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The Effect of Musk on Neurons in the Pattern of Ischemic Stroke

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Objectives: In this study, we investigated the neuroprotective effect of musk[1] after brain ischemia in rats. **Methods:** The middle cerebral artery occlusion model in rats was established by thread occlusion and reperfusion was performed 90 minutes after ischemia. The reperfusion was performed separately on the first, third, and seventh day of the week after brain ischemia. The ischemia area was detected and the expressions of Arg-1, BCL-2, and Iba-1 were detected by immunofluorescence staining. **Results:** The result of immunofluorescence showed that treatment with musk 50 mg/kg and 100 mg/kg for the first, third, and seventh days significantly improved the neurological function and increased the protein expression of Arg-1 and BCL-2 in the ischemic hemisphere of brains in rats. Also, the ischemic area was reduced. **Conclusions:** The research results confirms that Mongolian Badanga[2] musk in 100 mg/kg doses, has functions of protecting neuro cells, supporting neurogenesis against inflammation, improving neuro tissues regeneration, and reducing ischemic areas through increasing brain tissues Arg-1 and BCL-2 protein's expressions, and reducing Iba-1 proteins expressions.

Keywords: Ischemia, Reperfusion Injury, Immunofluorescence, Brain, Rats.

[1] Musk is a preputial gland in an egg shaped pouch with a size of 4-6 cm is located between the genitals and the umbilicus of the adult male (Giree). The musk is a biological substance with a persistent odor obtained from the sack.

[2] Mongolian Badanga musk: In Mongolia, Siberian musk deer (Moschus moschiferus) are also found in some parts of the country.

Introduction

In recent years, there is a continuously increasing trend of non-communicable diseases morbidity rates such as blood circulation system disease (21.7 per ten thousand people), not only in the world but also in Mongolia. Cardiovascular disease, cerebrovascular disease and ischemic stroke diseases are increasingly becoming one of the major causes of mortality [1].

An eepidemiological study was conducted on the Mongolian population cerebrovascular disease morbidity rate. From the study, the yearly incidence for ischemic stroke was 1.86 per 1000 person in 1986 and the ischemic stroke incidence has increased to 2.87 between 1998 and 1999 and to 2.97 per thousand people between 2000 and 2001, respectively. According to the research study of 2000 and 2001, the registered ischemic stroke mortality rate averaged 1.32 per thousand people (for male 1.49 and female 1.17) among the Ulaanbaatar city adult population. Out of this, the younger population rate up to 50 years old was 15.8%, which shows that Mongolia is a high ischemic stroke mortality rate country [2].

Stroke is an acute cerebrovascular disease characterized by a sudden onset of focal symptoms of partial or complete failure of cerebral function lasting more than 24 hours. It is caused by profound organic changes in the structure of the brain and is characterized by persistent focal neurological disorders [3]. Stroke, the same as myocardial infarction, also follows a circadian rhythm, and is very similar to ischemic heart disease. To estimate the start time of the stroke is not easy given that nearly a quarter of strokes occur during sleep [4]. Even taking this into account, a peak incidence has been observed between 6 am and 12 noon, when 55% of ischemic strokes occur [5,6].

In Mongolian traditional medicine, musk is used for brain blood clots, ischemic stroke and articulation numb. The research by Boldsaikhan (1993) showed that out of four thousand traditional medicines, musk is included in 537 traditional medicines [7]. Moreover, over 300 Korean and Chinese traditional medicines include musk and are used for sedation, restoration, cardiovascular, nerve, and respiratory diseases and also are used for treating impotency and improving mental function (Mills, 1998). Also, it has been proven that musk promotes central nervous system and hearth function and has an active function against snake poison and inflammation (Gaski and Johnson, 1994) [8].

Moreover, musk and the main functional substances of musk and muscone, has two cardio tonic functions of neuro cells revitalization in small dose and soothing function in big dose. Furthermore, via reducing prostaglandin E, prostaglandin F and vein's absorbable (leak able) characteristics, muscone shows functions against inflammation, bacteria, womb shrinking (contracting) and androgenic function. Musk has protecting functions of rat's brain's ischemia reperfusion, which is used for experimental purpose and protection of neuro cells during brain injury. Apart from these, it has been confirmed that musk also protects cardio myocytes cells [9-14].

Therefore, nowadays, as brain vascular disease derived ischemic stroke is increasing and its treatment and prevention issues are becoming of vital importance, we have tried to approve musk's function of protecting neuro cells during brain ischemia reperfusion in the experimental animals by creating cerebral ischemia, and reperfusion models.

This study is intended to discuss the neuroprotective effect of musk (Moschus moschiferus Linnaeus) on cerebral ischemia, and reperfusion injury model rats..

Materials and Methods

Suspension preparation

The suspension of dried musk, ethanol absolute, and desiccated or enriched goat liver was prepared to study its effect on recovery following ischemic stroke with reperfusion. The musk was milked from Musk deer, bred at The Institute of Traditional Medicine and Technology, located at Shar Khooloi of Gachuurt in Ulaanbaatar. It was mixed with 96% ethanol absolute and desiccated goat liver milled into a fine powder in a proportion of 1:5:49 by weight. Then, a suspension was prepared for the oral administration by dissolving the prepared musk in carboxymethyl cellulose (CMC).

Experimental animals and treatment groups

The research was done by experimental research design and there are 5 groups of rats involved in the experiment: One hundred fifty healthy Wistar rats weighing 180-220 grams were selected and fed at the laboratory. During the experimental period, the rats were housed under a consistent temperature of 200C ± 220C with illumination in 12 hours light/dark cycle). Food and water was available ad libitum.

Rats were divided into the following groups: 1. Control group (healthy), 2. Experimental group (cerebral ischemia and reperfusion), 3. Nimodipine group 10 mg/kg (cerebral ischemia and reperfusion + treated with nimodipine), 4. Musk administrated group 50 mg/kg, and 5. Musk administrated group 100 mg/kg (cerebral ischemia and reperfusion + musk 50 mg/kg and 100 mg/kg). Each group has 24 rats.

Establishment of focal cerebral ischemia model in rats

A 7-0 monofilament nylon surgical suture with a silicone coating at its tip was inserted into the middle cerebral artery of the experimental group rats (cerebral ischemia and reperfusion), nimodipine group10 mg/kg, musk administrated group 50 mg/kg and 100 mg/kg temporarily occluding the artery, and creating cerebral ischemia in the distribution of the middle cerebral artery, using the methodology of Longa [15].

10% chloral hydrate was used in a dose of 300 mg/kg intraperitoneal injection for anesthesia in rats, separating the right common carotid artery, external carotid artery, internal carotid artery, litigating and dissociating the main trunk of the external carotid artery. In the experimental group, cerebral ischemia mimicking an ischemic stroke was induced in the area of the middle cerebral artery by occluding the middle cerebral artery for 90 minutes followed by reperfusion. The control group had no surgical procedure. One of three oral treatments (musk 50 mg/kg, musk 100 mg/kg, or nimodipine 10 mg/kg) was given orally in each experimental group daily for one week and the results were studied in the first, third and seventh day of the week.

Triphenyl tetrazolium chloride (TTC) staining method for evaluating brain ischemic stroke area

Triphenyl tetrazolium chloride staining was used to determine the extent of brain injury. The cerebrum was frozen at -20^{0}C for 20 minutes then cut into 2 mm transverse sections. The cut sections were then heated in 1% TTC suspension for 10 minutes at 37^{0}C. This demarcated the healthy tissue, which was red in color, from the ischemic zone, which was seen white. Then, the stained cerebrum was stabilized for 24 hours in 4% formalin solution. The area of infarcted tissue was calculated by the Image J program and every group was compared and evaluated.

Analysing Arg-1, BCL-2 and Iba-1 of brain tissues using immunofluorescence histochemistry

The Arg-1, BCL-2 and Iba-1 proteins in the brain tissues were semi-quantitatively determined using immunofluorescence histochemical staining in which the brain tissue was washed in phosphate buffered saline (PBS) solution and kept in 10% formalin solution refrigerated at 4^{0}C for 24 hours. After this, the brain was kept in 96% ethanol absolute, in the refrigerator at 4^{0}C. Sections were then cut and put into water. Then followed the sequence of adding restore antigen, pouring limiting suspension, adding primary antibodies, then the second antibodies, then influencing by DAPI, then rinsing off, and sealing by anti-fluorescent quenching agent substances and observing by Fluorescence Microscope. Pictures were taken afterward. The absorbance was measured in Absorbance Units (AU). Each measurement was repeated three times.

Statistical analysis

The research results were analyzed by basic methods of biostatistics; arithmetic mean (M), standard deviation (SD), confidence interval (95% CI) calculation and the value of significance was checked by using all statistical comparisons were made by two-way ANOVA followed by Tukey's post hoc test. P-values less than .05 ($p<.05$) were considered statistically significant. Analyzed was done by using a SPSS 20.0 software program.

Ethical statement

The animal study was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. Formal approval to conduct the experiments was obtained from the Ethical Committee of the Mongolian National University of Medical Sciences (Protocol No.2018/3-11). All efforts were made to minimize the number of animals used and their suffering.

Results

Brain ischemia/reperfusion was created and evaluated on Wistar breed rat, by closing the Middle Cerebral Artery for 90 minutes as per Longa EZ (1989) methodology (Figure 1).

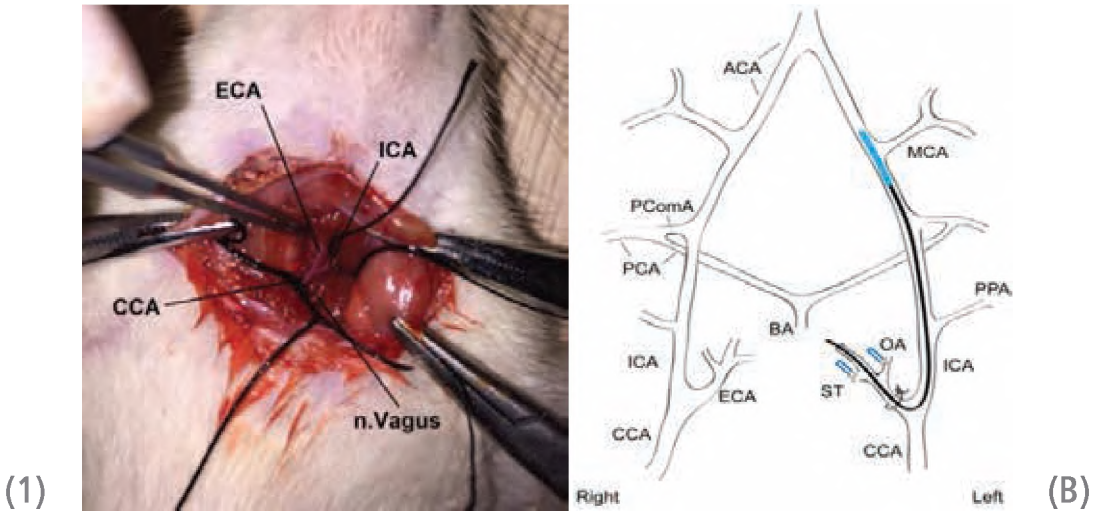

Figure 1. Method of creating left middle cerebral artery ischemic reperfusion injury. (A) Surgical microscope showing the dissection of the left common carotid artery (CCA), internal carotid artery (ICA), external carotid artery (ECA), and vagus nerve. (B) A plug on the end of a suture was passed via arteriotomy of the external carotid artery, through the internal carotid artery, occluding the middle cerebral artery for 90 minutes, and then was withdrawn, allowing reperfusion.

The results of analysis of evaluating brain ischemic stroke area by triphenyl tetrazolium chloride staining method

Following results were produced when comparing and evaluating the ischemic stroke area by Image J program in percent. Every group was compared and evaluated (Figure 2).

On the first day, after using Musk (50 mg/kg), the ischemic stroke area (10.92 ± 1.69) was compared to the experimental group's ischemic stroke area (21.86 ± 4.8). The result was the ischemic stroke area was 2 times smaller and is reduced by 50% ($p < .05$).

On the third day, after using the Musk (100 mg/kg), the ischemic stroke area (2.28 ± 0.74) (2.28%) was compared to the experimental group's ischemic stroke area (13.54 ± 3.29). The result was the ischemic stroke area was 6 times smaller ($p < .05$) and ischemic stroke center is reduced by 83.1 %.

On the seventh day, after using the Musk (100 mg/kg), the ischemic stroke area (1.60 ± 0.77) was compared to the (1.60%) Nimodipine 10 mg/kg group's ischemic stroke area. The result was the ischemic stroke area was 3 times smaller ($p < .05$) and ischemic stroke center is reduced by 64.1%. Depending on the doses ($p < .05$), Musk 50 mg/kg and Musk 100 mg/kg has reduced the ischemic stroke area by 3.3-6.2 times, compare to experimental groups (Table 1).

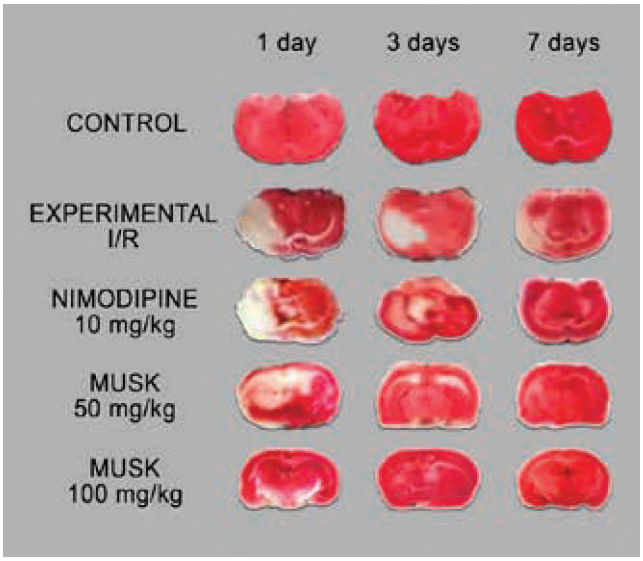

Figure 2. The results of staining coronal sections of the experimental rat brains with triphenyl tetrazolium chloride at first, third and seventh days after left middle cerebral ischemic reperfusion injury. The healthy tissue stained vivid red, while the infarcted tissue due to the ischemic stroke did not stain and appear white.

Table 1. Measurements of ischemic stroke area

	Groups				
	Control	Experimental	Musk 50 mg/kg	Musk 100 mg/kg	Nimodipine 10 mg/kg
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1st day	0	21.86 ± 4.80*	10.92 ± 1.69**	3.66 ± 1.81#	20.58 ± 5.93Δ
3rd day	0	13.54 ± 3.29*	5.16 ± 1.26**	2.28 ± 0.74#	7.04 ± 2.71Δ
7th day	0	9.92 ± 2.08*	2.64 ± 0.92**	1.60 ± 0.77#	4.52 ± 1.79Δ

*Control vs experimental at $p < .05$; **Experimental vs musk 50 mg/kg at $p < .05$; #Experimental vs musk 100 mg/kg at $p < .05$; Δexperimental vs nimodipine at $p < .05$

The effect of musk on Arg-1, BCL-2, Iba-1 proteins' brain tissue on the first, third, and seventh days after brain Ischemia.

The study of Arg-1, BCL-2, Iba-1 proteins' expression on the brain tissue of the ischemic reperfusion experimental group 150 rat analyzed the Musk effect by immunofluorescence method and compared the experimental group to control and Nimodipine groups. The study was conducted in the groups of musk dose small and musk dose high and showed following results: Iba-1 proteins size of the experimental groups rat's brain tissue was 33861 ± 2343 AU on the first day and it was 21725 ± 1805 AU and 34903 ± 4432 AU, respectively, on the third and seventh day. The BCL-2 protein size was 5276 ± 663 AU, 3488 ± 2424 AU and 4079 ± 1165 AU on the first, third and seventh day, respectively.

Arg-1 protein's expression was 4226 ± 582 AU, 2645 ± 598 AU and 4504 ± 1805 AU, on the first, third and seventh day, respectively. When comparing the above indicators to the first, third and seventh days of the control group's indicators, the Iba-1 protein size has increased with statistical significance ($p < .05$).

To compare the experimental group, BCL-2 protein size has reduced, with statistical significance, the first day, third day, and seventh day ($p < .05$). Whereas, Arg-1 protein's expression size has reduced with statistical significance of ($p < .05$), for all days.

In the ischemic stroke-reperfusion model, which was created by closing the middle cerebral artery, the Arg-1 and BCL-2 proteins have reduced significantly, on the contrary, Iba-1 protein has increased significantly.

In the experimental group, Arg-1 protein's content, which exudes from M2 phenotype macrophage cell, has significantly reduced for all days. This indicates that the function which protects neuro cells declined so that further neuro cells damage developed. During this period, Arg-1 protein has reduced probably due to increase of M1 phenotype macrophage, which is an inflammation reaction stimulator (Table 2).

When given the nimodipine 10 mg/kg for seven days after the ischemic reperfusion was formed, Arg-1 protein expression has increased with statistical significance ($p<.05$) on the first and seventh days and on the third day it has increased significantly ($p<.05$).

Table 2. The effect of Musk on Arg-1 protein expression in brain tissue on the first, third, and seventh days following left middle cerebral artery ischemic reperfusion injury as determined by immunofluorescence

	Groups				
Arg-1	Control	Experimental	Musk 50 mg/kg	Musk 100 mg/kg	Nimodipine 10 mg/kg
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1st day	21946 ± 992	4226 ± 582*	7370 ± 847	14600 ± 2653	15285 ± 4504
3rd day	20394 ± 1401	2645 ± 598*	4250 ± 52	18718 ± 4232#	17275 ± 1223
7th day	22543 ± 2297	4504 ± 1805*	12035 ± 2356*	13339 ± 7527#	13480 ± 296Δ

*Control vs experimental at $p < .05$; *Control and experimental vs musk 50 mg/kg at $p < .05$; #Control and experimental vs musk 100 mg/kg at $p < .05$; Δ Control and experimental vs nimodipine at $p < .05$

On the contrary, when giving the nimodipine 10 mg/kg for seven days, after the ischemic reperfusion was formed, Iba-1 protein expression has reduced on the first, third and seventh days, with statistical significance of (p<.05), compare to experimental group (Table 3).

When given the nimodipine 10 mg/kg for seven days after the ischemic reperfusion was formed, BCL-2 protein expressions were compared with the experimental group. On the first day, there was no difference, on the third day BCL-2 protein has increased slightly (p < .05), and on the seventh day has it

Table 3. The effect of Musk on Iba-1 protein expression in brain tissue on the first, third, and seventh days following left middle cerebral artery ischemic reperfusion injury as determined by immunofluorescence

	Groups				
Iba-1	Control	Experimental	Musk 50 mg/kg	Musk 100 mg/kg	Nimodipine 10 mg/kg
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1st day	9907 ± 1333	33861 ± 2343*	24063 ± 2742	13628 ± 569	12366 ± 960
3rd day	5790 ± 406	21725 ± 1805*	15467 ± 1252*	7031 ± 4130#	6230 ± 2052
7th day	8081 ± 1689	34903 ± 4432*	21648 ± 3827*	10424 ± 1149#	7117 ± 6340Δ

*Control vs experimental at p < .05; *Control and experimental vs musk 50 mg/kg at p < .05; #Control and experimental vs musk 100 mg/kg at p < .05; ΔControl and experimental vs nimodipine at p < .05

increased with statistical significance of (p < .05).

When measuring the Arg-1 and BCL-2 protein, after giving the Musk 50 mg/kg to the experimental group for seven days, there were no difference between first and third days (p>.05). However, Arg-1 and BCL-2 protein expressions have increased with statistical significance of (p<.05) on the seventh day.

Newsworthy, Arg-1 protein expression has increased with statistical significance of (p<.05) on the first, third and seventh days in the Musk 100 mg/kg group, compare to the experimental group. On the contrary, BCL-2 protein expression has increased slightly with statistical significance of (p<.05) (Table 2 and 4).

Discussion

Cerebral ischemia, reperfusion was created on "Wistar" breed rat, by closing the middle cerebral artery for 90 minutes as per the Longa

Table 4. The effect of Musk on BCL-2 protein expression in brain tissue on the first, third, and seventh days following left middle cerebral artery ischemic reperfusion injury as determined by immunofluorescence

	Groups				
BCL-2	Control	Experimental	Musk 50 mg/kg	Musk 100 mg/kg	Nimodipine 10 mg/kg
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1st day	31487 ± 12218	5276 ± 663*	7551 ± 8210	25146 ± 2138	29362 ± 9703
3rd day	15785 ± 6333	3488 ± 2424*	9198 ± 1379	15918 ± 4732	16063 ± 2504
7th day	34976 ± 3386	4079 ± 1165*	13381 ± 2146*	23169 ± 123#	22236 ± 1249Δ

*Control vs experimental at p < .05; *Control and experimental vs musk 50 mg/kg at p < .05; #Control and experimental vs musk 100 mg/kg at p<.05; ΔControl and experimental vs nimodipine at p < .05

E.Z (1989) methodology [15]. The experimental rat brain's ischemic stroke area was stained by the Triphenyl Tetrazolium Chloride (TTC) method and evaluated by Image J program. It shows 21.86±4.8 percent, 13.54±3.29 percent and 9.92±2.08 percent respectively on the first, third and seventh days and due to the ischemic stroke, which was created on the rat's left side brain, the rat developed movement disability on the right side. Other countries researches are shown also similar results, which is in the ischemic stroke model. The ischemic stroke area was around 30-40 percentage. Moreover, by movement evaluation, there is shown 2.5 points, 2.0 points and 1.5 points respectively on the first, third and seventh days [16].

A researcher Uranchimeg (2009) has studied brain and spleen tissues, on an ischemic stroke model group, created by closing middle cerebral artery. The study confirmed that 22 hours after closing the

middle cerebral artery, the inflammation cells had increased in spleen and the immune reaction settled down due to agmatine effect. This result is also similar to our research results especially, due to agmatine effect, there were signs of neuro protection against inflammation reducing the ischemic stroke area [17].

The effects on sensorimotor dysfunction were investigated by using balance beam test and rotarod test after brain ischemia. The expression of cyclooxygenase-2 (COX-2) was investigated by immunohistochemistry. Oral administration of musk at 300 mg/kg significantly reduced ($p<0.001$) the infarct volume by 32.4% compared with a vehicle-treated group [18].

A research study by Hu (2012) has found out that 24 hours after the ischemic stroke developed, M2 phenotype macrophage/microglia cells start to penetrate into ischemic center, then from the fifth day, the number of cells reaches at its maximum, then the number reduces from the 14th day [19].

On the other words, this study shows that there is a possibility of efficient musk influence before reducing a number of M2 cells, which has a function of protecting from inflammation, a neurotropic, and supportive neurogenesis after the 14 days.

From this study, a size of Arg-1 and BCL-2 which exude from M2 phenotype's macrophage/microglia cells, has increased with statistical significant on the seventh day, which confirms the effects of supporting neuro cell's regeneration, preventing from apoptosis, reducing ischemic stroke damages and widening blood veins.

Taylor in a 2013 article explains that M2 phenotype macrophage/microglia has increased in the ischemic stroke center in first week of the stroke, then, after 2 weeks, M1 phenotype macrophage/microglia cells increase [20].

Moschus compatible with borneolum synthcticum is a well-known herb pair in Traditional Chinese Medicine and the present study aims to assess the neuroprotective effect of a formula composed of this herb pair on ischemia stroke in rats. The middle cerebral artery occlusion model of focal cerebral ischemia in rat was performed by using an intraluminal suture method. The behavioral scores, infarct volume, and neuron ultrastructure of model and formula-treated rats were investigated after the 2 h of ischemia and 24 h of reperfusion. Meanwhile the expression levels of caspase-3, caspase-9, Bcl-2, and Bax were measured by western blot analysis. The formula treatment showed obvious neuroprotective effect by a significant decrease of the neurological scores ($p<0.01$) and the infarct volumes ($p<0.05$) when compared to the MCAO group. It observed that this formula had anti apoptosis activity on neuron cell under electron microscope. Furthermore, research result supported the idea that pro- and post administration of this formula had an anti-apoptosis effect by decreasing remarkably the expression of caspase-3 and caspase-9 ($p<0.05$) as well as increasing significantly the ratio of Bcl-2 to Bax ($p<0.01$). All evidence demonstrated the neuroprotective effect of this formula on ischemia stroke due to decrease of brain infract volume and modulation of the expression of apoptosis-related proteins [21]. It shows that the result of this study is similar to our result in terms of ischemia reperfusion model and increasing BCL protein expression, anti-apoptosis and protecting neural cells effect. Therefore, next time, there is a need to do research study of the musk effect in the longer run on the tenth, fourteenth, twenty first and twenty eighth days.

The limitation of this study is that it does not distinguish between monocytes and sedentary monocytes that enter the brain tissue from the blood vessel through inflammatory effect. Further, the side effects of musk and who can use it need to be studied. It is also necessary to clarify the effects of long-term use of musk on other organs. The musk has functions of protecting neuro cells, supporting neurogenic and preventing from inflammation, which approved by the musk 100 mg/kg group, where Arg-1 and BCL-2 proteins increased. When using musk 100 mg/kg, Arg-1 and BCL-2 protein has increased on the first, third and seventh days, which explains that the ischemic stroke area has reduced, and neuro cells regeneration has improved.

Conclusions

The research results confirms that Mongolian Badanga musk in 100 mg/kg doses, has functions of protecting neuro cells, supporting neurogenesis against inflammation, improving neuro tissues regeneration, and reducing ischemic areas through increasing brain tissues Arg-1 and BCL-2 protein's expressions, and reducing Iba-1 proteins expressions.

Conflict of Interest

The authors state no conflict of interest.

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