubated for 24 h.

MTT assay

There were 6 duplicate wells in each group, with 1 × 10⁵ cells/mL in 96-well plate. The procedures described in earlier sections were followed to establish a cell model of hypoxic injury, and the cells were treated with different doses of anisodamine as before. After the treatment was done, medium was removed from the wells, and 50 μL of 3-(4,5)-dimethylthiazolium bromide (MTT) reagent (Biosharp, China) was put in every well. After a

4-h incubation at 37 °C, the MTT medium was discarded, and the formazan crystals were solubilized in 100 µL of dimethyl sulfoxide (DMSO). The plate was shaken to allow complete dissolution of the purple formazan crystals. Absorbance values were measured at a wavelength of 490 nm using a microplate reader.

Trypan blue staining assay

Cells were seeded into six-well plates at a density of 1×10^5 cells/mL. The procedures described in earlier sections were followed to establish a cell model of hypoxic injury, and the cells were treated with different doses of anisodamine as before. Following treatment, the medium was removed from each well, and the cells were digested into a single cell suspension, followed by addition of 0.4% trypan blue solution (Biosharp, China). After thoroughly mixing via repeated aspirating/dispensing with the pipette tip, the cell suspension was examined under an optical microscope. Viable cells were colorless, while dead cells were blue in color. Cell survival (%) was calculated by identifying trypan blue-positive cells (dead cells) in relation to normal cells.

EdU staining assay for cell proliferation

Cells were plated in 35 mm dishes at a concentration of 1×10^3 cells/mL. The procedures described in earlier sections were followed to establish a cell model of hypoxic injury, and the cells were treated with different doses of anisodamine as before. Thereafter, EdU staining was carried out with EdU kit (Ribobio, China) in line with the protocol in the user manual. After staining, the cells were observed under the microscope (Olympus FluoView FV10i Live Cell Imaging System, Japan). The EdU-positive cells were red in color. For clearer view, the cell nuclei were counterstained blue with DAPI. Cell proliferation (%) was calculated by identifying EdU-positive cells (proliferating cells) in relation to unstained cells.

TUNEL staining assay

A cell model of hypoxic injury was established as described earlier, and the cells were treated with different doses of anisodamine as before. Thereafter, TUNEL staining was performed in accordance with the TUNEL kit (Roche, Switzerland) user manual, and the cells were observed under the microscope (Olympus FluoView FV10i Live Cell Imaging System, Japan). The TUNEL-positive cells were stained green. For clearer view, the cell nuclei were counterstained blue with DAPI. Percentage

apoptosis was calculated by identifying TUNEL-positive cells (apoptotic cells) in relation to normal cells.

Measurement of lactate dehydrogenase (LDH) levels

The cells were seeded in 24-well plates. When 80 - 90% confluency was reached, the cells were exposed to hypoxia in accordance with the procedure described earlier. Then, the newly-established hypoxic injury model cells were treated with anisodamine in line with the procedure depicted earlier. Then, 20 µL of cell culture supernatant was taken from each group, and LDH activity in the cell culture supernatant was measured in a colorimetric assay using LDH assay kit (Nanjing Jiancheng Biotechnology Co. Ltd, China) so as to assess the degree of cell injury. The measurement was performed in accordance with the LDH kit user manual protocol.

Enzyme-linked immunosorbent assay (ELISA)

The cells were seeded in 6-well plates. When 80 - 90% confluency was reached, the cells were exposed to hypoxia in accordance with the procedure described earlier. The newly-established hypoxic injury model cell were then treated with anisodamine doses as before. Cell culture supernatants were collected from each group, and kept in EP tubes at -20 °C prior to assay of MMP-9 levels using ELISA kit (Shanghai Yuanye Biotechnology Co. Ltd., China) in accordance with the user manual protocol. The MMP-9 level was calculated using the absorbance values at 450 nm.

Determination of protein expression levels

The cells were seeded in 6-well plates. When 90 % confluency was reached, the cells were exposed to hypoxia in accordance with the procedure described earlier. The newly-established hypoxic injury model cells were treated with anisodamine following the procedure depicted earlier. Thereafter, cell culture supernatant was collected from each group. A cell scraper was used to harvest cells from the plate. The cells were then incubated with RIPA lysis buffer (Beyotime, China) for 20 min, and centrifuged at 4°C (12,500 rpm; 10 min). The supernatant was collected into an EP tube, and the total protein concentration was measured using BCA kit (Beyotime, China). A sample of 50 µg total protein was resolved with SDS-polyacrylamide gel electrophoresis, and the protein bands were transferred from the gel to NC membrane. Following sealing with 5 % skim

milk for 1 h, the membrane was incubated with 1° antibodies (p-Akt and Caspase-3) for 12 h at 4 °C, followed by incubation with 2° antibody for 1 h. The expression levels of the proteins measured were determined using gel image analysis equipment (Odyssey, USA). The primary antibodies for p-Akt (Item # AF1546, dilution factor 1:1000), Caspase-3 (Item # AF1213, dilution factor 1:1000) and β -actin (Item # AF0003, dilution factor 1:1000), and the secondary antibody (Item # AF0239, dilution factor 1:500), were purchased from Beyotime Biotechnology Co. Ltd., China. The gray values of the bands were analyzed using Tanon software (β -actin was internal reference). The relative quantitative results were expressed as gray values/optical density values.

Statistical analysis

All experimental tests were repeated at least 3 times. Data were processed using GraphPad software. Two-group comparison was done with independent sample *t*-test, while multiple groups were compared with one-way ANOVA. Statistical significance was fixed at $p < 0.05$.

RESULTS

Influence of anisodamine on viability of microvascular endothelial cells after hypoxic injury

As shown in Figure 1 A, there was a high density of rat brain microvascular endothelial cells, and the cell growth was close to a short-shuttled state, like paving stones. Relative to control, the viability of cells in the hypoxic injury model was decreased to about 33 % ($p = 0.0003$; Figure 1 B). However, the attenuation in cell viability was reversed after treatment with anisodamine. After treatments of the model cells with anisodamine at doses of 3 mM and 10 mM, cell viability was increased about 3 times ($p = 0.0003$ and 0.0006 , respectively).

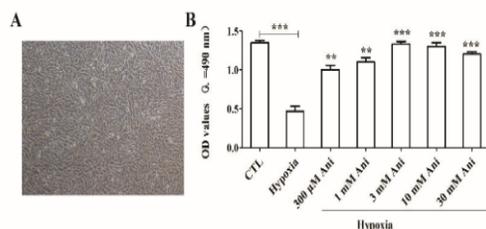


Figure 1: Effect of anisodamine treatment on the viability of brain microvascular endothelial cells under hypoxia in rats. (A) Cell morphology; (B) cell viability in each group.

Effect of anisodamine on % cell survival after hypoxic injury

The results of trypan blue staining assay are presented in Figure 2. Relative to control, % cell survival of the hypoxic injury model cells was reduced from 90 to 35 % ($p = 0.0001$). However, the attenuation in % cell survival was reversed after treatment with anisodamine. For example, treatment of the model cells with anisodamine at doses of 3 and 10 mM, increased the % survival from 35 to 85 % ($p = 0.0001$ and 0.0001 , respectively).

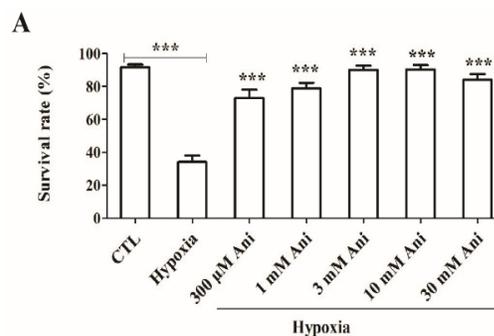


Figure 2: Effect of anisodamine treatment on the survival rate of brain microvascular endothelial cells in rats. (A) Percentage survival of hypoxia-injured brain microvascular endothelial cells in rats after anisodamine treatment

Effect of anisodamine on cell proliferation

The proliferation of hypoxic injury model cells was reduced from 70 to 45 %, relative to control ($p = 0.0114$). However, after treatment of the model cells with anisodamine at doses of 3 and 10 mM, the cell proliferation was increased to 65 and 55 %, respectively ($p = 0.0253$ and 0.0597 , respectively; Figures 3 A and 3 B).

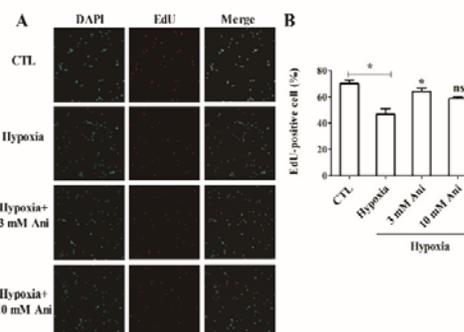


Figure 3: Effect of anisodamine treatment on cell proliferation after hypoxia injury, as determined using EdU stain (A). (B) Statistical analysis of data from EdU staining experiment

Effect of anisodamine on cell apoptosis following hypoxic injury

As shown in Figure 4, relative to control cells, the % apoptosis of hypoxic injury model cells was increased from 5 to 15 % ($p = 0.0013$). After treatment of the model cells with anisodamine at 3 and 10 mM, the apoptosis was decreased from 15 to 9 % and 12 %, respectively ($p = 0.0236$ and 0.0428, respectively; Figures 4 A and 4 B).

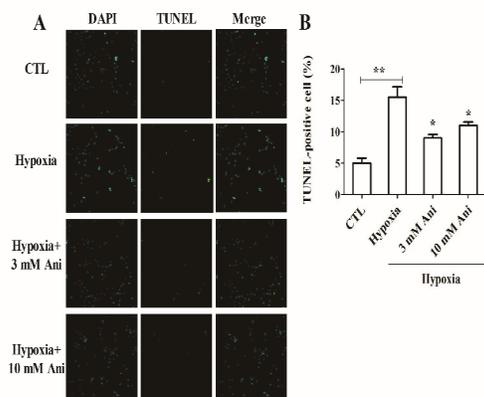


Figure 4: Effect of anisodamine on apoptosis of cerebral microvascular endothelial cells in rats. (A) TUNEL staining to detect apoptosis of cerebral microvascular endothelial cells in rats with hypoxia injury after anisodamine treatment; (B) Statistical analysis of data from TUNEL staining experiment

Effect of anisodamine on leaked LDH level of rat brain microvascular endothelial cells after hypoxic injury

As shown in Figure 5, the level of leaked LDH in cell culture supernatant was 55 U/mL in the control group, and it was increased to about 130 U/mL in the hypoxic cell injury model group ($p=0.002$). After treatments of the model cells with anisodamine at 3 and 10 mM, the level of leaked LDH in cell culture supernatant was reduced to about 75 and 95 U/mL, respectively ($p = 0.0096$ and 0.0197, respectively).

Effect of anisodamine on MMP-9 level of rat brain microvascular endothelial cells after hypoxic injury

As shown in Figure 6, compared with the control group, the level of leaked MMP-9 in the hypoxic injury model cell group was increased from 60 to 110 ng/mL ($p = 0.002$). After treatments of the model cells with 3 and 10 mM anisodamine, the level of leaked MMP-9 in the cell culture supernatant was reduced to 75 and 90 ng/mL, respectively ($p = 0.0074$ and 0.033, respectively).

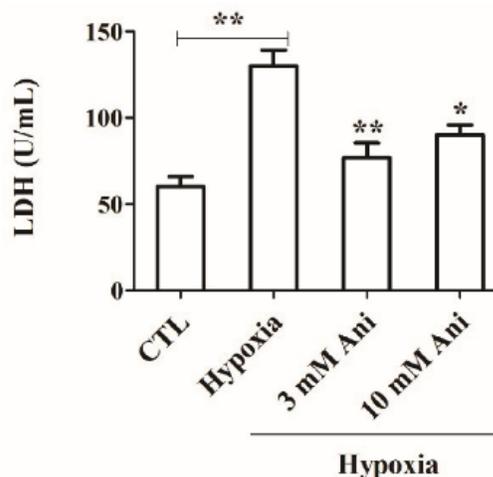


Figure 5: Effect of anisodamine on LDH leakage from cerebral microvascular endothelial cells in hypoxic rats. LDH leakage in rat brain microvascular endothelial cells after anisodamine treatment

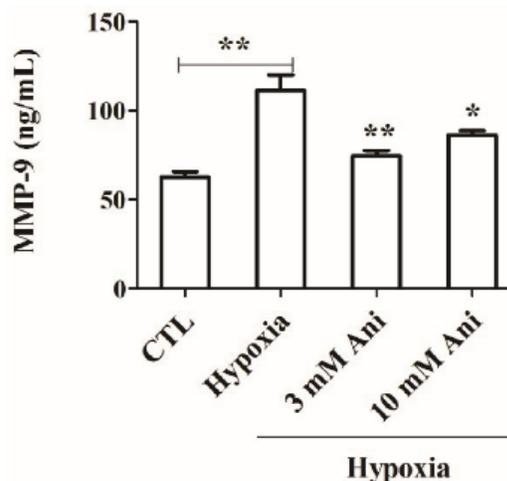


Figure 6: Effect of anisodamine on MMP-9 levels in rat cerebral vascular endothelial cells subjected to hypoxia

Mechanism of protection by anisodamine against hypoxic injury of rat brain microvascular endothelial cells

The hypoxic injury cell model group had significantly increased expressions of caspase-3 and Bax, relative to control cells (Figure 7 A and B). After treatment of the model cells with anisodamine at a dose of 3 mM, their levels were significantly decreased. In addition, the expression levels of Bcl2, p-Akt and PI3K in the model cell group were significantly lower than those in the control group, but after treatment with 3 mM anisodamine, their levels were significantly increased (Figure 7 C – E).

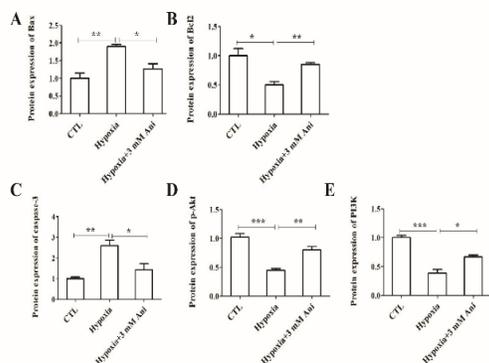


Figure 7: Levels of key proteins in rat brain microvascular endothelial cells after hypoxia injury after anisodamine treatment. (A) Expression level of Bax protein in cerebral microvascular endothelial cells of hypoxia injury rats treated with anisodamine; (B) expression level of Bcl2 protein in cerebral microvascular endothelial cells of hypoxia injury rats treated with anisodamine; (C) expression level of caspase-3 protein in cerebral microvascular endothelial cells of hypoxia injury rats treated with anisodamine; (D) expression level of p-Akt protein in cerebral microvascular endothelial cells of hypoxia injury rats after scopolamine treatment; (E) expression level of PI3K protein in cerebral microvascular endothelial cells of hypoxia injury rats treated with anisodamine

DISCUSSION

Brain microvascular endothelial cells are the main components that constitute the brain microvessels, and they are capable of synthesizing and releasing various vasoactive factors. The cells are crucial not only in maintenance of normal structure and function of brain blood vessels, but also in promoting angiogenic processes in the ischemic area and the permeability of the microvascular blood-brain barrier [16]. It has been reported that brain microvascular endothelial cells are subjected to severe injury due to hypoxia in the brain of stroke patients. Thus, alleviation of the hypoxic injury of brain microvascular endothelial cells is a key step in treatment of ischemic stroke [17].

In this study, a hypoxic injury cell model was established by incubating brain microvascular endothelial cells under hypoxic and sugar-free conditions. The newly established hypoxic injury model cells were then treated with anisodamine at various doses. Anisodamine-mediated improvements in hypoxic injury of brain microvascular endothelial cells were evaluated by comparing changes in cell viability, % survival, proliferation, apoptosis, and expression levels of LDH and MMP-9 in cell culture supernatants. Expressions of major proteins in the PI3K/Akt signal route were assayed as well, in order to

understand the mechanism through which anisodamine provided protection against hypoxic injury of rat brain microvascular endothelial cells.

Recently, studies reported that the neostigmine/anisodamine combination had therapeutic effects on acute ischemic stroke in rats via modulation of the $\alpha 7$ nAChR anti-inflammatory pathway in the downstream and the mitochondrial anti-apoptotic pathway. In this study, it was found that anisodamine reduced the % apoptosis of rat brain microvascular endothelial cells which were subjected to hypoxic injury, suggesting a protective role of anisodamine against hypoxic injury. Apparently, these results are consistent with the findings reported by Zhang. Moreover, anisodamine treatment increased cell viability, survival and proliferation, inhibited apoptosis and reduced leakage of LDH and MMP-9 in rat brain microvascular endothelial cells subjected to hypoxia injury. It has been reported that under the stimulation of hypoxia, the activity of matrix metalloproteinase-9 (MMP-9) is significantly increased, thereby causing the endothelial cells in the blood-brain barrier to shrink and die. As a result, extravasation of cellular components occurs. Therefore, MMP-9 is associated with permeability of the blood-brain barrier and survival of the endothelial cells [18].

In this study, the level of MMP-9 in the cell culture supernatant in the hypoxic injury model cell group was significantly higher than that in the control group. However, after treatment with anisodamine, the level of MMP-9 in the model cell group was significantly reduced. In further studies, it was also found that anisodamine decreased the expressions of caspase-3 and Bax, while increasing the expressions of Bcl-2, p-Akt and PI3K in the hypoxic injury cell model group. This finding suggests that anisodamine may exert significant protective effect against hypoxic injury of the endothelial cells through modulating the PI3K/Akt signaling pathway.

CONCLUSION

Treatment with anisodamine reverses the injury caused by hypoxia in rat brain microvascular endothelial cells. The findings in this study serve as preliminary results from an ongoing comprehensive and in-depth elucidation of the effect of anisodamine on the biological function of rat brain microvascular endothelial cells subjected to hypoxia injury. The findings also offer an insight into the prospects for clinical application of anisodamine for prevention and treatment of ischemic cerebrovascular diseases.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jing Wang and Youming Wang conceived and designed the study, and drafted the manuscript. Jing Wang, Qinghua Zhu, Xiaohui Duan, Lingyu Li, Shuyan Zhang and Jie Chen collected, analyzed and interpreted the experimental data. Qinghua Zhu and Youming Wang revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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