Original Article
Cent Asian J Med Sci. 2020 March;6(1):370-378. https://doi.org/10.24079/CAJMS.2020.03.006

CENTRAL ASIAN JOURNAL of MEDICAL SCIENCES
CAJMS

The Effect of Erdene urel on Microglia / Macrophage Distribution and Inhibition of Inflammatory Response after Brain Ischemia in Rats

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Submitted: February 3, 2020
Revised: February 23, 2020
Accepted: March 20, 2020

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Objectives: We studied the effect of Erdene urel on microglia/macrophage M1/M2 distribution and neuroinflammation using a cerebral ischemia-reperfusion injury model in rats. **Methods:** The middle cerebral artery occluded by thread occlusion, followed by reperfusion at 90 minutes after ischemia. The injury was studied at 1, 3, and 7 days after brain ischemia, and the neurological score was graded, after that, the brain tissue was collected by decollation. The phenotypes of M1 and M2 were detected by Iba1 and Arg-1 immunofluorescence, and the expressions of (TNF-α), (IL-1β), (IL-6), and (IL-10) were detected by ELISA. **Result:** The immunofluorescence showed that compared with the sham-operated group, the microglia/macrophage (Iba1) in the ischemic area of the control group's rats with middle cerebral artery occlusion and to which reperfusion was performed for 1, 3 and 7 days was increased ($p<.05$) and the number of M2 (Arg-1) ($p<.05$) was reduced. One day after reperfusion, the Erdene urel and nimodipine groups did not differ from the control group. However, after 3 and 7 days, the microglia/macrophage (Iba1) ($p<.05$) in the ischemic area of Erdene urel and nimodipine groups was reduced, while M2 (Arg-1) ($p<.05$) was increased. **Conclusion:** Erdene urel is likely capable of promoting the transformation of activated microglia/macrophages from M1 to M2.

Keywords: Microglia, Macrophage, Inflammation, Brain ischemia

Introduction

Brain ischemia is the most common central nervous system disease, which is also one of the leading causes of death and disability in adults. The pathogenesis of ischemic brain injury is very complicated and secondary inflammation plays an important role in ischemic brain injury [1, 2]. Microglia are a type of innate immunocyte in the brain. An early study suggests that the microglia activate promptly after brain ischemia, and gather to the site of injury-releasing inflammatory mediators, inducing other inflammatory cells to increase brain injury [3, 4]. The microglia are observed in either a resting state or activated state.

The activated microglia can be further divided into the classic activation-type, M1, and its alternative activation-type, M2. This M2 activation-type is so similar to the macrophage in shape and phenotype that it is difficult to distinguish. Recent studies have found that microglia/macrophages play a dual role in damaging and repairing tissue in ischemic brain injury [5, 6]. The M1 microglia/macrophages release pro-inflammatory factors, such as TNF-2, interleukin-1β (IL-1β), and IL-6, to increase the damage to nerves; however, M2 macrophages release anti-inflammatory factors like IL-10 to accelerate the reduction of inflammation and neural restoration. In 2012 Hu et al. found that the phenotype of activated microglia/macrophages changes dynamically after brain ischemia, and at the earlier phase, the M2-type is the predominant type and then transforming to predominately M1-type gradually [7]. The activated microglia/macrophages have a dual function of damaging and repairing, suggesting that the treatment of brain ischemia should be shifted from inhibiting microglia/macrophage activation to regulating the balance of the phenotype of microglia/macrophages.

Gan et al. found that 14 days after the reperfusion injury pretreated with Buyang Huanwu Decoction 13 mg/kg that immunofluorescence double-label staining showed the number of M1-type microglia/macrophages (CD16/32+) in the ischemic area was significantly reduced in the treatment group ($p<.01$), and the number of M2-type microglia cells (CD206+) was increased compared to controls ($p<.05$). The results of qRT-PCR showed that, compared with the control group, the expressions of M1-type microglia/macrophage surface markers CD86, iNOS, and pro-inflammatory factor TNF-α, IL-1β, IL-6 mRNA were significantly down-regulated in the treatment group ($p<.01$), and the expressions of M2-type microglia/macrophage surface markers CD206, Arg-1, and anti-inflammatory factors IL-10 and TGF-β mRNA were up-regulated ($p<.01$). The results suggested that Buyang Huanwu Decoction could inhibit the inflammatory response after brain ischemia in rats by promoting the conversion of activated microglia/macrophages from M1-type to M2-type [21].

The studies of Lianhua et al. [22] and Sarentuya et al. [23] found that the expression of BDNF, NGF, IGF-2, IGFbp2, TGF-b1, and the structural proteins vimentin and granulin in rats with middle cerebral artery occlusion reperfusion were up-regulated in the low-dose Erdene urel (125 mg/kg) and high-dose Erdene urel (500 mg/kg) groups by gavage 14 days compared with the control group. The expressions of GFbp2, TGF-B1, vimentin, and granulin, especially TGF-B1, were significantly up-regulated to protect or repair damaged nerves and reduce middle cerebral artery occlusion reperfusion injury [22, 23].

In Mongolian traditional medicine, Erdene urel is prescribed for the treatment of brain ischemia to invigorate repair and enhance blood circulation. A large number of clinical and animal tests have proved that Erdene urel has a protective and restorative effect following ischemic brain injury. Its actions are anti-oxidative, anti-apoptotic, anti-inflammatory, as well as inducing angiogenesis, neurogenesis, and so on [8-10]. However, we are unaware of previous studies investigating its effect on the regulation of microglia/macrophages following ischemic brain injury. The purpose of this study was to determine the effects high-dose Erdene urel (500 mg/kg) on the regulation of microglia/macrophages in the first week after a middle cerebral artery occlusion reperfusion injury in rats.

Materials and Methods

Reagents and Experimental Animals

One hundred ninety-two healthy male Wistar rats weighing 180-240g (provided by Animal Center of Shandong Traditional Chinese Medicine University, Animal certificate number: SCXK Lu20110007) were the animal model used in our study. They were housed at the Barrier Lab of Animal Experimental Research Center of Inner Mongolia University for Nationalities at 22 ± 2^0 C, 50%~60% relative humidity, altering light and dark for 12 hours daily, and had free access to food and water.

Erdene urel was provided by Affiliated Hospital of Inner Mongolia University for Nationalities M14022821. Nimodipine was purchased from Yabao Pharmaceutical Group Co, LTD H14022821, and Chloral hydrate from Sinopharm Group.

Rat TNF-α, IL-6, IL-10, IL-1β ELISA-kits (Item No. JM-01587R2, JM-01597R2, JM-01602R2, JM-02323M1)were provided by Jiangsu Biotechnology Co LTD. (Jiangsu, China). Iba1 was purchased from British Abcam Company (Item No. ab178847), Arg-1 from American CST Company (Item No. 93668S, Danvers, MA, USA), the goat anti-rabbit lgG by Cy3 (Item No. A0516) and DAPI (Item No. C1005) were purchased from China Beyotime Company (Shanghai, China), goat serum (Item No. SL038) and Antifluorescence quenching agent (Item No. S2100) were purchased from China Solarbio Company

(Beijing, China).

Key Instruments were Line switch (Jialing Biotechnology Co. LTD, Guangzhou, China), Freezing Microtome (Thermo Fisher Scientific Company, Model No. MH550, Waltham, MA, USA), Fluorescence Microscope (Olympus Company Model No. BX53, Tokyo, Japan), Microscopic Photographic System (Olympus Company Model No. DP73) and Ultrapure Water System (Heal Force Bio-meditech Holdings Limited Model No. NW10LVF, Hong Kong, China).

Establishment of Focal Brain Ischemia Model in Rats

A rat model of middle cerebral artery occlusion was established using Longa's method [11]. Anesthesia was established by injecting 10% chloral hydrate 350 mg/kg intraperitoneally. The right common carotid artery was dissected distally exposing the external carotid artery and internal carotid artery. The accessory branches of the external carotid artery were ligated. A small incision was made in the external carotid artery, and a plug on nylon suture was threaded into the internal carotid artery through the external carotid artery. The plug was advanced 18-20 mm until slight resistance was felt at the narrowing of the vessel characteristic of the origin of the middle cerebral artery, occluding the middle cerebral artery. After 90 minutes, the plug was pulled out to restore blood flow perfusion. The main branch of external the carotid artery was then ligated proximal to the incision, and the skin was sutured. The rectal temperature of the rats was maintained around 37^0C by heating a blanket and an incandescent lamp during the operation. Rats were placed in an incubator until they woke up after the operation.

Grouping and Administration of Rats

One hundred ninety-two rats were randomly divided into a sham-operated group, control group, Erdene urel group, and nimodipine group with a total of 48 rats in each group, 16 rats in each group at each time period. The dose of Erdene urel, and nimodipine administered was proportional to the human dose, based on body surface area, and was given by gavage. The rats in the Erdene urel group were given 500 mg/kg once a day, 24 hours after ischemia. The nimodipine group was given 10 mg/kg once a day, 24 hours after ischemia. The rats in the sham-operated group underwent the surgery but did not receive Erdene urel or nimodipine and were given water once a day. The rats in the control group did not have surgery and not receive Erdene urel or nimodipine and were given water once a day. Nimodipine was chosen as a comparison group to Erdene urel, due to its widely known therapeutic effect on treating brain ischemia [24].

The neurobehavioral evaluation of rats was conducted using the neurobehavioral injury scoring criteria of Bederson [4, 12] and was scored as follows as a grading scale: no neurological impairment symptoms scored 0 points; flexion adduction of the contralateral limb when lifting the tail (i.e., positive in lifting the tail suspension test) scored 1 point; decreased resistance of lateral thrust (i.e., positive lateral thrust test) and grade I symptoms scored 2 points; free movement time to the paralyzed side and grade II symptoms for 3 points; loss of consciousness, inability to walk scored 4 points.

Detection of the Phenotype of Microglia/Macrophages Using Fluorescent Immunoassay

On Days 1, 3, and 7 after surgery, the rats were anesthetized and intubated, and then their circulatory system drained while being rapidly flushed with 0.9% sodium chloride solution through the left ventricle, followed perfusion fixation by 4% paraformaldehyde. Their brain tissue was isolated, prepared into 8μm slices using a freezing microtome after fixation and dehydration.

The tissue was then prepared for indirect immunofluorescence staining as follows: the membranes were ruptured using room temperature 0.3% TritonX-100, the endogenous peroxidase was inactivated by 3% H2O2, and the plasma was then blocked Iba-1 antibodies were then added to the M1 cell surface markers on the microphages and Arg-1 antibodies to the microphage's M2 cell surface markers. The sample was overnighted at 4^0C and Cy3-goat anti-rabbit IgG fluorescent secondary antibodies (1:100) were added, incubated in the dark for 1 hour, and the slices sealed using a sealing reagent which contained DAPI. Those slices were examined using a fluorescence microscope and pictures taken. Each group's fluorescence diagram integral absorbance value was calculated using Image-pro Plus v 6.0 software to determine the expression intensity. The content of TNF-α, IL-6, IL-1β, IL-10 in the brain tissue was measured by ELISA following the kit's instructions.

The supernate of each group was gathered and the content of TNF-α, IL-6, IL-1β, IL-10 was measured in those supernates. Using ELISA, the absorbance was measured in Absorbance Units

(AU). Each measurement was repeated three times.

Statistical Analysis

SPSS 22.0 Statistical software was used for the statistical analysis, and the measurement data shown as mean ± SD. All statistical comparisons were made by two-way ANOVA followed by Tukey's post hoc test. p-values less than 0.05 ($p<.05$) were considered statistically significant.

Ethical Statement

The Medical Ethics Committee of the Affiliated Hospital of the Inner Mongolia University for The Nationalities approved the study protocol (NM-LL-2018-08-22-01).

Results

Erdene Urel's Effect on Neuroethology for Rats

The neurobehavioral evaluation of rats was evaluated using Bederson neurologic deficit scoring [12]. The results of the experiment showed that the neurobehavioral scores of rats in the control group after middle cerebral artery occlusion were significantly higher than those in the sham-operated group ($p<.05$), indicating that brain ischemia reperfusion has a significant effect on neurological dysfunction. One day after reperfusion, the scores of the Erdene urel group and nimodipine group were not different than the control group. However, on the third and seventh day after reperfusion, the neurobehavioral scores of the Erdene urel group and nimodipine group were significantly lower than that of the control group ($p<.05$). On the first and third days after reperfusion, the neurobehavioral scores of the Erdene urel group and nimodipine group were not different ($p>.05$). On the seventh day after reperfusion, the neurobehavioral scores of the Erdene urel group were lower than the nimodipine group ($p<.05$), suggesting that Erdene urel could improve the neurological function of rats with cerebral reperfusion injury (Table 1).

Effect of Erdene Urel on Phenotypic Distribution of Microglia/Macrophage in Rats After Brain Ischemia

Immunofluorescence double-label staining results showed that the expression of M1-type marker Iba-1 microglia/macrophage in the control group was significantly increased compared to the sham-operated group on the first (21628 ± 1627 AU), third (25922 ± 4690 AU), and seventh days (24291 ± 3829 AU). The Arg-1 expression was decreased on the first (4361 ± 454 AU), third (4268 ± 785 AU), and seventh days (4475 ± 811 AU) after middle cerebral artery occlusion reperfusion in rats ($p<.05$) (Figure 1, 2).

On the first day after middle cerebral artery occlusion reperfusion in rats, the staining of the Erdene urel group and nimodipine group were not statistically different from the control group ($p>.05$). On the third and seventh days after the middle cerebral artery occlusion reperfusion, the expression of Iba-1 in microglia/macrophage in the Erdene urel group and the nimodipine group were significantly decreased ($p<.05$), and the Arg-1 was increased compared with the control group ($p<.05$) (Table 2, 3).

The Effects of Erdene Urel on TNF-α, IL-6, IL-1β, IL-10 in Brain Tissue on the First, Third, and Seventh Days After Brain Ischemia

The ELISA results showed that on the first, third, and seventh days after reperfusion injury, the expressions of TNF-α, IL-1β, IL-6 was significantly up-regulated ($p<.05$) and IL-10 was down-regulated ($p<.05$) in the control group compared with the sham-operated group. On the first day after reperfusion, there was no significant difference between Erdene urel group and the nimodipine group and the control group (Table 4).

Table 1. Erdene urel's effect on neuroethology for rats (mean ± SD).

Groups	Neurological Score			
	0 day	1 day	3 day	7 day
Sham-operated	-	-	-	-
Control	2.29 ± 0.69	2.33 ± 0.63	2.25 ± 0.67	2.12 ± 0.74
Erdene urel 500 mg/kg	2.45 ± 0.65	1.95 ± 0.75	1.45 ± 0.6*	1.29 ± 0.55*
Nimodipine 10 mg/kg	2.37 ± 0.57	2.04 ± 0.75	1.62 ± 0.71*	1.58 ± 0.65*

*$p<.05$ vs control; n=16 in each group each day.

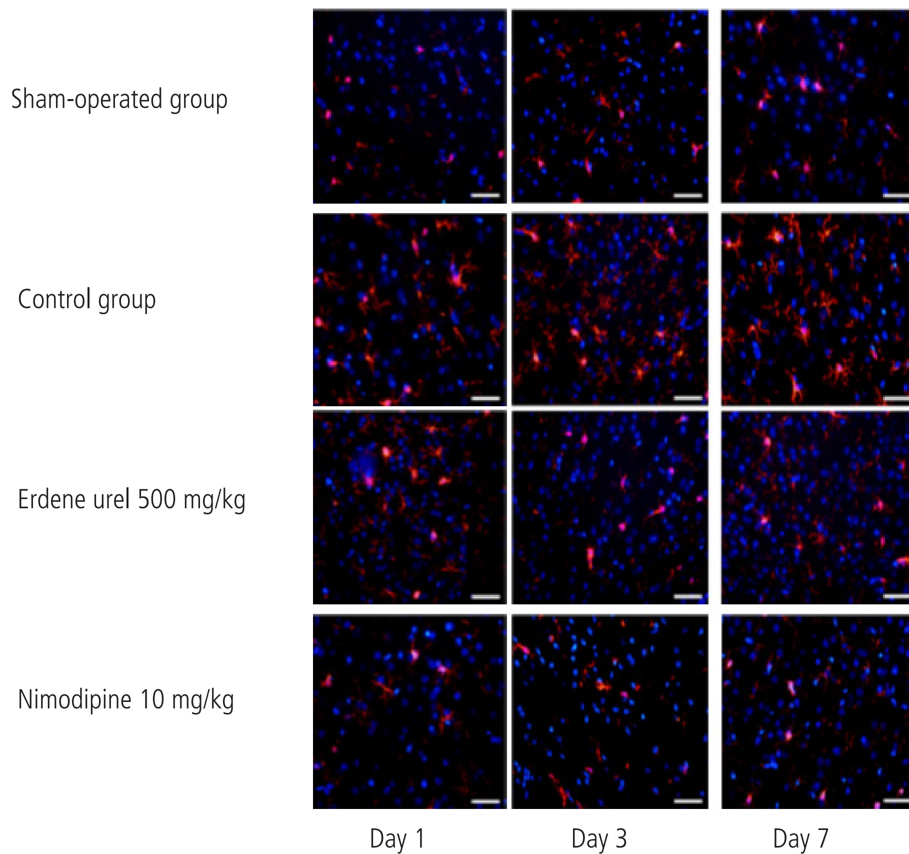

Figure 1. Effect of Erdene urel on M1-type distribution of microglia/macrophage in rats after brain ischemia (measuring scale: 50μm, 400×) Red indicates Iba-1 protein and blue is DAPI staining. As the number of pathological cells increases, the cell body becomes larger, and the cell axon thickens and becomes shorter. At a dose of Erdene urel 500 mg/kg the number of positive cells on the seventh day was noticeably reduced, and the cell shape became smaller.

Table 2. Effect of Erdene urel on M1-type distribution of microglia/macrophage in rats after brain ischemia (mean ± SD, AU).

Groups	Iba-1		
	1 day	3 day	7 day
Sham-operated	9910 ± 819	9673 ± 1199	9560 ± 1176
Control	21628 ± 1627*	25922 ± 4690*	24291 ± 3829*
Erdene urel 500 mg/kg	20096 ± 1695	20003 ± 1703**	18603 ± 3011**
Nimodipine 10 mg/kg	20258 ± 1574	20615 ± 1790**	18991 ± 1570**

*p<.05 vs sham-operated; **p<.05 vs control; n=16 in each group each day.

Table 3. Effect of Erdene urel on M2-type distribution of microglia/macrophage in rats after brain ischemia (mean ± SD, AU).

Groups	Arg-1		
	1 day	3 day	7 day
Sham-operated	19996 ± 1941	21199 ± 21504	21504 ± 2230
Control	4361 ± 454*	4268 ± 785*	4475 ± 811*
Erdene urel 500 mg/kg	4634 ± 864	16843 ± 4379**	19782 ± 1343**
Nimodipine 10 mg/kg	4870 ± 1098	15139 ± 4104**	18193 ± 2194.**

*p<.05 vs sham-operated; **p<.05 vs control; n=16 in each group each day.

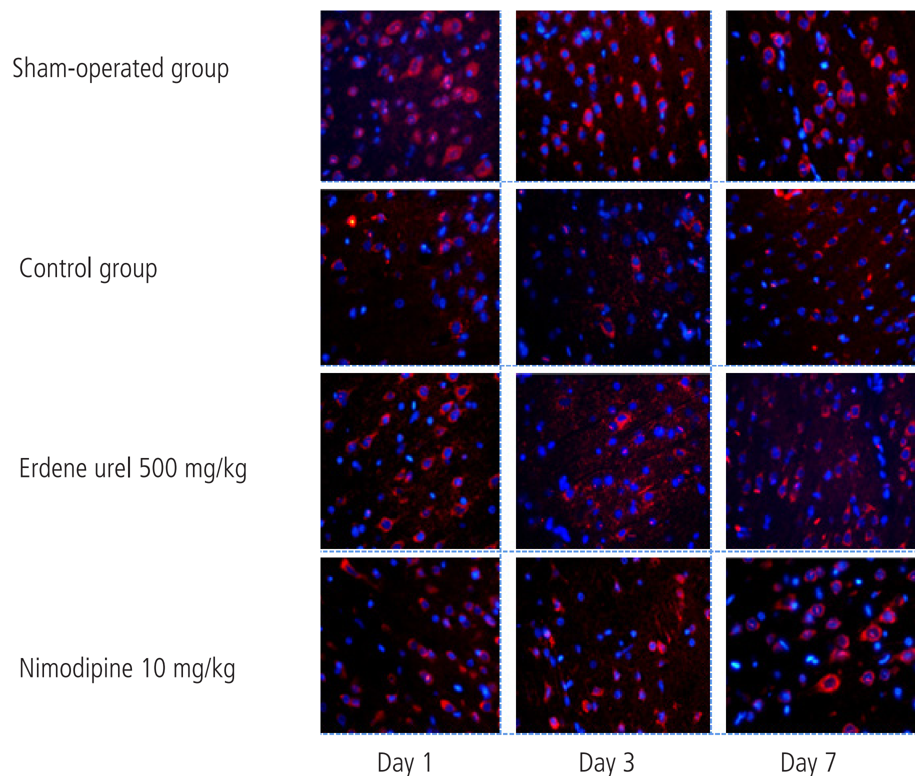

Figure 2. Effect of Erdene urel on M2-type distribution of microglia/macrophage in rats after brain ischemia (measuring scale: 50μm, 400×). Red indicates positive Arg-1, BCL-2 expression, and blue positive DAPI staining. As the number of pathological cells decreases, the cell body becomes small, thin, and the cell axon becomes clear. At a dose of Erdene urel 500 mg/kg, the number of positive cells on the seventh day increased significantly, and the cell shape became larger and rounder.

Table 4. The effects of Erdene urel on TNF-α, IL-6, IL-1β, IL-10 in the brain tissue one day after brain ischemia (mean ± SD, pg/ml).

Groups	TNF-α	IL-6	IL-1β	IL-10
Sham-operated	219 ± 18	105 ± 6.53	28 ± 2.57	64 ± 5.13
Control	425 ± 31*	159 ± 14*	58 ± 2.37*	30 ± 3.49*
Erdene urel 500 mg/kg	393 ± 31	149 ± 9.03	54 ± 5.59	35 ± 5.53
Nimodipine 10 mg/kg	394 ± 28	152 ± 6.16	57 ± 3.23	34 ± 5.14

*p<.05 vs sham-operated; n=16 in each group each day.

On the third and seventh days after reperfusion injury, the TNF-α, IL-1β, IL-6 were significantly down-regulated (p<.01) and IL-10 was up-regulated (p<.01) in Erdene urel group and nimodipine group compared with the control group. On the first, third and seventh days after reperfusion injury, the expressions of TNF-α, IL-1β, IL-6 were not significantly down-regulated in Erdene urel group compared with the nimodipine group (p>.05). On the first and third days after reperfusion injury, the expression of IL-10 was not up-regulated in Erdene urel group compared with the nimodipine group (p>.05). On the seventh day after reperfusion injury, the expression of IL-10 was up-regulated in Erdene urel group compared with the nimodipine group (p<.05) (Table 5, 6).

Discussion

Erdene urel can promote the recovery of nerve function and reduce the size of infarction after brain ischemia, and its mechanism is related to the inhibition of nerve cell apoptosis, the inhibition of oxidative stress and inflammatory response, and the promotion of neurogenesis and angiogenesis. In recent years, research on the effects of Erdene urel after brain injury

Table 5. The effects of Erdene urel on TNF-α, IL-6, IL-1β, IL-10 in the brain tissue three days after brain ischemia (mean ± SD, pg/ml).

Groups	TNF-α	IL-6	IL-1β	IL-10
Sham-operated	215 ± 16	112 ± 16	29 ± 4.19	63 ± 2.75
Control	382 ± 22*	200 ± 22*	56 ± 6.39*	32 ± 1.98*
Erdene urel 500 mg/kg	339 ± 12**	149 ± 1.72**	33 ± 6.30**	48 ± 4.16**
Nimodipine 10 mg/kg	343 ± 13**	153 ± 6.64**	35 ± 3.58**	47 ± 6.39**

*p<.05 vs sham-operated; **p<.01 vs control; n=16 in each group each day.

Table 6. The effects of Erdene urel on TNF-α, IL-6, IL-1β, IL-10 in the brain tissue seven days after brain ischemia (mean ± SD, pg/ml).

Groups	TNF-α	IL-6	IL-1β	IL-10
Sham-operated	218 ± 16	106 ± 6.78	30 ± 3.16	66 ± 5.13
Control	367 ± 17*	184 ± 15*	46 ± 2.65*	38 ± 2.23*
Erdene urel 500 mg/kg	312 ± 18**	138 ± 5.96**	31 ± 5.32**	59 ± 1.49**
Nimodipine 10 mg/kg	314 ± 23**	143 ± 8.94**	32 ± 2.23**	51 ± 2.05**

*p<.05 vs sham-operated; **p<.01 vs control; n=16 in each group each day.

has been gradually increasing [8-10]. In our study, we found that Erdene urel promoted the transformation of microglia/ macrophages from M1-type to M2-type after brain ischemia, and inhibited the inflammatory response of nerves. Microglial cells are branched protrusions that constantly expand to monitor the microenvironment in the brain and maintain the homeostasis of the central nervous system physiologically. After brain injury, microglia cells are rapidly activated, their cell body becomes larger, and protuberance becomes amoeboid and migrate to the injured area [3, 4]. Like macrophages, activated microglia have both M1 and M2 phenotypes. The M1-type microglia cells highly express CD16/32, CD86, major histocompatibility complex II and i-NOS, and release pro-inflammatory factors TNF-α, IL-1β, IL-6, NO and reactive oxygen species to aggravate nerve damage. In contrast, the M2-type highly expresses Arg1, releasing anti-inflammatory factor IL-10, and thus promotes inflammation reduction and nerve repair [5, 6]. Therefore, in this study, Iba-1, TNF-α, IL-1β, and IL-6 were selected as M1-type microglia/ macrophages markers, and Arg-1 and IL-10 were selected as M2-type microglia/macrophages markers. Hu et al.[7] first found in the control of focal brain ischemia in rats that the number of M2-type microglia/macrophages began to increase 1-3 days after ischemia, peaked 3-5 days and decreased after seven days. On Day 14, the number of M2-type microglia/macrophages returned to the pre-injury level, while M1-type microglia/macrophages number began to increase three days after ischemia, continuing to 14 days after ischemia. In traumatic brain and spinal cord injury, they also found similar phenomena [12]. Therefore, they developed a new way of thinking about ischemic brain damage, in which the conversion from microglia/macrophages from M1 to M2-type opened the door to new treatment possibilities [13]. Then a large number of studies identified drugs such as curcumin [14], Rhodiola [15], metformin [16], sildenafil [17] etc. Our ELISA test results showed that on the first, third and seventh days after middle cerebral artery occlusion reperfusion injury compared with the control group, the microglia/macrophages of M1 markers, the expressions of TNF-α, IL-1β, and IL-6 were reduced, and expressions of Arg-1 M2-type markers, IL-10 Iba were increased in the Erdene urel group.

The above studies suggest that Erdene urel can promote the transformation of microglia/ macrophages from M1-type to M2-type after brain ischemia, to inhibit the inflammatory response. In addition, recent research has found that activated microglia/ macrophages have dual effects of damage and promotion [5, 6] in which M1-type microglia/macrophages impair neurogenesis by releasing TNF-α, IL-1β, and IL-6 [18], while M2-type microglia/ macrophages promote the neurogenesis by releasing IGF-1 and TGF-β [19]. Lianhua's previous study has found that Erdene urel can promote the neurogenesis after brain ischemia and neural functional recovery [20], whether the mechanism is related to its promotion of transforming the microglia/macrophages to M2-type remains a subject for further study.

In conclusion, our study investigated the regulation of microglia/macrophages in the high-dose group (500 mg/kg) at different time points on the first, third and seventh days after oral gavage, and its inhibition of inflammatory response

after middle cerebral artery occlusion reperfusion in rats. Even though our study had a small number of rats, a limited number of days since the reperfusion injury, the results still suggest that Erdene urel likely promoted the transformation of microglia/macrophages from M1-type to M2-type after middle cerebral artery occlusion reperfusion, thus inhibiting the inflammatory response after cerebral ischemia-reperfusion injury and thus repair of cell damage. However, both the molecular mechanism and significance of the regulation of microglia/macrophage distribution by Erdene urel after brain ischemia require further study. In our next study, our research group will investigate cell survival rate at the gene level using Erdene urel water extract at different time points after hypoxic injury and further infer the molecular target of Erdene urel's action.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgments

The authors express their sincere gratitude to the College of Mongolian Medicine, Inner Mongolia University for Nationalities for providing the laboratory facilities to conduct our experimental research.

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