A Model of Focal Ischemic Stroke in the Rat: Reproducible Extensive Cortical Infarction

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SUMMARY In the search for a more reproducible focal ischemic stroke model in the rat, we systematically interrupted blood flow to the right middle cerebral artery territory by ligating the right middle cerebral artery, and the right and left common carotid arteries in succession. Using a laser-Doppler flowmeter, we found that the relative surface blood flow in cerebral cortex supplied by the right middle cerebral artery decreased to 62, 48, and 18% of baseline respectively after successive ligation of the right middle cerebral artery, and the right and left common carotid arteries. A focal infarct in the cerebral cortex supplied by the right middle cerebral artery was consistently noted after ligation of the right middle cerebral and the right common carotid arteries and temporary clip occlusion of the left common carotid artery for 60 min. The surface areas of infarction measured 100 ± 6 mm² and the maximal cross-sectional area of infarction was 10.4 ± 1.1 mm² (N = 10). The mortality rate was 7% (N = 70). The characteristic changes of ischemic necrosis were limited to the cortex with sparing of subcortical structures. No motor deficits occurred. Occlusion of the right middle cerebral artery alone or together with the right common carotid artery did not consistently cause gross infarction and the maximal cross-sectional area of infarction was smaller (the right middle cerebral artery, 1.7 ± 0.8 mm², N = 10; the right middle cerebral artery plus the right common carotid artery, 4.8 ± 1.9 mm², N = 10). Permanent ligation of the right middle cerebral artery and both common carotid arteries had a high mortality (60% in 3 days, N = 10). Results from this study showed that a more consistent cortical infarction in the right middle cerebral artery territory with low mortality can be achieved by interruption of collateral circulation following the right middle cerebral artery ligation.

The advantages of using the rat for stroke study include the similarity of its intracranial circulation to that of man,¹ the abundant neurochemical data derived from rat brain,² and the relatively low animal cost which is important for large scale studies for statistical analysis. The several models of cerebral ischemia developed in the rat³-¹³ can be classed by topography as global or focal and by chronology into reversible and irreversible. These methods entail intravascular embolization or extravascular ligation.¹⁴-¹⁶ For studies of the molecular events in cerebral ischemia, an animal model of focal and irreversible ischemia allowing only partial reperfusion to create a reproducible infarction of predictable size and location would be of considerable value. Intravascular embolization can cause focal irreversible ischemia without need for craniectomy but precise control of the ultimate site of the emboli is impossible and the infarction produced is usually multifocal and variable in size and location.¹⁴-¹⁶ The extensive intracranial collateral circulation in the rat provided by the circle of Willis, leptomeningeal anastomoses and dorsal collateral junctions¹⁷ constrains the use of either unilateral or bilateral common carotid artery (CCA) ligation to produce consistent ischemic lesions unless a systemic insult such as hypotension⁶ is added. The additional systemic variable may cause generalized metabolic derangement and/or compromise cardiopulmonary function.

The procedure of Tamura et al⁹ of ligation of the proximal middle cerebral artery (MCA) through a subtemporal approach has been reported to result in consistent ischemic changes, but the procedure is technically difficult and sufficiently invasive that the survival of hours limits it to acute experiments.¹⁰ Coyle used a less invasive surgical approach with MCA ligation above the rhinal fissure but did not produce cerebral infarction in young Wistar rats.¹¹ We have undertaken to develop a reliable infarction model reasoning that the collateral circulation to the MCA territory is decisive in infarct occurrence after MCA ligation and that graded interruption of this collateral circulation would determine the lesion size in the bed of the occluded MCA. A systematic approach employing a relatively non-invasive surgical procedure has resulted in the development of a predictable large cortical infarct.

Materials and Methods
Laser Doppler Flowmetry

To determine that the blood flow in the right MCA territory after right MCA ligation was further reduced by occlusion of CCAs, we first measured the cerebral blood flow in the cortex supplied by the right MCA with a laser Doppler flowmeter¹⁸-²² (Periflux, Perimed, Sweden) before and after successive ligation of the right MCA, right CCA and left CCA. Long Evans rats (250-350 gm) were anesthetized by intraperitoneal injection of ketamine HCl (100 mg/kg body weight). Body temperature was monitored by a rectal probe and maintained at 37.5 ± 0.5°C with a heat lamp regulated by a thermostat (VeraTherm, Model 2156, Chicago, Il.) Aterial pressure was continuously monitored with...
an intra-arterial catheter in the right femoral artery which was connected to a pressure transducer (Gould, P23ID, Oxnard, Ca.) and Grass polygraph recorder (Grass, Model 79D, Quincy, Ma.). Animals with hypotension requiring resuscitation were discarded. A tracheostomy was done. Ventilation was maintained with a Harvard small animal ventilator (South Hatck, Ma.) at a constant inhalation pressure of 6-7 mmHg and rate of 80/min using 95% O₂ and 5% CO₂. A 1.5 cm midline incision was made on the scalp, connective tissue was removed and a 2.5 mm burr hole was prepared using a dental drill with the aid of a Bausch and Lomb Stereo Zoom 7 microscope (Rochester, N.Y.) over the cortex supplied by right MCA: 5 mm lateral to the sagittal suture and 7 mm caudal to the coronal suture. The drill pit was constantly cooled with saline at ambient temperature and care was taken to preserve a thin bone layer (that was later removed gently with forceps) at the depth of the hole in order to avoid thermal and physical injury to the cerebral cortex. A plastic holder made from cast acrylic was mounted around the burr hole, secured by two screws and then reinforced with dental cement. A standard laser Doppler probe (Pflöd, PeriMed, Sweden) was inserted into the burr hole through the holder, which fit the probe tightly preventing it from moving. The principles of measuring the tissue surface blood flow with a laser Doppler flowmeter have been described in detail elsewhere. In brief, light from a helium-neon laser is carried by an optical fiber to a probe and illuminates a 1 mm³ volume of tissue. Moving red blood cells backscatter the light and cause a frequency shift which is determined by photodetectors and displayed after digitized processing. In this study, relative blood flow was measured. The blood flow recorded prior to any arterial ligation was allowed to stabilize for at least 30 min. The flow then obtained served as baseline and was designated as 100%. Measurements were then made after successive ligations of the right MCA, right CCA, and left CCA and were calculated as percent of the baseline. The minimum interval recorded was 30 minutes after blood flow became stable following each step of arterial ligation described below. Five rats were used in this study. They were sacrificed at the end of blood flow measurement.

Ligation of Right MCA

A 1.5 cm scalp incision was made at the midpoint between the right eye and the right ear. The temporalis muscle was separated in the plane of its fiber bundles and retracted in order to expose the zygoma and squamosal bone. Using microsurgical techniques, a burr hole, 2 mm in diameter, was made with a dental drill 1 mmrostal to the anterior junction of the zygoma and the squamosal bone. Care was taken as described above to avoid thermal or physical injury to the cortex during preparation of the burr hole. The dura mater was carefully pierced with a #11 scalpel. The exposed area was openings and ligated with a square knot using a 10-0 suture. The craniotomy was covered with a small piece of gelfoam, the temporalis muscle and overlying skin were allowed to fall back and were sutured separately.

Ligation of CCAs

The CCAs were isolated via a ventral midline cervical incision and both the CCAs were ligated. Either the right or both CCAs were ligated as specified.

Procedures for the Development of Focal Infarction

Five groups of animals underwent different procedures during the search for an optimal stroke model. Each group consisted of 10 animals except for Group 4 which included 13 animals.

Group 1: Sham operation. The animals underwent isolation of both CCAs and passage of a needle beneath the right MCA without ligation.

Group 2: Ligation of the right MCA only.

Group 3: Ligation of the right MCA and right CCA.

Group 4: Ligation of the right MCA and right CCA plus temporary clip compression of the left CCA with a pair of non-traumatic micro-aneurysm clips for 60 min. In this group of animals, the existence of blood flow in the left CCA was checked after clip compression was released. Animals without established flow through left CCA were discarded.

Group 5: Ligation of the right MCA and both CCAs.

Histopathological Studies

Three days after operation and 24 hours after intraperitoneal injection of 0.75 cc 2% Evans Blue the rats were anesthetized with ketamine HCl. Following intracardiac perfusion of 150 ml normal saline, the animals were fixed by perfusion with 200 ml of 10% neutral buffered formalin. The brain was kept in this fixative for at least 3 days before section. The cerebral hemispheres were cut in the coronal plane and paraffin embedded. Sections 6-7 μm thick were stained with hematoxylin and eosin, and examined by light microscopy. Areas of infarction were determined using the morphometric procedures described below. For the purpose of photographic illustration, two rats in Group 4 were perfused with 150 ml normal saline and 50 cc triphenyl tetrazolium chloride (TTC). Animals dying within 3 days after surgery were autopsied as soon as the death was noted.

Morphometric Studies

The area of infarction on the cortical surface was measured following the procedure described by Jones and Coyle in 10 rats in Group 4. One animal in Group 4 was excluded because of death in less than 3 days and two were perfused with TTC for photographic illustration. Scaled photographs of the dorsal and lateral views of each fixed brain were taken before section. Prints with 10× magnification were prepared to measure the lesion size on a Videoplan interactive image analysis system (Carl Zeiss, West Germany). The perimeter of the lesion was traced on a digitizing pad. The intersection between dorsal and lateral projections was determined by common correspondence points. The traced area of the dorsal view was multiplied by a
constant (1.55) developed by Jones and Coyle to correct for the effect of curvature. The corrected dorsal area is added to the lateral area to give the total surface area of the lesion. Similar morphometric procedures were used to measure the maximal infarct size by cross-section area in each group of animals except for Group 5. The majority of animals in Group 5 died before day 3. (See Results).

Statistical Analysis
The data are expressed as mean ± SEM. The significance of differences between group means was determined using Student’s t test except that paired t test was used in the statistical analysis of the results from measurement of the relative blood flow using laser Doppler flowmetry. A p value less than 0.05 was considered significant.

Results
Laser Doppler Flowmetry
Ligation of the right MCA produced a fall of the relative surface blood flow in the cortex supplied by the right MCA to 62 ± 11% (mean ± SEM, N = 5) of the baseline (100%). Additional ipsilateral CCA occlusion produced a further flow reduction to 48 ± 10%. The relative blood flow declined to 18 ± 4% following ligation of the right MCA and both CCAs. The reduction in relative blood flow following each successive ligation was significant by paired t test. The mean arterial pressures (MAP) were 121.5 ± 8.5 mm Hg before and 123 ± 8.6 after right MCA and right CCA ligation. The difference was not significant. After additional left CCA ligation, the MAP was 136.5 ± 14.7 which was significantly elevated when compared to that either before or after right MCA and right CCA ligation.

Mortality and Morbidity of Surgery
There was no mortality in Group 1, 2 or 3. In Group 4, 1 of the 13 rats died before 3 days. In subsequent biochemical studies using the Group 4 procedure, we noted 4 deaths in a total of 57 rats which were followed for 3 days or longer in an overall mortality in 70 rats of 7% in Group 4. Of a total of 124 rats in Group 4, four were discarded because of failure to reestablish blood flow in left CCA after release of the clip or the need for resuscitation during surgery. The mortality rate was 60% (N = 10) in Group 5. Death among Group 4 and 5 animals occurred within 2 days after surgery. Severe brain swelling with cerebellar tonsillar herniation was noted at autopsy. Judging by the ability of the animal to walk and run normally with no asymmetry of muscle tone, there was no apparent impairment of motor function 24 hours after operation when all surviving animals had begun to eat and drink. Follow up motor examination for up to 2 weeks after surgery revealed no deficit.

Morphological Studies
None of the 10 rats in Group 1 or 2 had grossly visible infarction, while 3 out of 10 in the Group 3 did.
were seen in Group 2 and variable, but usually larger, infarcts were seen in Group 3. All animals in Group 4 had large infarcts in the right MCA territory and occasional small infarcts in the left MCA territory. The cerebral infarcts from Group 4 were manifested histologically by large confluent area of coagulation necrosis (fig. 4) infiltrated by a few macrophage (fig. 5). In addition to the coagulated appearance of the tissue, neurons and glia in various stages of cellular necrosis were apparent throughout the infarcted zone. Such cells exhibited shrinkage, intense eosinophilic homogenation of cytoplasm and pyknotosis, karyorrhexis and karyolysis especially well seen in neurons (fig. 6). All these changes were limited to the cortical grey matter with the rare occurrence of edema and disintegration of a few nerve fibers in the underlying white matter. Subcortical structures and basal ganglia were spared (fig. 4). The maximum cross-sectional infarction areas measured 1.67 ± 0.90 mm²; 5.09 ± 1.69 mm²; and 10.38 ± 1.10 mm² in Group 2, 3 and 4 respectively. The difference between any two groups was significant.

Discussion

This study derived from reasoning that a sufficient reduction of the collateral circulation following MCA ligation in the rat would produce severe cerebral ischemia with resultant infarction in the MCA territory. Estimation of blood flow by laser Doppler flowmetry has been applied to skin, kidney, testis, and cerebral cortex and this correlated well with the Xenon clearance method for skin blood flow measurement in human forearm. In this study, relative surface blood flow by laser Doppler flowmetry in the cortex supplied by the right MCA decreased significantly after successive occlusion of the right MCA, right CCA and left CCA. It fell to 18% of the baseline after right MCA and bilateral CCA occlusion. The
critical level of blood flow for cerebral electrophysiological failure is approximately 30% (15-18 ml/100 gm min) of the normal in several species including human.23 The flow threshold for infarction has not been clearly established, but may be lower than that for electrophysiological failure. In our study, occlusion of the right MCA and both CCAs appeared to reduce blood flow to a level lower that that sufficient for neuronal activity to cease. A similar principle has been employed in the dog model to produce cerebral infarction by ligation of the MCA and the ipsilateral intracranial internal carotid artery.26

We found that ligation of the right MCA above the rhinal fissure by itself was insufficient to cause consistent cerebral infarction as has also been found by Coyle.11 Additional ligation of the ipsilateral CCA did not establish a large and consistent infarct. This is not surprising in view of the relatively minor impact of right CCA ligation on blood flow to cerebral cortex supplied by the right MCA (reduction from 62 to 48% of baseline). Ligation of the right MCA and both CCAs which reduced relative blood flow in the right MCA territory drastically (to 18% of baseline), was not compatible with long-term survival (60% mortality). The Group 4 procedure, (ligation of the right MCA and CCA plus clip occlusion of the left CCA for 1 hour) offered an optimal outcome in this search for a chronic focal ischemia model. The mortality rate was low (7%) and a grossly-visible infarct was consistently observed (96%). Microscopically, lesions with typical ischemic changes were limited to the cortex and the basal ganglia and other subcortical structures were spared probably due to the occlusion distal to the lateral lenticulostriate arteries. While restoration of flow in the left CCA after occlusion for 1 hour reduced the mortality in Group 4, reperfusion might contribute to the development of an infarct in the right MCA territory.27 The mean surface area of infarction in our stroke model group (Group 4) is larger than that reported by Coyle et al in spontaneously hypertensive stroke-prone rats after MCA ligation (100 ± 6 mm² vs 62 ± 11 mm²).28 Furthermore, our model obviates the need to use hypertensive rats.

Ligation of the MCA above the rhinal fissure by Robinson et al in adult Sprague-Dawley rats produced a circular lesion 1-5 mm in diameter that was usually adjacent to the craniotomy site.29,30 Coyle attempted this in young Wistar rats and was not able to establish an infarct.11 The lesions in both the experimental and sham-operated rats11 resembled those reported by Robinson et al and were considered to be the consequence of the surgical exposure and ligation of the MCA.31 The lesion size (1-4 mm in diameter) in our sham-operated animals was the same and was felt to result from the surgical trauma during isolation and ligation of the right MCA. Another approach was that of Tamura et al who ligated the proximal MCA just lateral to the olfactory tract.9 Access was made by excision of the temporalis muscle, zygoma, and coronoid process and this allowed exposure of the MCA stern. Ischemic damage was reported to be variable in the cortex and basal ganglia but the procedure was so extensive that postoperative feeding was a problem. Thus their pathological studies were limited to several hours post-surgery. Evolution of the ischemic changes and estimation of infarct size at a later stage were not reported. They suggested that a postoperative survival of several days could be achieved by less extensive exposure without removal of any bone or muscle.9 Our technique is simpler and less invasive. There is no difficulty in feeding because bone and muscle have not been excised. This model enables long term survival and facilitates study of the chronology of biochemical changes in cerebral infarction. For example, we have completed a study of the evolution of neurofilament protein degradation paralleling the accumulation of brain calcium content32 for up to 14 days post surgery using this model.

In this model as in the gerbil,32 no impairment of motor function occurs with a large cortical infarct probably because the primary motor cortex in the rat is located medially34 and is spared. Or, the primary motor cortex may be needed only for complex motor performance and subcortical nuclei may suffice for ambulation. The lack of motor impairment eliminates behavior analysis or grading of neurological deficits for the assessment of therapeutic intervention. Neurological deficits such as hemiparesis are difficult to assess in small laboratory animals such as the gerbil and the rat and the other neurological dysfunction such as respiratory abnormalities, seizures and impairment of consciousness which do occur35 are not common with human focal ischemic stroke and difficult to assess quantitatively. The morphometric measurement of infarct sizes in Group 4 and the mortality rate in Group 5 appear to be useful parameters which can be used to objectively evaluate the efficacy of drug treatment in moderate and severe focal ischemia respectively. This model, however, bears little resemblance to human stroke. The analogous events of unilateral MCA and CCA occlusion and clipping of contralateral CCA are distinctly rare in the human.

In conclusion, employing a procedure of systematic interruption of blood supply to the right MCA territory we were able to produce consistently a large cortical infarct by ligating the right MCA and right CCA plus clip occlusion of left CCA for 1 hour. The surgical procedure is relatively simple, mortality is low, and long term survival is usual. With this model, biochemical studies of the evolution of cerebral infarction can be carried out. Morphometric analysis of infarct size in this model offers a quantitative measure of the efficacy of pharmacological intervention.

References
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