Modulation of muscarinic receptors by anisodine hydrobromide in cerebral ischemia

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ABSTRACT

Ischemic cerebrovascular diseases pose significant challenges due to their high mortality, disability rates, and recurrence risk, imposing substantial societal and healthcare burdens. Current treatment modalities, including medication and surgical interventions, have limitations. This study explores the therapeutic potential of anisodine hydrobromide, a neuroprotective compound, with a focus on its interaction with muscarinic receptors (M1-M5) in cerebral ischemic diseases, employing a middle cerebral artery occlusion (MCAO) rat model, and microglial HM cells and astrocytes SVG12 as models. Immunohistochemistry comprehensively assessed M1-M5 receptor expression in cerebral arteries, hippocampus, and parenchymal tissues in MCAO rats before and after anisodine hydrobromide administration. Additionally, a hypoxia/reoxygenation (H/R) model validated our findings using SVG12 and HM cells. M receptor mechanisms under hypoxia, including calcium ion influx, reactive oxygen species (ROS) levels, and aspartate expression were explored. Anisodine hydrobromide effectively reduced exacerbated M1, M2, M4, and M5 receptor expression in hypoxia/reoxygenation (H/R)-treated brain tissues and M2 receptors in H/R-treated cells. Concentration-dependent inhibition of calcium ion influx and ROS levels was observed, elucidating its neuroprotective mechanisms. Under H/R conditions, HM cells exhibited decreased aspartate levels by anisodine hydrobromide, Atropine, and M2 inhibitor treatments. These findings shed light on the modulation of muscarinic receptors, particularly the M2 subtype, by anisodine hydrobromide in cerebral ischemia. The neuroprotective effects observed in this study highlight the promising clinical prospects of anisodine hydrobromide as a potential therapeutic agent for ischemic brain diseases, warranting further investigation into its mechanisms of action.

Introduction

Ischemic stroke, characterized by localized ischemic necrosis or cerebral infarction due to insufficient blood supply and oxygen in brain tissues, represents the most prevalent form of stroke (1). Clinically, it is associated with a high incidence, disability rate, and mortality rate (2). Presently, drug therapy is a pivotal approach for stroke treatment. Thrombolytic therapy stands out as a highly effective intervention during the acute phase of ischemic stroke. However, factors such as delayed patient presentation for medical care have contributed to the low utilization rate of thrombolytic treatment for acute ischemic stroke (3). Studies have demonstrated that hypoxia/reoxygenation can lead to cerebral injury through various mechanisms, resulting in phenomena like cellular apoptosis, neuronal demise, and microglial cell activation (4, 5). Consequently, the development of innovative therapeutic strategies targeting neurodegenerative diseases induced by hypoxia/reoxygenation is of significant importance.

In ischemic brain diseases, astrocytes (AS) and microglia (MG) play crucial roles in neuroinflammation and neuroprotection (6). AS provides vital support within the normal adult central nervous system (CNS). They envelop neuronal cell bodies and neurites, establishing a structural framework for neural tissue. Additionally, they secrete neurotrophic factors, exerting substantial influence over neuronal generation, development, differentiation, and regeneration (7). MG are innate immune-active cells and CNS phagocytes. In their quiescent state, MG are widely distributed in the brain's gray matter and lack phagocytic capabilities. Following cerebral ischemia-reperfusion, AS are rapidly activated, undergoing significant morphological and functional changes. The role of activated AS is multifaceted, contributing to brain edema and acute inflammation, and mediating various pathways in ischemic brain injury. Moreover, excessive proliferation of AS in later stages can lead to the formation of glial scars, mechanically impeding axonal regeneration and impacting the structural and functional recovery of neural tissue. On the other hand, resting microglia transform from a ramified morphology to cells with fewer processes, migrating towards damaged neurons, and assuming amoeboid-like cells with phagocytic capabilities (8). In summary, both types of glial cells have both beneficial and detrimental effects in ischemic brain diseases. A more comprehensive...
understanding of their roles may lead to innovative therapeutic approaches.

As previously mentioned, drug therapy is a primary strategy for stroke treatment, and the quest for new drugs is essential for effectively managing cerebral hypoxic injury. Muscarinic receptors, a type of ion channel, are involved in various biological processes, including neural signal transmission, cellular apoptosis, and inflammatory responses (9). Muscarinic receptors are considered key molecules in cellular death and inflammatory responses following cerebral hypoxia/reoxygenation (10). Excessive activation of these receptors has been linked to neuronal damage and the excessive proliferation of neuroglial tumor cells (11). Consequently, drugs targeting muscarinic receptors have emerged as a promising avenue for treating neurological disorders induced by hypoxia/reoxygenation (12). Muscarinic receptors belong to the G-protein-coupled receptor family and play pivotal roles in various biological processes. The agonists and antagonists of muscarinic receptors in clinical shock may be closely associated with their regulation of the immune and nervous systems. For instance, research by Sapronov has shown that applying the cholinergic receptor antagonist atropine (ATP) 30 minutes before inducing a shock model can effectively alleviate heart rate irregularities, restore B cell function in the spleen, and regulate serotonin levels (13). Additionally, A.F. van den Heuvel found that the muscarinic receptor inhibitor, atropine, has a significant cardioprotective effect. It enhances blood flow in coronary arteries, and reduces lactic acid production in the myocardium during atrial pacing, leading to improved myocardial ischemic conditions (14). Furthermore, some studies have found that the activation of muscarinic receptors can protect nerve cells from oxidative stress and DNA damage, exerting a neuroprotective effect. This opens new research avenues for the treatment of neurological diseases (15). Therefore, the role of muscarinic receptors in ischemic stroke is highly complex, likely attributed to the diversity of muscarinic receptors and the intricacies of the disease's pathogenesis. A more profound investigation is required to uncover the potential implications of muscarinic receptors in neuroprotection and therapeutic interventions for these diseases.

Anisodine hydrobromide (AT3) is a well-known selective antagonist of muscarinic receptors and holds substantial potential for the treatment of neurological disorders (16). Previous studies have suggested that AT3 can ameliorate symptoms of neurological disorders by reducing neuronal overactivation (16). However, most studies have focused on neurons, and the role of muscarinic receptors in MG and AS remains unclear.

In this study, we employ a rat middle cerebral artery occlusion (MACO) model and neuronal cell lines, as well as astrocytes under hypoxic conditions, as in vivo and in vitro models, respectively. AT3 will be utilized as a therapeutic/intervention approach to evaluate its impact on neurofunctional recovery and damage to MG and AS. The objective is to delve into the mechanisms of action of muscarinic receptors, providing novel insights and approaches for treating neurological disorders induced by hypoxia/reoxygenation. This research is expected to furnish more substantial evidence for the use of this drug in neurological disorder treatments, offering more effective therapeutic strategies for individuals with neurological disorders.

Materials and Methods

Animal Model Establishment and Drug Intervention

Healthy male Sprague-Dawley (SD) rats weighing 220-250 g, of Specific Pathogen-Free (SPF) grade, were procured from the Sichuan University Animal Experiment Center. A one-week adaptation period was allowed for the animals, which were subsequently housed under controlled conditions with temperatures maintained at 22-26°C, humidity levels between 50-70%, and a 12-hour light-dark cycle. Prior to surgery, the rats underwent a 12-hour fasting period but had access to water and food before and after the procedure. Ethical approval for the animal experiments was granted by the Sichuan University Animal Ethics Committee.

Under pentobarbital sodium anesthesia (50 mg/kg), the rats were positioned supinely and secured on an operating table. The neck area was aseptically prepared with iodine disinfection, followed by a surgical incision to expose the neck. Gentle dissection exposed the common carotid artery and external carotid artery, and the internal carotid artery was ligated using a vascular clip. A small incision was made in the common carotid artery, and a specialized thread occluder was carefully inserted toward the internal carotid artery until resistance was encountered. The precise time of thread occluder insertion was recorded, followed by local disinfection, suturing of tissues, and skin closure. After a 2-hour occlusion period, the thread occluder was slowly withdrawn to the marked point to initiate reperfusion. The normal group underwent identical surgical procedures to the MACO model group, with the exception that the thread occluder was not inserted. Subsequently, the MCAO model rats were randomly divided into two groups: the MCAO group and the AT3 group. The latter received an injection of AT3 at a dose of 0.6 mg/kg via the caudal vein during reperfusion. Both the normal group and the MCAO group received an equivalent volume of normal saline. Following the operation, the rats were allowed to recover, kept warm, and housed individually with access to normal food and water.

Cell Culture

Human microglial (HM) cells and SVG p12 cells were obtained from the BeNa Culture Collection (BNCC), China. HM cells were cultured using Prigrow III medium (Applied Biological Materials Inc., BC, Canada.) on extracellular matrix-coated culture plates (Corning Incorporated, NY, USA). For SVG p12 cells, DMEM was used as the culture medium, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The culture plates were maintained at 37°C in a 5% carbon dioxide incubator with 100% humidity. The culture medium was refreshed every 2-3 days, and cells were passaged at a 1:2 ratio. To validate the role of M2 receptors, gallamine triethiodide (68 μM, Selleck, China) was used.

Cell Model Establishment

HM and SVG p12 cells were transferred to a glucose-free DMEM medium containing 10% fetal bovine serum at a density of 1×10^6/ml. The culture plates were placed in hypoxia using the AnaeroPouch pack (C-11, Mitsubishi Gas Chemical Company, Inc., Japan) at 37°C for 3 hours. Following hypoxia, the medium was aspirated, and the cells were supplemented with regular DMEM medium and...
further incubated at 37°C in an incubator with 5% CO2 and 95% air for an additional 3 hours. In addition to the H/R (hypoxia/reoxygenation) group, other model groups required the addition of various substances to the glucose-free DMEM medium. Specifically, the Anisodamine hydrobromide (Ani HBr) 10 group received 10 μg/mL Ani HBr, the Ani HBr 20 group received 20 μg/mL Ani HBr, the AT3 10 group received 10 μg/mL AT3, the AT3 20 group received 20 μg/mL AT3, and the ATP group received 20 μg/mL ATP. Imaging and quantification of positive staining was performed under a microscope. The negative control employed tap water. Hematoxylin was used for counterstaining. The aspartic acid content in the samples was measured using the BCA method, and protein samples with identical concentrations were stored at -20°C. SDS-PAGE (12%) electrophoresis was performed, followed by transfer of proteins to a membrane. Subsequent steps included primary antibody incubation with CHRM1 polyclonal antibody (1:200, A16819, Abclonal, China), CHRM2 Polyclonal Antibody (1:200, A1567, Abclonal), CHRM3 Polyclonal Antibody (1:200, A1602, Abclonal), CHRM4 Polyclonal Antibody (1:200, A2866, Abclonal), and CHRM5 Polyclonal Antibody (1:200, A5367, Abclonal) overnight at 4°C, followed by incubation with a secondary antibody (HRP-conjugated secondary antibody, 1:5000, Cell Signaling Technology, USA) at room temperature for 1 hour. Chemiluminescent film development was conducted to visualize protein expression intensity, with β-actin serving as an internal reference for the analysis of each protein's expression intensity in individual samples.

Western Blotting

Brain tissues were sliced into small pieces and then added to PMSF and RIPA lysis buffer (final PMSF concentration: 1 mM) on ice. The tissue was homogenized using an ultrasonic homogenizer and then centrifuged at 14,000 rpm for 5 minutes in a low-temperature centrifuge. The resulting supernatant was collected and stored in EPP tubes at -20°C. The protein concentration was determined using the BCA method, and protein samples with identical concentrations were prepared based on the original protein concentrations. These samples were stored at 120°C. SDS-PAGE (12%) electrophoresis was performed, followed by transfer of proteins to a membrane. Subsequent steps included primary antibody incubation with CHRM1 polyclonal antibody (1:200, A16819, Abclonal, China), CHRM2 Polyclonal Antibody (1:200, A1567, Abclonal), CHRM3 Polyclonal Antibody (1:200, A1602, Abclonal), CHRM4 Polyclonal Antibody (1:200, A2866, Abclonal), and CHRM5 Polyclonal Antibody (1:200, A5367, Abclonal) overnight at 4°C, followed by incubation with a secondary antibody (HRP-conjugated secondary antibody, 1:5000, Cell Signaling Technology, USA) at room temperature for 1 hour. Chemiluminescent film development was conducted to visualize protein expression intensity, with β-actin serving as an internal reference for the analysis of each protein's expression intensity in individual samples.

ROS Detection

After cell treatment, cells were seeded in culture plates and exposed to a DCFH-DA probe (1 μM, Beyotime, China) solution for 20 minutes at 37°C. Subsequently, cells were washed three times with serum-free culture medium to remove unbound probes. Cells loaded with probes were directly observed under an LSM710 laser confocal microscope (Carl Zeiss, USA) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Calcium Influx Detection

HM cells and SVG p12 cells were cultured separately in their respective culture media until they reached 70% confluence. Cells were divided into groups and treated with different AT3 concentrations. The fluo-3 probe (S1056, Beyotime, China) was prepared with a serum-free culture medium, and after treating cells in each group, cell samples were observed using a confocal microscope. Fluorescence signal intensity was recorded and analyzed for differences among different concentration treatment groups.

Aspartic Acid Content Measurement

An Aspartic Acid Content kit (ml805102, mlbio, China) was used to measure the level of Aspartic Acid in HM cells and SVG p12 cells. After cell treatment, cell lysis buffer was added, and cells were scraped from the bottom of the bottle. After complete cell lysis, centrifugation was performed (4°C, 12,000×g, 10 minutes), and the supernatant was collected for subsequent analysis. Following the assay kit instructions, the aspartic acid standard solution was used to establish the relationship between absorbance values and aspartic acid, creating a standard curve. The cell lysis supernatant served as the test sample, and sample absorbance values were obtained following the assay kit's instructions. The aspartic acid content in the samples was
calculated using the standard curve.

**Data Statistical Analysis**

Statistical analysis of all research results was conducted using SPSS 17.0 statistical software (IBM Corporation, Armonk, NY). Data were presented as “mean ± standard deviation,” and one-way ANOVA was applied to the test result database, followed by Tukey’s post hoc test for pairwise comparisons. The level of significance was set at \( p<0.05 \). All experiments were repeated at least three times.

**Results**

**Effects of AT3 on M1-M5 muscarinic receptors expression in MCAO rat brain tissues**

Utilizing immunohistochemistry, we assessed the expression profiles of M1-M5 receptors in distinct brain regions, including cerebral arteries, hippocampus, and brain parenchyma (Figures 1-3). In cerebral artery tissues, hypoxia-reoxygenation (H/R) treatment (MCAO) induced a noticeable upregulation of M1, M2, and M4 receptors, which was subsequently ameliorated following AT3 administration. However, the expression levels of M3 and M5 in various rat tissue groups exhibited no statistically significant differences (Figure 1).

In hippocampal tissues, akin to the outcomes observed in cerebral artery tissues of model animals, M1, M2, M4, and M5 receptors exhibited upregulation following H/R treatment, and this upregulation was subsequently reversed by AT3 treatment. Conversely, M3 expression remained relatively stable across diverse experimental groups (Figure 2).

In brain parenchymal tissues, M1 receptor expression was significantly augmented in the MCAO group, and post-treatment with AT3, it was significantly downregulated, accompanied by concomitant reductions in M2 and M5 expression. However, M2 and M5 displayed no significant differences in expression levels between the sham surgery group and the MCAO group (Figure 3).

**Effects of AT3 on M1-M5 muscarinic receptors expression in HM and SVG12 cells under H/R conditions**

To further substantiate the response of M1 to M5 receptors to AT3, we conducted in vitro experiments employing a hypoxia/reoxygenation (H/R) model in microglia (HM cells) and astrocytes (SVG12 cells). We meticulously quantified receptor expression levels. As depicted in Figure 4, M1, M3, M4, and M5 receptors exhibited negligible changes in expression levels in response to AT3 treatment. In contrast, post-H/R treatment, M2 receptor expression showed a pronounced increase, which was subsequently downregulated upon exposure to AT3 (Figure 4A and Figure 4B).

**Effects of M2 receptor inhibitors and AT3 on ROS and intracellular calcium in SVG P12 and HM cells**

To elucidate the potential mechanistic actions of M receptors under hypoxic conditions, we harnessed the H/R in vitro model and introduced Atropine as the M receptor inhibitor. Consequently, we measured calcium ion influx in HM cells and assessed levels of reactive oxygen species (ROS) in SVG12 cells. As evidenced in Figure 5, H/R treatment of HM cells significantly augmented calcium ion influx, substantiated by the intensified red signal distribution observed in Figure 5A. Administration of various
Concentrations of AT3 led to a concentration-dependent reduction in calcium ion influx, denoted by a diminished red signal. Although atropine treatment also yielded a reduction in calcium ion influx, it was less pronounced than the effect observed with 20 μg/mL AT3 treatment. Additionally, no significant differences were observed between the impacts of the M2 inhibitor and 20 μg/mL AT3 treatment (Figure 5A). In SVG P12 cells, H/R treatment significantly elevated ROS levels. Furthermore, an escalation in the concentration of AT3 led to a more potent inhibition of ROS levels (Figure 5B). Both atropine and the M2 inhibitor demonstrated varying degrees of inhibitory effects on ROS levels (Figure 5B).

Effects of M2 receptor inhibitors and AT3 on aspartic acid expression in SVG12 and HM Cells

Microglia within the brain are acknowledged as the primary producers of aspartate. In our endeavor to comprehend the underlying mechanisms in these cells, we quantified the aspartate content within distinct groups of HM cells. As depicted in Figure 6A, H/R treatment significantly elevated aspartate levels within HM cells. Remarkably, both AT3 and Atropine, as well as the M2 inhibitor, demonstrated a substantial reduction in aspartate levels, with no appreciable distinctions among them (Figure 6A).

Astrocytes, conversely, are primarily responsible for the elimination of aspartate in brain tissue. To gain insights into the potential mechanisms of action of AT3 within astrocytes, we measured aspartate content across various groups of SVG12 cells. As delineated in Figure 6B, following H/R treatment, a significant reduction in aspartate content was observed in the cells, indicative of impaired

Figure 3. Impact of AT3 on M1-M5 receptor expression in brain parenchymal tissues of MACO rats. In the MCAO group, there was a significant surge in M1 receptor expression, which was subsequently downregulated following treatment with AT3. Additionally, a decrease in M2 and M5 expressions was observed post-treatment. Interestingly, there were no significant differences in M2 and M5 expression between the sham surgery group and the MCAO group.

Figure 4. Examination of M1 to M5 receptor response to AT3 in H/R model in HM cells and SVG12 cells. (A) While the expression levels of M1, M3, M4, and M5 remained stable following AT3 treatment, M2 exhibited significant upregulation after H/R treatment, which was subsequently downregulated upon AT3 administration. (B) Detailed expression patterns of M2 following H/R treatment and its response to AT3 treatment are depicted.

Figure 5. Effects of M1-M5 Receptor Inhibitors and AT3 on ROS and Intracellular Calcium in SVG P12 and HM Cells. (A) Intracellular calcium levels in HM cells significantly increased following H/R treatment, as indicated by the red signal distribution. Treatment with various concentrations of AT3 led to a concentration-dependent decrease in calcium ion influx. (B) In SVG P12 cells, H/R treatment resulted in a significant increase in ROS levels, which were more strongly inhibited as the concentration of AT3 increased. Atropine and the M2 inhibitor also had varying inhibitory effects on ROS levels.

Figure 6. Evaluation of aspartate levels in HM cells and SVG12 cells following various treatments. (A) H/R treatment induced a significant increase in aspartate content in HM cells, which was noticeably downregulated upon treatment with AT3, ATP, and M2 inhibitors. (B) In SVG12 cells, aspartate content was not significantly decreased by H/R treatment, and treatment by AT3, and M2 inhibitors post H/R.
aspartate elimination. However, subsequent treatment with AT3, Atropine, and the M2 inhibitor did not produce significant alterations in their ability to absorb aspartate. These findings necessitate further experiments to elucidate the precise underlying mechanism.

**Discussion**

The aging population in China has brought about a growing burden of ischemic cerebrovascular diseases, characterized by high mortality, disability rates, and recurrence rates, leading to significant societal and healthcare costs (17). Current clinical treatments primarily involve medication and surgical interventions. While medication therapy is generally safe, it may require long-term use and has limited efficacy (18), and surgical interventions carry inherent risks and potential complications (2). In this study, we investigated the expression patterns of M1-M5 receptors and assessed the impact of AT3 on their expression levels in cerebral ischemia models, both in vivo and in vitro. Additionally, we examined the effects of AT3 on calcium influx, ROS levels, and other aspects in microglia and astrocytes, while utilizing receptor inhibitors. The objective of this research was to explore the potential therapeutic role of AT3 by targeting M receptors in cerebral ischemic diseases.

Researchers have increasingly recognized the critical roles of microglial cells and astrocytes in the context of ischemic cerebral stroke. The integrity of the blood-brain barrier (BBB) relies on the normal function of endothelial cells and glial cells. Microglial cells, known for their high plasticity, adopt M1 or M2 phenotypes in response to brain diseases, exerting pro-inflammatory or anti-inflammatory effects, respectively. In addition, astrocytes can transition from a resting to an activated state, referred to as reactive astrocytes, under ischemic conditions. Microglial M1 polarization and astrocytic activation can exacerbate ischemic stroke, leading to brain injury (19-21). The Muscarinic Receptor, particularly the M2 receptor, is closely associated with the behavior of microglial cells and astrocytes in ischemic stroke (22, 23). Our study revealed an upregulation of M2 receptor expression in both animal and cell models, suggesting its potential involvement in the regulation of hypoxic injury through brain glial cells.

Muscarinic receptors, a subtype of G protein-coupled receptors (GPCRs), are primarily activated by acetylcholine and are widely distributed in the central and peripheral nervous systems. They play crucial roles in various physiological processes and serve as targets for multiple drugs used to treat various conditions. For example, muscarinic receptor M2 expression is inhibited in offspring from diabetic mother mice, and insulin treatment effectively upregulates M2 receptors, countering the effects of diabetes (24). In type 1 diabetes mouse models, there was a significant increase in M2 expression in both pancreatic and myocardial tissues (25). Acetylcholine has been found to exhibit antioxidant effects on oxidative stress in cardiomyocytes, primarily mediated through the M2 acetylcholine receptor (M2 AChR) (26). Changes in the expression of muscarinic M2 receptors due to hypoxia have also been observed in the brainstem (27). In our study, the expression of M2 receptors increased in response to hypoxia and significantly decreased following AT3 treatment. This supports the notion that muscarinic receptors, particularly M2, play a role in cerebral ischemic diseases.

The muscarinic M2 receptor mediates various biological functions. For instance, M2 receptor agonists have been found to inhibit cell cycle progression, reduce cell survival rates, downregulate stem cell markers, and modulate the hypoxia response in glioblastoma multiforme (GBM) (28). Knocking down the M2 receptor in zebrafish influenced heart rate and cardiac β-adrenergic receptor expression under hypoxic conditions, highlighting its role in modulating cardiac responses (29). Additionally, the M2 receptor influences the activity of L-type calcium channels, regulating calcium influx in smooth muscle cells (30). In our study, we demonstrated the involvement of M2 receptors in cerebral blood vessels, hippocampus, and brain parenchymal tissues after hypoxia. In vitro experiments revealed that targeting the M2 receptor effectively controlled calcium influx and ROS production while reducing glutamate secretion or enhancing cellular uptake. This underscores the critical role of M2 receptors in cerebral ischemic diseases and suggests the potential therapeutic effects of AT3 targeting M2 receptors.

AT3, an alkaloid isolated from plants of the Solanaceae family, shares a similar mechanism of action with atropine. It has shown promise in treating central nervous system disorders by enhancing cerebral microcirculation in ischemic stroke patients and exhibiting therapeutic effects such as anti-neuronal apoptosis, improved cerebral tissue perfusion, and antioxidative properties (31). Our study confirmed the neuroprotective effects of AT3 against brain injury in vivo and in vitro hypoxia-reoxygenation models. Considering its similarity to atropine, the experimental results suggest that AT3 possesses protective properties akin to atropine. However, further research, especially microcirculation-related research is needed to elucidate the mechanisms of AT3.

**Conclusions**

This study has provided initial insights into the expression patterns of muscarinic receptors M1-M5 in hypoxia-reoxygenation models, both in vivo and in vitro. It has also validated the neuroprotective effects of AT3, particularly through its modulation of the M2 receptor, in brain glial cells by controlling calcium ion influx and ROS production. This study emphasizes the crucial role of AT3 in ischemic brain diseases and underscores its potential clinical significance.

**Interest conflict**

The authors have no conflicts of interest.

**Consent for publications**

The author read and proved the final manuscript for publication.

**Availability of data and material**

All data generated during this study are included in this published article.

**Authors’ Contribution**

Y.Z. designed the studies, all authors contributed to the data analysis and manuscript writing.

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