External Carotid Artery Territory Ischemia Impairs Outcome in the Endovascular Filament Model of Middle Cerebral Artery Occlusion in Rats

Michael Dittmar, MD; Thilo Spruss, PhD; Gerhard Schuierer, MD; Markus Horn, MD

Background and Purpose—Middle cerebral artery occlusion (MCAO) by an intraluminal filament is a widely accepted animal model of focal cerebral ischemia. In this procedure, cutting of the external carotid artery (ECA) is a prerequisite for thread insertion. However, the implications of ECA transsection have not yet been described.

Methods—After 90 minutes of filament MCAO or sham surgery, rats were evaluated for up to 14 days in terms of body weight development, core temperature, and motor performance. Repeated in vivo MRI of the head and neck was performed for quantification of brain edema and infarct volume. The temporal muscles were histologically analyzed postmortem.

Results—In 47% of all rats, ischemic tissue damage to the ipsilateral ECA area, including temporal, lingual, and pharyngeal musculature, was detectable by MRI. Histology of temporal muscles confirmed acute ischemic myopathy. Animals with ECA territory ischemia (ECA-I) showed delayed body weight development and poorer recovery of motor function. There was no difference in the extent of brain edema or final cerebral lesion size between ECA-I–affected and unaffected rats.

Conclusions—Filament MCAO was complicated by the consequences of ECA ischemia in approximately half of all rats. Impaired mastication and swallowing functions restricted ingestion and resulted in postsurgical body weight loss and worse motor performance. Impaired cerebral microperfusion resulting from dehydration and reduced spontaneous motor activity resulting from reduced food and water uptake might have contributed to poorer neurological recovery in ECA ischemic rats. Thus, adverse effects caused by extracerebral ischemia with potential impact on outcome have to be considered in this stroke model. (Stroke. 2003;34:2252-2257.)

Key Words: ischemia ■ magnetic resonance imaging ■ middle cerebral artery occlusion ■ outcome ■ rats

Animal models of cerebral ischemia represent an important contribution to both our understanding of human stroke and the development of new therapies. Currently, the minimally invasive endovascular filament model is widely accepted to induce transient or permanent middle cerebral artery occlusion (MCAO) in rats. Since first introduced by Koizumi and coworkers,1 it has been applied on a huge scale to elucidate the pathophysiological basis of stroke and to evaluate neuroprotective strategies. This model allows us to study focal brain ischemia within the intact skull free from changes in cerebrospinal fluid dynamics or damage to the surrounding nerve plexus of the MCA. Nevertheless, some pitfalls of the intraluminal filament model have also been described such as insufficient MCAO, inadvertent premature reperfusion, and subarachnoid hemorrhage.2

The present study describes a different adverse effect of this model that has not been noticed previously. Because in most technical modifications, ligation and transsection of the external carotid artery (ECA) are needed to insert the filament via the ECA stump, blood flow to the ipsilateral mastication and neck muscles, pharynx, tongue, salivary glands, face, external and middle ear, and the meninges is inevitably interrupted.3 Besides the ECA trunk, several branches are frequently cut before filament insertion2,4-11 (Figure 1). Despite the broad acceptance of the endovascular filament model, the consequences of interrupted blood supply to the ECA territory have not yet been investigated. The present study evaluates the incidence of ECA territory ischemia (ECA-I) after MCA filament occlusion surgery and its impact on postischemic recovery.

Materials and Methods
Animal care and all experimental procedures were carried out in accordance with guidelines of the German law governing animal care and the European Communities Council Directive (86/609/EEC). Protocols were approved by the Ethics Committee for Animal Research of the Bavarian government.
MCA Occlusion
Forty-five male Wistar rats (250 to 300 g, Charles River) were subjected to MCAO (n=37) or sham surgery (n=8). Anesthesia was induced with 5% isoflurane inhalation and then maintained with 2% isoflurane in nitrous oxide/oxygen 70%/30% after endotracheal intubation and mechanical ventilation by a small animal respirator (RSBiomedtech). Rectal temperature was monitored continuously and was maintained at 37.0°C by use of a thermostatically feedback-regulated heating pad (RSBiomedtech). After cannulation of the tail artery to provide monitoring of blood pressure and blood gases, rats were placed in a stereotaxic frame. The bregma was exposed by a midline incision, and the skull bone was countersunk bilaterally, except for a thin layer of the internal lamina for bilateral monitoring of local cortical blood flow in both MCA supply territories by laser Doppler flowmetry (MBF3D, Moor Instruments). After a midline incision at the neck, the right carotid bifurcation was exposed, and the following ECA branches were cut after electrocoagulation: the occipital, the cranial thyroid, and the ascending pharyngeal artery. The pterygopalatine artery was exposed but not intercepted. After occlusion of the common carotid artery (CCA) by a microclip, the right ECA was ligated and cut distally to the cranial thyroid artery. A silicone-coated 4-0 nylon monofilament (Prolene, Ethicon) was introduced into the ECA and gently advanced through the internal carotid artery until its tip occluded the origin of the MCA. Through this, local cortical blood flow in the right MCA territory dropped to ~20% of baseline. The endovascular suture remained in place until reperfusion was allowed by withdrawal of the filament and removal of the microclip at the CCA after 90 minutes of ischemia. Sham-operated rats were processed identically except for MCAO.

Physiological Variables
Arterial blood pressure and heart rate were recorded every 15 minutes throughout the experiment. Arterial blood gases (PO2, PCO2) were measured at least 4 times, and plasma glucose and hemoglobin were measured after establishment and before removal of the arterial catheter. Body temperature was documented before surgery and 24 and 48 hours and 8 days after surgery.

MRI Protocols and Lesion Quantification
Twenty-four hours and 2 and 8 days after surgery, rats were reanesthetized for quantification of infarct volume and brain edema by in vivo MRI. Measurements were performed on a 1.5-T clinical routine MR scanner (Magnetom Symphony, Siemens) similar to the approach described by Guzman et al.12 A T2-weighted TSE sequence (repetition time, 2500 ms; echo time, 96 ms; echo train length (ETL), 7; time of acquisition (TA), 6:04; acquisitions, 8; slice thickness (SL), 2 mm; gap, 0; matrix, 128×256, 4/8 rectangular (rec) field of view; field of view, 84 mm) and a heavily T1-weighted inversion recovery sequence (repetition time, 3000 ms; echo time, 60 ms; time of inversion, 150 ms; ETL, 11; TA, 5:33; acquisitions, 10; SL, 2 mm; gap, 0; matrix, 121×256, 4/8 rec field of view; field of view, 109 mm) were applied in the axial and coronal orientations for scanning the rat brain. Brain edema (T2) on days 1 and 2 and infarct volume (T1/IR [inversion recovery]) on day 8 were determined by tracing lesion contours from printed MR images onto standard transparent foil to be cut out and weighed. The 10-mm scale bar on the MR prints served as a volume standard. The arithmetic mean of values for coronal and axial plains of 1 sequence represented the total lesion size.

Body Weight Measurement and Neurological Assessment
All rats were weighed and neurologically examined before surgery and on days 1, 2, and 8 after surgery according to Menzies et al13 (0=no apparent deficits, 1=contralateral forelimb flexion, 2=decreased grip of contralateral forelimb, 3=contralateral circling if pulled by tail, 4=spontaneous contralateral circling). Furthermore, 16 rats were additionally weighed and neurologically scored 14 days after surgery. Body weight change was expressed as percentage difference to baseline value before surgery.
Histology
Five days after surgery, 3 rats subjected to MCAO and 2 sham animals were killed by an overdose of pentobarbital (100 mg/kg body weight IP). Both temporal muscles were removed, fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for at least 7 days, and paraffin embedded. Microtome sections (5 μm) were deparaffinized and stained with hematoxylin-eosin.

Statistical Analysis
Physiological variables, MR lesion volumes, and ratios of hemispheric diameters were expressed as mean±SEM and compared by Student’s t test. Body weight change was tested for differences by 1-way analysis of variance with posthoc correction for multiple comparisons (least significant differences). The neurological score at each time point was expressed as the median and was compared by the Mann-Whitney U test. Values of P<0.05 were considered statistically significant; values of P<0.01 were considered highly significant.

Results
Frequency of ECA-I
In 21 of 45 animals (47%), T2-weighted MRI detected hyperintense signal changes in the right ECA territory. Rats subjected to MCAO (18 of 37, 49%) and sham animals (3 of 8, 38%) were comparably affected by ECA-I. In addition to the right mastication muscles, the ipsilateral pharynx and tongue were frequently involved (Figure 2a and c). In none of the cases was ischemic damage to the contralateral ECA supply territory visible. MR signal changes in the ECA territory were most striking on days 1 and 2 after surgery but were still detectable on day 8 in most rats (Figure 2a, c, and e).

Histology
Histological examination of the right temporal muscle from 2 rats showing pathological MR signaling in the right ECA territory (1 MCAO, 1 sham) displayed varying stages of degeneration and regeneration of striated muscle fibers (Figure 2). Predominantly, the integrity of the sarcolemma was preserved, whereas the sarcoplasmic structures showed different stages of disintegration. An abundant loss of myofibrils led to pronounced atrophy with reduced fiber diameters. Occasionally, the damaged fibers were associated with inter-
Physiological Variables and Extent of Brain Lesions in Animals After MCAO With and Without ECA-I

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No ECA-I (n=17)</th>
<th>ECA-I (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>267±2</td>
<td>272±3</td>
</tr>
<tr>
<td>Core temperature, °C</td>
<td>35.8±0.2</td>
<td>35.7±0.2</td>
</tr>
<tr>
<td>Blood glucose, mmol/L</td>
<td>8.0±0.6</td>
<td>8.9±0.7</td>
</tr>
<tr>
<td>Hemoglobin, mmol/L</td>
<td>8.4±0.1</td>
<td>8.2±0.1</td>
</tr>
<tr>
<td>During MCAO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>87±2</td>
<td>86±2</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>188±4</td>
<td>194±5</td>
</tr>
<tr>
<td>PaO2, mm Hg</td>
<td>98±3</td>
<td>102±5</td>
</tr>
<tr>
<td>PaCO2, mm Hg</td>
<td>45±1</td>
<td>43±1</td>
</tr>
<tr>
<td>End of surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose, mmol/L</td>
<td>6.2±0.2</td>
<td>6.6±0.2</td>
</tr>
<tr>
<td>Hemoglobin, mmol/L</td>
<td>7.9±0.1</td>
<td>7.8±0.1</td>
</tr>
<tr>
<td>Duration of anesthesia, h</td>
<td>5.0±0.2</td>
<td>5.0±0.2</td>
</tr>
<tr>
<td>Follow-up</td>
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<td></td>
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<tr>
<td>Core temperature, °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>37.2±0.1</td>
<td>37.1±0.1</td>
</tr>
<tr>
<td>Day 2</td>
<td>37.0±0.1*</td>
<td>36.6±0.1*</td>
</tr>
<tr>
<td>Day 8</td>
<td>37.0±0.1</td>
<td>36.7±0.2</td>
</tr>
<tr>
<td>Lesion volume, mm³</td>
<td></td>
<td></td>
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<tr>
<td>Day 1</td>
<td>305±25</td>
<td>307±21</td>
</tr>
<tr>
<td>Day 2</td>
<td>319±27</td>
<td>311±22</td>
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<td>Day 8</td>
<td>183±20</td>
<td>197±18</td>
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<tr>
<td>Ratio of hemispheric diameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1.21±0.02</td>
<td>1.21±0.02</td>
</tr>
<tr>
<td>Day 2</td>
<td>1.20±0.02</td>
<td>1.20±0.01</td>
</tr>
<tr>
<td>Day 8</td>
<td>1.02±0.01</td>
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</tr>
</tbody>
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Values are expressed as mean±SEM. *Statistically significant differences (P<0.05, Student’s t test).

There were no statistically significant differences in physiological variables between animals with and without ECA-I except for body temperature on day 2, which was significantly lower in ECA-I rats (Table 1). Four animals (1 with, 3 without ECA-I) presented with a body temperature >38.0°C on days 1 to 8 after surgery (maximum, 38.2°C). Three rats subjected to MCAO died on days 7 and 9 after surgery (2 with, 1 without ECA-I). There was no significant difference between ECA-I and nonaffected animals in terms of brain edema and final cerebral lesion (Table 1).

All rats showed a loss of body weight after surgery. Shams without ECA-I recovered soon, regaining baseline weight before day 8, and presented highly significantly differently from rats with MCAO at each tested time point (P=0.001) except for rats with MCAO and intact ECA territory on day 8 (P=0.056). In shams with ECA-I, body weight development nearly coincides with the respective curve of the MCAO group without ECA-I. In rats with MCAO and ECA-I, body weight was significantly lower on days 2 and 8 compared with rats with MCAO without ECA-I (P=0.026). Although statistically not significant, there was a trend to different body weight in the 2 groups on day 1 after surgery (P=0.079; Figure 3).

Motor performance (median) was found to be poorer in ECA-I rats throughout postsurgical recovery except for day 8. On day 14, rats with ECA-I performed significantly worse (P<0.05). All sham animals presented unsuspicious regarding motor performance at each time point (Table 2).

**Discussion**

The intraluminal filament model of MCAO is widely used in experimental stroke research. Prerequisite for insertion of the filament is access to the CCA or internal carotid artery, which afterward can be surgically closed. This is provided by the stump of the ECA at the cost of hypoperfusion of the ECA supply territory, ie, mastication and neck muscles, pharynx,

<table>
<thead>
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<th>Parameter</th>
<th>No ECA-I</th>
<th>ECA-I</th>
</tr>
</thead>
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<tr>
<td>Surgery Day 12</td>
<td>4.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Day 2</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Day 8</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Day 14</td>
<td>1.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Poorer motor performance was found in ECA-I rats at each tested time point during postsurgical recovery except for day 8. On day 14, this difference was statistically significant (*P<0.05, Mann-Whitney U test).
tongue, salivary glands, and face. In the present study, we describe for the first time that almost half of all animals suffered from ischemic lesions of the mastication and swallowing system, which was independent of the cerebral lesion size. Conspicuous MR signaling was correlated to histopathological findings of acute ischemic myopathy. ECA-I was accompanied by a significantly lower body weight on days 2 and 8 after surgery and poorer motor performance on day 14.

Because all animals were subjected to the identical surgical procedure by a single operator and no relevant differences in physiological variables were found between rats with and without ECA-I, development of ischemic changes most likely was correlated to differences in vascular architecture of individual rats. Animals with marked collateral blood supply to the ECA territory are probably less susceptible to ischemia after ECA ligation, whereas littermates with poorer collateralization might tend to develop ischemic damage after the experimental procedure.

ECA-I presumably leads to pain and to impairment of mastication and swallowing in affected rats, thereby probably resulting in reduced food and water consumption and consequently delayed body weight regain after surgery.

Although there are no experimental data on the effects of food and fluid restriction after focal brain ischemia, clinical observations suggest a negative effect of both malnutrition and dehydration on functional outcome after stroke.\(^1^4\),\(^1^5\) In the present study, reduced food and water uptake resulting from impaired mastication and swallowing could cause delayed rehabilitation for 2 reasons. First, dehydration leads to hemoconcentration with impaired cerebral microperfusion.\(^1^6\) Second, rats with ECA-I were less trained in motor activity because they fetched food from the roof of the cage less often than animals with unaffected mastication and swallowing function. Such restricted motor activity might be responsible for poorer motor rehabilitation.\(^1^7\) Because no dissimilarities were found between MCAO rats with and without ECA-I regarding cerebral infarct size or brain edema, the ischemic brain damage itself most probably could not have contributed to different functional recovery after surgery.

Traumatic injury, eg, resulting from fixation in the stereotaxic frame or wound retraction, denervation, or local infection may also lead to morphological alterations of head and neck muscles. However, because in all cases the ischemic changes in the extracranial tissue were clearly restricted to the right side, specifically to the vascular territory of the ECA, and manipulations of supplying nerves (eg, mandibular branch of the trigeminal nerve) were avoided, the observed injuries must have been caused by ischemia and not by other unspecified side effects.

The described effects of ECA-I on outcome after experimental stroke raise several implications for the planning and interpretation of further studies using the MCAO filament model. Because body weight development is correlated to histopathologically observed ischemic brain damage,\(^1^8\) weight change is a frequently used measure for recovery.\(^1^9\),\(^2^0\),\(^2^1\) Therefore, 2 important aspects have to be considered. First, because ECA-I appeared in \(~50\%\) of all animals and was not predictable, increased variance of data possibly prohibits statistically proven differences between experimental treat-

ment groups. Second, experimental therapies such as anti-inflammatory compounds or analgesics also work on the ischemic musculature and thus might improve functional recovery after surgery for extracerebral reasons. Thus, in the presence of ECA-I, a drug-associated benefit may be found that is not related to cerebral effects and would not be reproducible in the absence of ECA-I.

In addition, different food and water consumption also influences the oral uptake of substances offered for therapeutic or diagnostic purposes. Resulting differences between rats with and without ECA-I therefore lead to distortions of experimental data. Moreover, dysphagia caused by ischemic damage of the swallowing system might result in aspiration of food, water, or secreta with consecutive development of pneumonia. Pulmonary infections are usually accompanied by hyperthermia, reduced general constitution, and decreased motor activity. In combination, these conditions may affect both ischemic brain damage and functional recovery. However, in this study, only 1 animal with ECA-I presented with a body temperature \(>38.0\,^\circ\mathrm{C}\), and mean body temperature of ECA ischemic rats was found to be even lower than in rats without ischemic muscle changes.

Finally, ischemic lesioning of skeletal muscles is associated with increased levels of intracellular proteins in the peripheral blood such as creatine kinases, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, and troponin T.\(^2^2\),\(^2^3\) Because creatine kinases and troponin T also have been found to be elevated in human blood after stroke,\(^2^4\),\(^2^5\) confirmation of these findings in animal experiments might be misleading in cases of ischemic muscle damage.

In the present study, ischemic injury to the ECA territory has been demonstrated to be a frequent side effect of the intraluminal filament model of MCAO in rats. ECA-I influences poststroke body weight development, neurological outcome, and presumably the concentration of muscle proteins in the peripheral blood. Hypothetically, a modification of the model with protection of the ECA might solve the problem but does not seem to be technically practical if reperfusion through the CCA is desired. A systematic review of rat strains other than Wistar rats is required to evaluate strain-related differences in extracranial vascular architecture and susceptibility to ECA-I in this model.

In conclusion, our data indicate that ECA-I should be identified by in vivo imaging techniques to exclude affected rats from further analysis. Therefore, early MRI investigation after surgery, capable to detect ischemic lesions in both the MCA and the ECA territory, is recommended to optimize the reliability of the endovascular filament model.

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References


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