

# Research Paper: Optimization of Transient Focal Cerebral Ischemia Model by Middle Cerebral Artery Occlusion

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## ABSTRACT

**Introduction:** Cerebral ischemia is one of the most common causes of death in human populations in the industrial communities. The need for animal models is inevitable to study the pathophysiology and treatment of cerebral ischemia in human. The current study aimed at evaluating the strengths and weaknesses of different techniques used to create ischemia in previous studies and optimizing the transient focal cerebral ischemia model by Middle Cerebral Artery Occlusion (MCAO).

**Methods:** In the current study, 35 male Wistar rats were divided into 4 groups. The weight of rats in the groups 1 and 2 ranged 250 to 270 g with ischemic induction duration of 60 and 90 minutes, respectively. The weight of rats in the groups 3 and 4 ranged 250 to 310 g with ischemic induction duration of 60 and 90 minutes, respectively. The ischemia was performed by the MCAO method using a special filament. Motor neurological disorders were assessed 24 hours after ischemia. The ischemic area was appeared using Triphenyl-Tetrazolium Chloride (TTC) staining. Histopathological examination of the ischemic area was performed using the Nissl staining.

**Results:** In a similar duration of ischemia, the ischemic lesion volume was significantly greater in the group with lower weight. The mortality rate was lower in the groups with lower weights compared with other groups.

**Conclusion:** Based on the results of the present study, it is better to use rats with lower weights to create transient focal cerebral ischemia model using MCAO method.

## Key Words:

Middle cerebral artery, Ischemia, Stroke

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## 1. Introduction

**S**troke means a sharp reduction or complete cessation of blood flow in a brain region, preventing needed oxygen and metabolic materials delivery, and removal of waste products from the brain [1]. Stroke can be divided into 2 types: ischemic (artery occlusion) and hemorrhagic (artery rupture). Stroke causes and risk factors are: cerebral thrombosis, cerebral embolism, subarachnoid hemorrhage, and intracerebral hemorrhage. The cerebral thrombosis and cerebral embolism known as ischemic strokes account for 70% to 80% of all cases [2]. Cerebral ischemia is one of the most common causes of death in industrialized communities [3]. The need for animal models is inevitable to study the pathophysiology and treatment of cerebral ischemia in human. The most important features of animal ischemic models are repeatability and similarity to human models [4].

Several approaches can be utilized to create focal cerebral ischemia such as permanent Middle Cerebral Artery Occlusion (MCAO) through craniotomy, embolic MCA occlusion, photothrombosis model, and intra-arterial suture occlusion of MCA [5]. Previous studies showed that high mortality rate of animals in researches, incomplete blockage of the arteries, various degrees of ischemic lesions, and various volumes of cerebral lesions generated in the ischemic area are the disadvantages of these methods [6, 7]. Technical details are also varied among the methods. For example, rat weight or ischemia time is different in methods employed in studies. In general, various techniques with different variables and results indicated the need to introduce a standard procedure in order to create certain focal ischemic lesions. One of the main variables raised in different studies was the weight range of laboratory animals. Given that, the diameter of vessels would be definitely larger by the elevation of body weight; so if heavier rats are used, a specific filament probably does not make the same ischemic volume in rats.

In the present study, the best method was applied to overcome the limitations and variables of MCAO, taking into account the results as well as the advantages and limitations of previous studies in creating focal ischemia. Therefore, by applying the changes, the level of technical success increased as well as a standard and repeatable method was introduced to create focal ischemia in an animal model.

## 2. Materials and Methods

In the present study, 35 male Wistar rats were divided into 4 groups. The weight of the rats ranged from 250 to

310 g. The animals, before and during the testing, were maintained under standard conditions of 12:12 hours light/dark cycle with proper humidity, temperature of 25°C and free access to food and water. All experiments were performed in accordance with the ethical principles of animal use approved by the Research Council of Baqiyatallah University of Medical Sciences, Tehran, Iran.

The rats were tested in the following groups: 1. Ischemia Group (IG1): Weight range of 250 to 270 g with ischemic induction duration of 60 minutes; 2. Ischemia Group (IG2): Weight range of 250 to 270 g with ischemic induction duration of 90 minutes; 3. Ischemia Group (IG3): Weight range of 250 to 310 g with ischemic induction duration of 60 minutes; 4. Ischemia Group (IG4): Weight range of 250 to 310 g with ischemic induction duration of 90 minutes; and 5. The sham group: All surgical procedures were carried out to establish cerebral ischemia, but filament placement and ischemic induction not performed in this group.

### Cerebral focal ischemic induction

For cerebral transient focal ischemic induction, MCA was blocked by MCAO, as follows: The rats anesthetized by 4% isoflurane were placed in supine position and their shaved neck area was cleaned and disinfected. The 2.5% isoflurane was used to induce anesthesia. A longitudinal incision was performed in the midline of the anterior part of the neck. After removing the skin, salivary glands, muscles on the right side of the sectioned area, common carotid artery, external carotid, and internal carotid right were observed. The vagus nerve and carotid body were carefully separated from them. Connective tissue and adjacent elements of the internal carotid artery were pushed until the origin of this branch to prevent the entry of filaments into pterygopalatine artery. The pterygopalatine artery was temporarily blocked using surgical thread.

Occipital artery and superior thyroid artery, the branches of the external carotid artery, were carefully blocked. Then, the black braided nylon, 0-3 gauge (ethicon) with rounded head through the heat was directed into the internal carotid artery via external carotid artery to deal with the resistance and block the middle cerebral artery. After creating ischemia, the filament was removed and reperfusion was established for 24 hours. During the surgery, rectal temperature was measured by a digital thermometer and maintained at about 37°C using heat lamps and pads.

### Assessment of neuromotor dysfunction

Animals 24 hours after creating the ischemia were evaluated for the incidence of Neurological Deficit

Score (NDS) caused by ischemia. A specific test introduced by Pelseniyala et al. was used to this end. In this test, score 1 was given to rats with natural motion; score 2 to the animal that bended the front leg on the opposite side of the lesion or reduced the ability of gripping in that organs while hangs down from the tail; score 3 to the animal that began to turn toward the ischemic hemisphere (left) at the start of motion on a flat surface, which was at normal state at the rest time; score 4 to the animal that had lost righting reflex or had little resistance against the righting by pushing to lateral positions; score 5 to the animal with no spontaneity motion.

### Measurement of brain lesion volume

After evaluation of neuromotor dysfunction, the animals were anesthetized by ether. The brain was removed after creating cervical dislocation. Then, 2-mm coronal sections were prepared using brain matrix. For staining, the samples were placed in 2% Triphenyl-Tetrazolium Chloride (TTC) solution at 37°C for 20 minutes. The ischemic areas appeared in white and healthy areas in red. The sections were placed in 10% buffered formalin to be fixed for 24 hours.

After final preparation, the images of sections were taken separately through a digital camera (Cannon, Japan). The surface area of the damaged parts of the cortex and striatum was measured for each section using the NIH image analyzer software. The lesion volume was calculated as a percentage of total volume for all sections.

### Histopathologic studies

To evaluate the histopathological changes, 5-micron sections were prepared from tissue samples. Cresyl violet acetate staining was utilized to evaluate the morphology of cells and changes following ischemia.

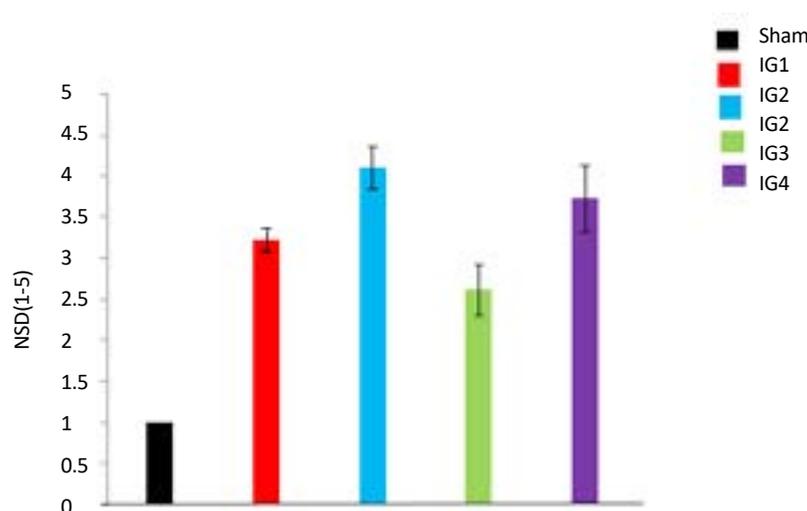
### Statistical analysis

The obtained results were expressed as mean±Standard Error of Mean (SEM). The Student t test was used for intergroup comparison and  $P<0.05$  was considered as level of significance.

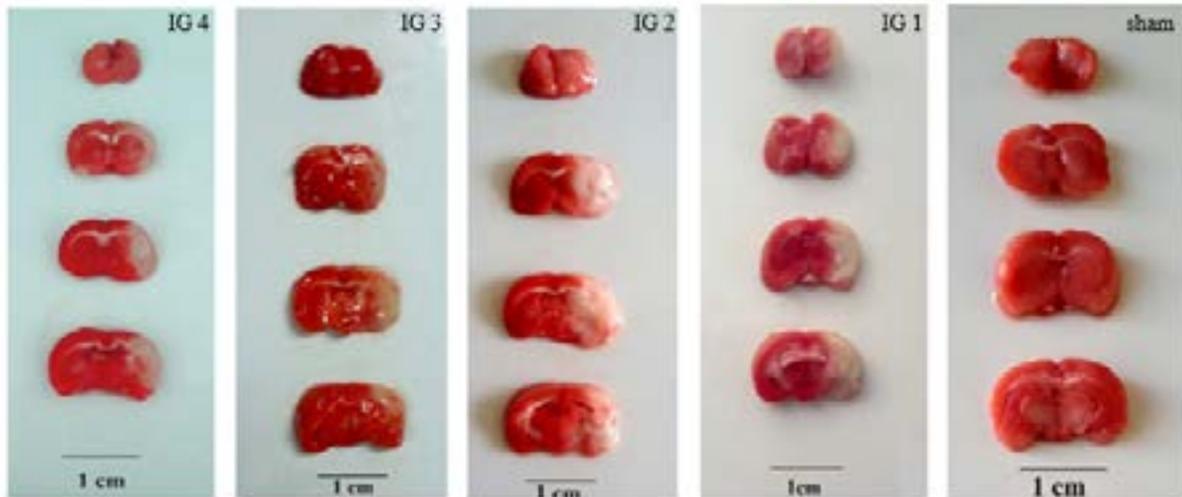
## 3. Results

No motor dysfunction was observed in the control group 24 hours after ischemia (NDS=1), but neuromotor dysfunctions were found in other ischemic groups and neurologic test scores were higher in the intervention groups, compared to the controls. Assessment of neuromotor dysfunctions among the ischemic groups showed that NDS was higher in groups with 90-minute ischemic durations compared with the ones with 60 minutes duration. Between the 2 groups with 60-minute ischemic duration, the NDS score was higher in the animals with lower weights. As seen in Figure 1, the NDS score distribution among the groups with higher weights was more than the lower weight ones. The mortality rate in the groups 1, 2, 3 and 4 was 3.6%, 5.2%, 5.5% and 9%, respectively.

Results of the current study also indicated that the ischemic lesion volume in group 2 was higher than



**Figure 1.** Neurological dysfunctions scoring in the sham and ischemic animals 24 hours after reperfusion. Data are shown as mean±SEM.



**Figure 2.** Coronal sections stained with TTC in the sham and ischemic groups

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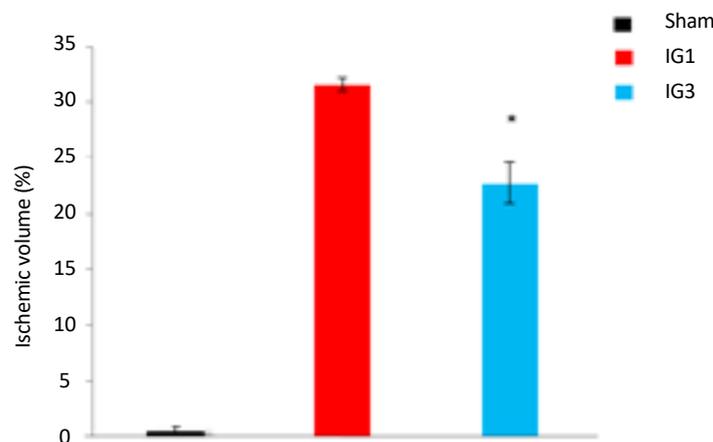
other groups (Figure 2). The ischemic areas are shown in white and the non-ischemic areas in red. As shown in Figure 3, the ischemic lesion volume in 60-minute ischemic groups was significantly higher in the animals with lower weights; the same results were obtained for 90-minute ischemic groups (Figure 4). The results also showed that the lesion volume among the groups with higher weight had more changes.

In histopathological studies, the Nissl bodies were stained using cresyl violet acetate solution. As seen in Figure 5, no pathological changes were observed in the control group. In ischemic groups, neurons had dense and shrunken nuclei. The numbers of cells in the IG1, IG2, and IG3 groups were fallen sharply compared with the control group and neurons with dense nuclei were visible. The cell damage rate was less in the group IG4

and healthy neurons were obvious alongside the damaged and dense neurons.

#### 4. Discussion

The high prevalence of stroke disease in the industrialized countries indicated the need for efforts to identify risk factors and methods to treat the disease. The use of similar animal models to simulate cerebral ischemia and evaluate the therapeutic strategies can be very beneficial. Extensive clinical studies on stroke in humans indicated that the infarct size in ischemic stroke was very small and limited in most cases. A human clinical trial showed that brain infarct size often ranges from 28 to 80 cubic millimeters [8]. Another clinical research revealed that malignant infarction in human ischemia is observed only in 10% of the cases [9]. The most advantageous point

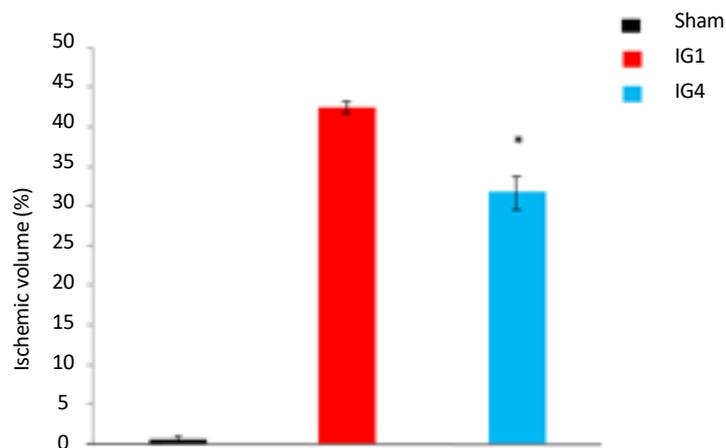


**Figure 3.** Brain ischemic volume in sham and 60-min ischemic groups 24 hours after reperfusion

Data are shown as mean±SEM.

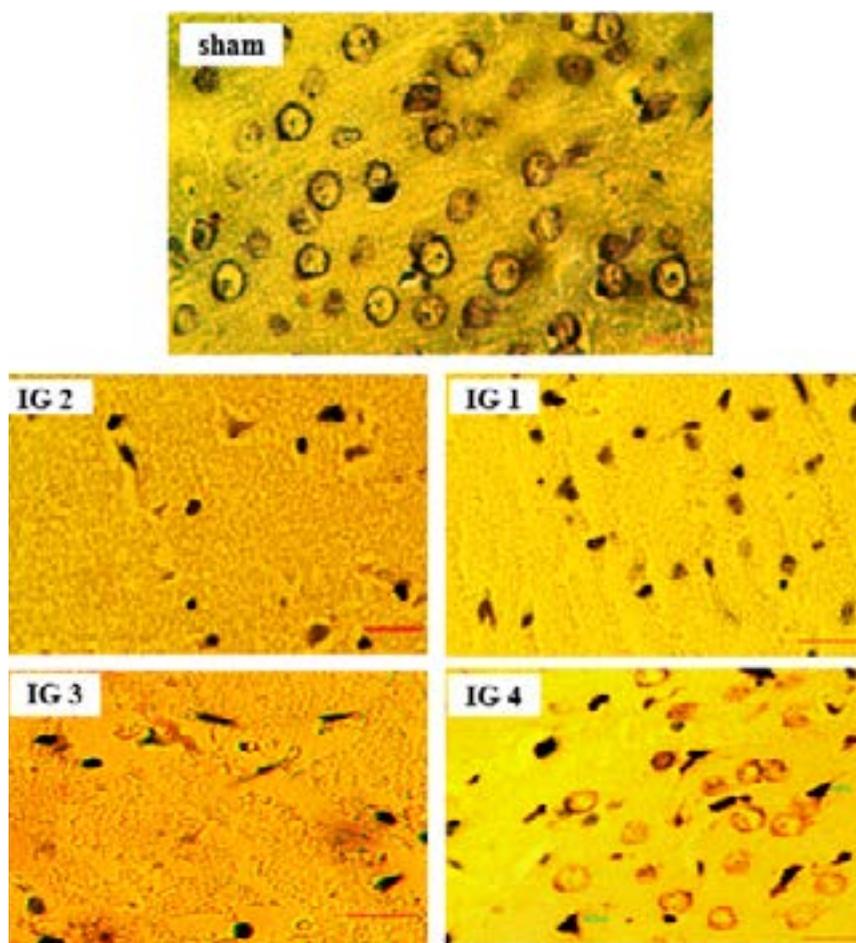
\*: Significant difference; P<0.05

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**Figure 4.** Brain ischemic volume in sham and 90-min ischemic groups 24 hours after reperfusion  
Data are shown as mean±SEM.  
\*: Significant difference; P<0.05

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**Figure 5.** Histological sections of the brain in the sham and ischemic groups using cresyl violet acetate staining  
Eosinophilic neurons are not found in the control group, but in ischemic groups are seen in abundance. Scale bar=20 μm

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in the advanced animal models is their similarity to the human models; hence, the use of methods to create permanent ischemia does not seem desirable. In addition to this advantage, high mortality rate, complicated surgical procedures, and extensive changes in the size and location of brain lesions limited the application of such models in cerebral ischemia studies.

In subsequent studies, the transient MCAO by intra-arterial filament placement method were used; one of the advantages of this method is the reversibility of artery occlusion. In addition, the transient MCAO is a convenient technique with shorter surgical time and lower cost. This technique was gradually changed. For example, the heating and forming the filament tip technique was used to better blockage in the artery [10, 11]. The filament tip covered by silicone and polymers is a technique applied in recent studies. Although the use of silicon in forming filaments increased the efficiency of ischemic lesions, some researchers believe that the separation of the silicon particles can lead to the incidence of vascular occlusion. Ethicon 0-3 filament was used in the current study. The filament tip was formed using heat in a particular way. The shaped form was studied carefully under a light microscope to ensure regular and uniform shape.

The results obtained from the method of intra-arterial filament placement for focal ischemia in animal models are very different because of various variables and variation in details. In the current study, different variables were evaluated in order to introduce a standard and repeatable approach for inducing transient focal ischemia. The variables of previous studies were the ischemic duration, weight of the rats, anesthetics, etc. The researchers utilized the periods of 60, 90 and 120 minute in the intra-arterial filament placement method. Garcia and Li demonstrated that the periods longer than 60 minutes could lead to create larger ischemia, especially in the hippocampal and hypothalamic areas [12]. Damage to cells in hypothalamus caused hyperthermia in rats within the 24 hours after ischemia [13]. The incidence of hyperthermia resulted in increased cell death and animal mortality [14]. In addition, studies suggest that damage to the hypothalamus was rarely observed in human ischemic. Animal models should be designed in such an extent to do not damage the vast area of hypothalamus.

It is also important to note that striatal damages begin in a short interval of ischemia and generally cause necrosis in cells, but cortical damages start with delay and gradually result in cell apoptosis [15]. Therefore, reperfusion reduces cell damage in the cortex. Moreover, hypoxia-induced genes such as heat shock protein 70,

Bcl-2, Bcl- XL, and Bax with neuroprotective role can exclusively be expressed in the cortex [16, 17].

Animal model that in addition to the limited damage to ischemic core area can cause a delayed death in the cortex has acceptable similarity to human ischemia [18]. According to these reasons, the 60-minute ischemic duration is more appropriate for creating ischemia similar to humans in animal models. Results of the current study also confirmed this issue. In histopathological assessments of cerebral ischemic samples, the pathological changes were reported including the formation of eosinophilic neurons (neurons with pyknotic, shrieked, dense, and triangular nuclei with highly acidophilic cytoplasm).

In the current study, the histopathological changes were clearly observed in the ischemic groups. Another variable in intra-arterial filament placement method was the weight range of rats. Given that, the diameter of vessels would be larger definitely by increasing the body weight, so if heavier rats are used in a group, a specific filament probably does not give the same ischemic volume in rats. For example, Oliff et al., studied rats weighed 276 to 346 g. The success rate of intra-arterial filament placement method was 70% in their study; a lower efficiency compared with the studies on rats with lower weights was observed [19, 20]. The results of the current study clearly showed that using smaller rats increased the accuracy and repeatability of intra-arterial filament placement method and reduced mortality.

The method of inducing anesthesia in rats was another variable considered in other studies. Some studies used a combination of ketamine and xylazine for animal anesthesia that the mortality rate of the method was very high due to respiratory and cardiovascular disorders alongside of a long recovery time. The use of isoflurane in animal anesthesia caused a significant reduction in mortality and recovery time. Moreover, studies show that isoflurane has neuroprotective effects on rats' recovery [21]. The focal ischemia model was developed in rats and mice. Hypothalamic ischemia caused hypothermia in mice unlike rats, and required intensive care during the recovery period [22]. Also, unlike rats, the damage levels caused by ischemia varied in different murine races. For example, the damage levels caused by ischemia were higher in BALB/c mice than that of C57BL/6 mice [23].

In different races of mice, collateral arteries had various patterns. In addition, the sensitivity level of cells to ischemia varied in different races of mice. In the murine model, ischemic lesion volume was directly related to the artery occlusion and followed it dramatically. For ex-

ample, ischemic lesion volume caused by artery occlusion in C57BL/6 mice for 30 minutes was about 5 times higher than the occlusion for 15 minutes [24], or bilateral common carotid artery occlusion for 3 minutes did not create visible ischemic lesion, whereas obstruction for 5 minutes caused ischemic lesion in the hypothalamus and the striatum. Considering these characteristics, using rats in order to create optimal models of focal ischemia seems more reasonable.

The present study aimed at making a comprehensive review of previous studies on creating animal models of focal cerebral ischemia in order to introduce a standard method with high repeatability and efficiency. Based on the results of the current study, it is suggested to use rats with lower weights for creating animal ischemia model that can be generalized to ischemia in humans. The use of isoflurane for animal anesthesia was more appropriate compared with other anesthetic agents. In addition, results of the study suggested that it is better to create transient focal ischemia model by MCAO using Ethicon thread formed for 60 minutes.

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### Conflict of Interest

The authors declared no conflicts of interest.

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