Abstract. – OBJECTIVE: This study aims at investigating the neuroprotective role of Vitamin D3 (VitD3) in rats with cerebral infarction and its molecular mechanisms.

MATERIALS AND METHODS: Male Sprague-Dawley (SD) rats were selected and randomly divided into sham operation group, middle cerebral artery occlusion (MCAO) model group, and VitD3 treatment group. The therapeutic effect of VitD3 was evaluated via neurobehavioral scoring and triphenyltetrazolium chloride (TTC) staining. For the evaluation of VitD3 influence on cerebral blood perfusion, Micro-PET imaging technique was applied. The mRNA levels of vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) gene were detected via Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Immunofluorescence staining assay was employed to determine the changes in micro-vessel density. Bromodeoxyuridine (Brdu) assay was used to count the number of new vascular endothelial cells. Protein expressions of key genes in the Shh signaling pathway were detected by Western blotting.

RESULTS: Our results showed that VitD3 improved the score of neurological function and decreased the size of cerebral infarction in MCAO rats. VitD3 improved cerebral perfusion in the ischemic area after MCAO. VitD3 up-regulated levels of vascular growth-related factors. VitD3 elevated micro-vessel density after cerebral infarction and promoted the proliferation of vascular endothelial cells in the ischemic cortex. The sonic hedgehog (Shh) signaling pathway in the ischemic cortex of MCAO rats was activated after VitD3 treatment.

CONCLUSIONS: We showed that VitD3 improves cerebral perfusion and reduces neurological impairment in MCAO rats via activating the Shh signaling pathway.

Key Words: Vitamin D3, Cerebral infarction, Angiogenesis, VEGF, Ang-1, Shh signaling pathway.

Introduction

Stroke is a common neurological disease with high incidence, mortality and disability rates, of which about 87% cases are ischemic stroke (also known as cerebral infarction)1,2. Treatment for cerebral infarction aims to restore cerebral reperfusion in the ischemic area and save the ischemic penumbra as early as possible1. Currently, recombinant tissue plasminogen activator (rt-PA) thrombolytic therapy is the unique treatment method based on evidence-based medicine. However, not so many ischemic stroke patients are benefited from rt-PA therapy due to its narrow therapeutic time window4,5.

In recent years, the collateral circulation has been recognized as an effective way to improve blood perfusion in cerebral ischemic zones6. Revascularization and angiogenesis are the basic conditions for protecting neurovascular unit (NVU) and promoting neurofunctional remodeling. These two phenomena run throughout the entire processes of the acute, subacute and chronic phases of cerebral ischemia, with a relatively long therapeutic time window7-10. Currently, angiogenesis promotion directly stimulates the establishment of collateral circulation mainly by transplanting genes or recombinant proteins into ischemic zones11-13. However, it is hard to be applied in clinical practice because it is limited by...
the immunogenicity of heterologous proteins and genes as well as difficult procedures.

Wong et al. have pointed out that Vitamin D3 (VitD3) is able to promote revascularization. For normal population, VitD3 supplementation can increase the number of circulating angiogenic myeloid cells (AMCs) that promote revascularization. Similarly, VitD3 is also capable of promoting the revascularization of damaged blood vessels in animal models. The effect of VitD3 on promoting angiogenesis in ischemic brain damage is rarely reported. Therefore, this study explored the neuroprotective effect of VitD3 on rats with cerebral infarction by establishing MCAO model. We further investigated its molecular mechanisms in promoting micro-angiogenesis and improving cerebral perfusion, which provides new ideas for the treatment of ischemic stroke.

Materials and Methods

Experimental Animals

Specific pathogen-free (SPF) male Sprague-Dawley (SD) rats weighing 250-300 g provided by the Xinjiang University Animal Center were used in this investigation. During experiment, animals were housed under specific pathogen-free (SPF) conditions in the Xinjiang Medical University Animal Center, with a 12 h light on/off cycle, an ambient temperature of 21-22°C and a humidity of 40%-55%. Rats were given free access to water and food. This study was approved by the Animal Ethics Committee of Xinjiang Uygur Autonomous Region People's Hospital Animal Center.

Establishment of MCAO Models

MCAO rat model was established as previously described. After rats were anesthetized, they were fixed on a rat board in supine position. The neck hair was cut off for disinfection with iodophors at the operative region. Next, a longitudinal incision was made on the surface of the neck for exposure of the right common carotid artery, internal carotid artery, and external carotid artery. Subsequently, the distal end of the external carotid artery was ligated with a thin line, and the common carotid artery and internal carotid artery were clipped with artery clamps. After that, an incision was made on the external carotid artery, through which a tether was inserted into the external carotid artery, followed by clipping with artery clamps. The external carotid artery was snipped and the artery clamp on internal carotid arterial was removed. The catheter was reversed and inserted into the internal carotid artery, with a depth of approximately 18-22 mm from the origin of the external carotid artery to the anterior cerebral artery (ACA), so as to block blood supply to the middle cerebral artery (MCA). Then, the incision was sutured layer by layer after the tether was inserted, and the tether was pulled out for reperfusion after 2 h of ischemia. After operation, rats were transferred to a warm, dry and clean cages individually, and their recovery condition was monitored until emergence from an anesthesia. Rats in sham operation group (Sham group) received the same procedures without tether insertion.

Experimental Grouping and Scheme

Experimental rats were randomly divided into three groups, namely Sham group (administration with normal saline via gavage), MCAO model group (MCAO group, MCAO + intragastrical administration with an equivalent amount of normal saline) and VitD3 treatment group (VitD3 group, MCAO + administration with 1000 IU/kg/d VitD3 through gavage).

Evaluation of Neurological Function

The modified Longa method was used for scoring at 12 h, 1, 3, 7, and 14 d after MCAO operation in rats. The specific criteria for scoring were applied: 0 points: the actions of the four limbs were normal without neurological impairment; 1 point: the left forepaw of the rat was flexed and cannot be extended when its tail was lifted vertically; 2 points: the rat leaned to the left and may rotate to the left when walking on the flat ground; 3 points: the rat walked unstably, and the whole body leaned to the left; and 4 points: the rat had consciousness disturbance or was not capable of walking autonomously.

Triphenyltetrazolium Chloride (TTC) Staining

At 7 and 14 d after MCAO operation, rats were anaesthetized and decollated to collect their brains. Brain sample was continuously cut into sections in the sagittal plane with 2 mm in thickness. After that, brain sections were soaked in 3% TTC solution (Oxoid, Hampshire, UK), put in an incubator at 37°C for 30 min of staining in the dark and fixed with 4% paraformaldehyde solution for 2 h. Cerebral infarction foci were pale, and normal brain tissues were red.
Neuron Staining
Rats were anaesthetized at 14 d after MCAO operation, followed by perfusion with normal saline and paraformaldehyde into the heart. Then, rats were decollated to collect their whole brains. Brain sample was subjected to dehydration with graded sucrose and cut into brain sections (2 μm in thickness) using a freezing microtome. After membrane permeation with 0.2% Phosphate-buffered saline and Tween®20 (PBST) for 20 min, brain sections were blocked with 1% bovine serum albumin (BSA) (AMRESCO, Solon, OH, USA) at room temperature for 1 h. Primary antibody diluted with BSA was added for incubation in the refrigerator at 4°C overnight. On the next day, brain sections were incubated with diluted secondary antibody at room temperature in dark for 2 h. Neuron staining was observed using a fluorescence microscope.

Micro-PET
Rats were anesthetized and fixed on an examining table at 1 and 14 d after MCAO operation. Fludeoxyglucose (18F-FDG) was injected through rat tail vein. The injection time, dose, measured residual dose, and time of residual measurement were recorded. The injection interval between every two rats was about 15 min. At 1 h after injection, anaesthetized rats were gently placed on a scanning table for scanning and data collection, whose four limbs were fixed with medical tapes.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)
Ribonucleic acid (RNA) was extracted from the ischemic cortex of rats and reversely transcribed into complementary deoxyribonucleic acid (cDNA) according to the instructions of a reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). Step One Plus™ Real-Time PCR system was employed for amplification in accordance with the instructions of a SYBR green kit. The relative expression levels of various target genes were counted using the 2ΔΔCT method. Primer sequences used in this study were as follows: VEGF, F: 5'-TCGGGCCTCGGAACCATGA-3', R: 5'-CCTGAGAGATCTGGAAC-3'; Ang-1, F: 5'-CACCGTGAGGATGGAAGCCT-3', R: 5'-TTCCTAAGGGAACCACTGC-3'; GAPDH, F: 5'-CGCTCTGCTCCTGTT-3', R: 5'-ATCCGTTGACTCGACCTC-3'.

Western Blotting
Brain tissues from the ischemic cortices of rats in each group were fully disintegrated with an ultrasonic disintegrator and centrifuged at 4°C, 12,000 rpm/min for 30 min. Then, the supernatant was collected. Protein quantification was carried out using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA), followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Next, membrane transfer was performed, after which the gel was discarded. Thereafter, the membrane was cut according to the molecular weight of the target protein according to the signs of the marker. Cut bands were blocked with 5% nonfat milk at room temperature for 1 h, followed by incubation with target protein primary antibody at 4°C overnight. On the next day, the bands were incubated with horseradish peroxidase (HRP)-labeled secondary antibody for 2 h at room temperature. Finally, enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) was performed for color development.

Double-Immunofluorescence Staining of Cluster of Differentiation 31 (CD31) and Alpha-Smooth Muscle Actin (α-SMA)
The whole brain tissue of rats was sectioned into 20 μm-thick brain sections using the freezing microtome, followed by membrane permeation with 0.2% Phosphate buffered saline and tween 20 (PBST) for 20 min. Then, brain sections were incubated with 2% bovine serum albumin (BSA) at room temperature for 1 h. CD31 primary antibody diluted with 2% BSA was added for incubation at 4°C overnight. Sections were taken out on the next day, added with diluted fluorescent secondary antibody and incubated at room temperature for 2 h in dark. Subsequently, sections were incubated with 2% BSA-diluted α-SMA primary antibody and corresponding secondary antibody as the previously described. Sections were mounted with anti-quencher and observed under the fluorescence microscope.

Statistical Analysis
All data were expressed as mean ± standard deviation and analyzed using Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA). The t-test was used for comparisons of data meeting normal distribution between two groups, and those did not conform to normal distribution were compared via non-parametric test. One-way analysis of variance (ANOVA) was applied for comparisons among multiple groups, followed by Post-Hoc Test (Least Significant Difference). *p*<0.05 suggested that the difference was statistically significant.
Results

VitD3 Improved the Score of Neurological Function in MCAO Rats

Rats had evident neurological impairment after MCAO operation, manifesting as motion disturbance in the left limbs. No neurological impairment was found in rats of Sham group. The difference in the score of neurological function was not evident between VitD3 group and Sham group at 12 h after reperfusion. The Longa score was gradually declined after operation in a time-dependent manner. The degree of improvement in neurological function in VitD3 group was overtly superior to that in MCAO group from the postoperative 3rd day, which last for 14 days (Figure 1A).

VitD3 Decreased the Size of Cerebral Infarction in MCAO Rats

The infarct size was detected via TTC staining at 7 d after operation. Clear necrotic brain tissue (white area) was observed after establishing MCAO model. Smaller infarct size was observed in VitD3 group compared with that of MCAO group (Figure 1B). At 14 d after the operation, neuron fluorescence staining assay was conducted to determine the degree of neuronal loss. The results indicated that the neuron staining positive area in VitD3 group was remarkably larger than that in MCAO group at 14 d after the operation, indirectly reflecting the protective effect of VitD3 on the ischemic stroke (Figure 1C).

VitD3 Improved Cerebral Perfusion in the Ischemic Side After MCAO

Micro-PET/CT scanning was used to detect cerebral perfusion in ischemic side of rats at 1 and 14 d after MCAO operation. The results revealed that at 1 d after the operation, the ischemic hemisphere had evident lower blood perfusion compared with that in Sham group. At 14 d after the operation, the cerebral perfusion in VitD3 group was clearly better than that in MCAO group, suggesting that the continuous administration of VitD3 after MCAO operation can significantly increase the collateral circulation of the rat brain (Figure 1D). It is considered that the protective role of VitD3 in ischemic stroke is exerted through improving the neurological function.

VitD3 Up-Regulated Levels of Vascular Growth-Related Factors

RT-PCR results manifested that the messenger RNA (mRNA) level of vascular endothelial growth factor (VEGF) after MCAO operation was continuously increased with time prolongation. Significant difference in VEGF level was found between VitD3 group and MCAO group at 3 d, which last for 14 days. The mRNA expression of angiopoietin-1 (Ang-1) was also upregulated in VitD3 group than that of MCAO group (Figure 2).

VitD3 Elevated Micro-Vessel Density After Cerebral Infarction

In this study, CD31 and a-SMA were used as an endothelial cell marker and a pericyte marker, respectively. Cerebral ischemic penumbra was
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subjected to double-labeling immunofluorescence to measure micro-vessel density at different time points after the operation. The results suggested that ischemia and hypoxia induced massive angiogenesis around the lesion. Micro-vessel density was peaked at 7 d after cerebral infarction, which was gradually decreased at 14 d. VitD3 group had higher CD31/a-SMA-positive micro-vessel density at 7 and 14 d in comparison with MCAO group (Figure 3).

**VitD3 Promoted the Proliferation of Vascular Endothelial Cells in the Ischemic Cortex**

To further verify the impact of VitD3 on proliferation of vascular endothelial cells in the rat ischemic cortex after MCAO operation, rats were intraperitoneally injected with Brdu that can be synthesized into S phase of DNA mitosis. Cells that were positively dual-stained with CD31 and Brdu were deemed as new endothelial cells. New endothelial cells were detected in the ischemic penumbra after MCAO operation. Continuous administration of VitD3 for 14 d could significantly increase the number of new endothelial cells, indicating that VitD3 can overtly promote angiogenesis in the ischemic cortex of MCAO rats (Figure 4).

**VitD3 Activated the Sonic Hedgehog (Shh) Signaling Pathway in the Ischemic Cortex of MCAO Rats**

Numerous studies have proved that Shh signaling pathway participates in angiogenesis after cerebral infarction. To further state the mechanism of VitD3 in promoting angiogenesis after cerebral

![Figure 2. Vitamin D3 up-regulated levels of vascular growth-related factors in MCAO rats. A, Analysis of mRNA level of VEGF by RT-PCR in different time points. B, Analysis of mRNA level of Ang-1 by RT-PCR in different time points. *p<0.05 vs. MCAO group.](image)

![Figure 3. Vitamin D3 elevated micro-vessel density after cerebral infarction in MCAO rats. A, Representative images of micro-vessel density in brain tissues by double-labeling immunofluorescence. B, Analysis of CD31/a-SMA positive micro-vessel in different time (magnification 200×). *p<0.05 vs. MCAO group.](image)
infarction, protein expressions of key genes in the Shh signaling pathway were detected by Western blotting. The results indicated that VitD3 could significantly increase the protein expressions of Shh, Ptch-1, and Smo in the ischemic cortex at 7 d after MCAO (Figure 5A, 5B). MCAO rats were intraperitoneally injected with cyctopamine (CY, a Shh blocker) to observe whether VitD3 increased the expressions of VEGF and Ang-1 by activating the Shh signaling pathway. The data showed that inhibition of the Shh signaling pathway distinctly reduced the protein expressions of VEGF and Ang-1 in MCAO rats (Figure 5C). Rescue experiments found that the promotive role of VitD3 in up-regulating the expression levels of VEGF and Ang-1 was significantly reversed after CY treatment (Figure 5D).

Discussion

Ischemic stroke is a common cerebrovascular disease resulted from focal neurological impairment due to acute cerebrovascular occlusion caused by various causes. With the aggravated aging of the society and increased multiple risk factors such as hypertension, hyperglycemia, and hyperlipidemia in China, the incidence rate of cerebral infarction becomes increasingly higher. In addition, stroke in young population is very common. The high disability, fatality, and recurrence rates of cerebral infarction bring a heavy burden on the affected families and the society. Currently, the most effective treatment method for cerebral infarction is thrombolytic therapy or thrombectomy within the ultra-early time window. For those patients missing the time window, they are mainly treated with antithrombotic therapy, cerebral protection, and improvement of circulation, together with prevention and control of complications at the same time. There are evident differences in clinical manifestations and treatment effects of such patients. One of the key factors is the conditions of collateral circulation.

After onset of acute cerebral ischemia, degeneration and necrosis of neurons are detected in the ischemic core zones, with focal irreversible neurological impairment. Neurons in the ischemic penumbra surrounding the infarct foci can survive for a certain period of time under the condition of low blood supply. It is urgent to establish collateral circulation, improve blood supply to the ischemic penumbra and rescue moribund neurons as early as possible, so that to reduce ischemic brain damage and improve its prognosis. We found that continuously intraperitoneal injection of VitD3 is able to evidently improve the score of neurological function and reduce the size of cerebral infarction of MCAO rats. Micro-PET scanning results suggested that continuous administration of VitD3 for 14 d is capable of significantly improving the cerebral perfusion in the ischemic side. Neuron fluorescence staining revealed that the degree of neuron loss is overtly reduced, implying that the improvement of cerebral blood flow is an important condition for the reconstruction of neurological function. The improvement of blood supply in the ischemic penumbra is mainly dependent on micro-angiogenesis. It is noteworthy that VitD3 has no evident neuroprotective effect in the early stage of ischemic stroke. The protective effect of VitD3 on stimulating angiogenesis was an event occurring in the relatively late stage after brain damage.

Scholars have confirmed that angiogenesis is observed after ischemic stroke, and new vessels can effectively improve cerebral blood perfusion,
Vitamin D3 promotes cerebral angiogenesis after cerebral infarction in rats which is closely related to nerve regeneration and reconstruction of neurological function. Moreover, various adhesion factors, cell molecules, and extracellular matrix are involved in angiogenesis after cerebral infarction. VEGF and Ang systems play important roles in promoting lumen formation and vascular maturation. Experimental studies have verified that VEGF and Ang systems interact on each other in angiogenesis after cerebral ischemia, jointly promoting angiogenesis. In this study, it was found that VitD3 treatment could significantly increase micro-vessel density co-marked by CD31/a-SMA in the ischemic cortex at 7 and 14 d after operation. VitD3 can not only promote angiogenesis, but also strengthen the integrity of the structure of new vessels. In addition, the expression levels of VEGF and Ang-1 in the ischemic cortex were also examined in this work. The results indicated that the expression levels of VEGF and Ang-1 were overtly increased at different time points after ischemia, suggesting that VitD3 can up-regulate the expressions of vascular growth factors and facilitate micro-angiogenesis.

The expression of VEGF in the central nervous system is regulated by multiple signaling pathways. Recent works have confirmed that Shh signaling pathway can co-regulate the expressions of VEGF and Ang-1 systems. Shh is a member of the Hedgehog family, exerting its role in promoting differentiation of nerves and blood vessels during the embryonic period and repairing damaged vessels. The activation of Shh pathway promotes angiogenesis in ischemic tissues via regulating the expressions of downstream target genes VEGF and Angs. We also observed the activation of Shh signaling pathway in brain tissue after VitD3 treatment in MCAO rats. The Shh signaling blocker further verified that VitD3 protects MCAO in rats through activating the Shh signaling pathway.

Conclusions

We showed that VitD3 increases the micro-vessel density in the ischemic side of the brain of MCAO rats and promotes angiogenesis after cere-

![Figure 5](image-url)
bral infarction, thus improving cerebral perfusion and reducing neurological impairment in MCAO rats. The protective role of VitD3 in MCAO is mainly dependent on the Shh signaling pathway.

Conflict of Interest
The Authors declare that they have no conflict of interest.

References


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