Neuroprotective Effects of MK-801 in Different Rat Stroke Models for Permanent Middle Cerebral Artery Occlusion
Adverse Effects of Hypothalamic Damage and Strategies for Its Avoidance

T. Gerriets, MD; E. Stolz, MD; M. Walberer, DVM; M. Kaps, MD; G. Bachmann, MD; M. Fisher, MD

Background and Purpose—Permanent middle cerebral artery occlusion (MCAO) with the use of the suture technique causes hypothalamic damage with subsequent hyperthermia, which can confound neuroprotective drug studies. In the present study the neuroprotective effects of dizocilpine (MK-801) were compared in different permanent MCAO models with and without hypothalamic damage and hyperthermia.

Methods—Sixty Sprague-Dawley rats were treated with MK-801 or placebo, beginning 15 minutes before MCAO, and assigned to the following groups: suture MCAO (group I), macrosphere MCAO without hypothalamic damage (group II), or macrosphere MCAO with intentionally induced hypothalamic infarction (group III). Body temperature was measured at 3, 6, and 24 hours. Lesion size was determined after 24 hours (2,3,5-triphenyltetrazolium chloride staining).

Results—Hypothalamic damage was present in animals in group I and was intentionally induced in group III with the use of a modified macrosphere MCAO technique. Body temperature was significantly increased 3, 6, and 24 hours after MCAO in these 2 groups of animals. Hypothalamic damage and subsequent hyperthermia could be avoided effectively by limiting the number of macrospheres (group II). MK-801 provided a highly significant neuroprotective effect in group II but not in groups I and III.

Conclusions—Hypothalamic damage with subsequent hyperthermia masked the neuroprotective effect of MK-801. This side effect can be avoided by using the macrosphere MCAO technique with a limited number of spheres. This model therefore may be more appropriate to study the effects of neuroprotective drugs in permanent focal cerebral ischemia than the suture method. (Stroke. 2003;34:2234-2239.)

Key Words: animal models — cerebral infarction — MK-801 — neuroprotection

Animal studies are an important link between the bench and bedside in the development of new neuroprotective drugs. The intraluminal suture technique is the most widely used animal stroke model and can be applied to induce both permanent and transient middle cerebral artery (MCA) occlusion (MCAO). In this model, a monofilament is advanced into the internal carotid artery (ICA) until the tip obstructs the orifice of the MCA. This model induces highly reproducible focal cerebral ischemia in rodents and can simulate reperfusion by withdrawing the suture at defined time points.1 The most important drawback of this technique, however, is the pathological increase of body temperature (>39°C) when this model is used for permanent MCAO.2–6 It is widely accepted from experimental and clinical studies that hyperthermia increases infarct volume and worsens functional outcome.4,7–10 Hyperthermia can influence the evaluation of neuroprotective compounds in experiments in which the suture occlusion model is used.11 The recently developed “macrosphere model” is a new intravascular technique for permanent MCAO in rats that, in contrast to the suture model, does not cause artificial hyperthermia.6

The present study was conducted to determine the impact of different stroke models on the effect of a neuroprotective compound. The N-methyl-d-aspartate (NMDA) antagonist dizocilpine (MK-801) was used for this study because this drug has been studied widely and has demonstrated efficiency.12,13 The neuroprotective effects of MK-801 were evaluated versus placebo in the permanent suture MCAO model (group I), which induces hypothalamic damage and hyperthermia, and in the macrosphere MCAO model (group II), which avoids these side effects. In a third experiment, MK-801 was tested with the use of a modified macrosphere MCAO technique that causes hypothalamic damage (group III).
Materials and Methods

Animal Preparation
All procedures used in this study were in accordance with our institutional guidelines and the German animal protection legislation. Male Sprague-Dawley rats weighing 290 to 350 g were anesthetized with 5% isoflurane delivered in air at 3.0 L/min for 2 minutes. Anesthesia was maintained with 1% to 2% isoflurane delivered in air at 0.5 L/min. PE-50 polyethylene tubing was inserted into the femoral artery for monitoring of blood pressure and for obtaining blood samples to measure pH, PaCO₂, PaO₂, and glucose. During the surgical procedure, body temperature was monitored continuously with a rectal probe and maintained at 36.5°C to 37.0°C with a thermostatically controlled water flow system.

Induction of Focal Brain Ischemia
The right ICA and external carotid artery (ECA) were exposed through a midline incision of the neck. The ECA was isolated, and the superior thyroid and the occipital arteries were ligated and transected. Then the distal portion of the ECA was ligated and transected to create an ECA stump with a length of approximatively 5 mm. The pterygopalatine branch of the ICA was also transected to create an ECA stump with a length of approximatively 5 mm. The pterygopalatine branch of the ICA was also transected to create an ECA stump with a length of approximatively 5 mm. Then the tubing was removed, the wounds were closed, and the animals were allowed to recover from anesthesia.

Suture Model (Group I)
A 4-0 silicone-coated nylon suture with a thermically rounded tip was introduced through the ECA stump as described previously. The occluder was advanced into the ICA 16 to 18 mm beyond the carotid bifurcation until mild resistance indicated that the tip was lodged properly in the anterior cerebral artery (ACA) and thus blocked blood flow to the MCA.

Macrosphere Model (Groups II and III)
PE-50 tubing, filled with saline and TiO₂ macrospheres of 0.315 to 0.355 mm in diameter (BRACE GmbH), was inserted into the ECA stump. Six macrospheres per animal were used in group II and 10 in group III. The tip of the tubing was placed in the carotid bifurcation without affecting the blood flow to the ICA and fixed with a 5-0 suture. Then the macrospheres were advanced separately into the ICA by a slow injection of approximately 0.05 mL saline each until they were moved passively into the cerebral circulation by the blood flow. After the macrosphere injection, the ICA was flushed carefully with 0.5 mL saline under simultaneous inspection of the carotid artery. Excessive dilatation of the ICA, indicating high flushing pressure, was avoided. Then the tubing was removed, and the ECA stump was ligated.

Experimental Protocol
For each group, 20 animals were randomly assigned to placebo or MK-801 treatment. Physiological parameters (temperature, glucose, mean arterial blood pressure, and arterial blood gas analysis including PO₂, PCO₂, and pH) were measured. Thereafter, MK-801 (1 mg/kg in 1 mL saline) or placebo (1 mL saline) was injected intraperitoneally by an investigator who was blinded for group assignment. MCAO was performed 15 minutes after drug injection. Physiological parameters were measured again 15 minutes later. Then the tubing was removed, the wounds were closed, and the animals were allowed to recover from anesthesia.

Drug injection (1 mg/kg MK-801 in 1 mL saline or 1 mL saline) was repeated at 3 and 6 hours after MCAO. Neurological evaluation was performed 3, 6, and 24 hours after induction of ischemia and scored on a 6-point scale: 0 = no neurological deficit, 1 = failure to extend left forepaw fully, 2 = circling to the left, 3 = falling to the left, 4 = no spontaneous walking with depressed level of consciousness, and 5 = death. Body temperature was measured before each neurological evaluation with a rectal probe.

Twenty-four hours after induction of ischemia, the animals were anesthetized deeply with the use of isoflurane and killed by decapitation. The brains were removed quickly, and the localization of the macrospheres in the basal cerebral arteries was determined with the use of a magnifying glass. The brains were sectioned coronally into 6 slices, each 2 mm in thickness, incubated in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C, fixed by immersion in 10% buffered formalin solution, and scanned with a computer scanner (ScanJet 3400C, Hewlett Packard; resolution 600×600 dpi).

The unstained areas of the fixed brain slices were defined as infarcted. With the use of image analysis software (ImageJ 1.25s; National Institutes of Health), the areas of both hemispheres and of the infarcted regions covering the cortex and the subcortical region were calculated for each slice. The percent hemispheric lesion volume (%HLV) was calculated to compensate for the space-occupying effect of brain edema with the use of the equation

\[ \%HLV = \frac{LV}{HV_i} \times \%HLV \]

where LV is the direct lesion volume and HV_i is the ipsilateral hemisphere volume, both calculated by multiplying the area by the slice thickness and summing the volumes. Cortical and subcortical %HLV values were calculated likewise. Hypothalamic damage was determined from the TTC-stained brain slices as described previously (Figure 1). Determination of infarct size and hypothalamic injury was performed by an experienced investigator blinded to group assignment and clinical assessment.

In the macrosphere model, appropriate MCAO was considered present if 1 or more macrospheres directly blocked the MCA mainstem or if the distal ICA and the ACA were occluded, blocking blood flow into the MCA. Animals were excluded if no macrospheres were lodged in the MCA mainstem and blood flow to the MCA was possible via cross flow from the ACA. Animals were excluded from this study if intracerebral or subarachnoidal hemorrhage was present and if hypothalamic infarction was present in group II or absent in group III.

Figure 1. TTC staining. Ischemic lesions (pale) included the hypothalamic region in group I (suture MCAO) and in group III (injection of 10 macroospheres) (with hypothalamic infarction [H+]). In group II (embolization of 6 macrospheres) (without hypothalamic damage [H−]), the hypothalamic region was not affected. Treatment with MK-801 (bottom) provided a significant neuroprotective effect only in group II. Arrows indicate hypothalamic infarction; arrowheads, unaffected hypothalamic region.
Statistical Analysis
The Kolmogorov-Smirnov test was used to test for normal distribution of the parametric data, followed by a 1-way ANOVA. Then the individual groups were subjected to a pairwise post hoc comparison with the Scheffé procedure. Nonparametric scores were analyzed with the Kruskal-Wallis test followed by a nonparametric pairwise post hoc analysis based on median ranks (Tukey-Kramer method). To determine a correlation between body temperature and the presence of a hypothalamic infarction, data from all experimental groups were pooled and analyzed with the Spearman rank correlation on ranks.

Data are presented as mean±SD or median. The level of probability of $P<0.05$ was regarded as significant.

Results
Physiological Variables and Model Failure Rate
Nineteen animals had to be excluded and replaced because of technical problems, most of them in group III (Table 1).

The physiological variables of the remaining animals did not differ between placebo- and MK-801-treated rats in the macrosphere MCAO and the suture MCAO groups ($P>0.05$; data not shown). Body temperature, however, increased significantly after surgery in groups I and III, while the animals in the macrosphere MCAO group (without hypothalamic damage) remained normothermic (Figure 2).

Clinical Outcome
All animals were awake 5 to 15 minutes after isoflurane anesthesia was terminated. Fifteen animals died between hours 6 and 24 (Table 2). These animals were not excluded because quality of the TTC staining was sufficient for determination of lesion size. Animals treated with MK-801 demonstrated severe ataxia at 3 and 6 hours after MCAO. This previously described side effect of MK-801 was observed in all groups and resulted in significantly decreased clinical scores compared with placebo

Postmortem Examination and Lesion Size
The macrospheres were lodged tightly in the basal cerebral arteries, blocking blood flow to the MCA mainstem. In group III, the macrospheres were found lined up in the ICA, occluding almost the entire intracranial portion of the vessel, including the origin of the hypothalamic artery, while only the most distal part of the ICA was filled with macrospheres in group II.

In groups I and III, hypothalamic damage was confirmed in all animals on TTC staining. In these animals, TTC-derived lesion volumes were not reduced significantly by MK-801 compared with placebo.

In contrast, MK-801 provided a robust and highly significant neuroprotective effect in rats subjected to the macrosphere technique without hypothalamic infarction (group II), with an almost 60% reduction of total, cortical, and subcortical lesion size (Figure 3, Table 2).

The presence of hypothalamic infarction was correlated significantly with body temperature measured at 3 ($r=0.55$; $P<0.001$), 6 ($r=0.58$; $P<0.001$), and 24 hours after MCAO ($r=0.57$; $P<0.001$) but not with temperature during anesthesia (before MCAO: $r=0.08$; $P=0.53$; 15 minutes after MCAO: $r=-0.11$; $P=0.40$).

Discussion
Hypothalamic Infarction
The neuroprotective effect of MK-801 was tested in 3 different models for permanent MCAO. No hypothalamic damage was present in group II, in which the recently introduced macrosphere MCAO model was used. In contrast, infarction of the hypothalamic region could be confirmed in all animals subjected to suture MCAO (group I) and was intentionally induced in group III, in which a modified

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**TABLE 1. Model Failure Rate**

<table>
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<tr>
<th>Group</th>
<th>Suture</th>
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<tr>
<td></td>
<td>Placebo</td>
<td>MK-801</td>
<td>Placebo</td>
<td>MK-801</td>
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<td>SAH</td>
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* Nineteen animals were excluded and replaced due to technical problems.

(H+/H−): macrosphere model with/without hypothalamic infarction.

SAH/ICH indicates subarachnoidal/intracerebral hemorrhage; HI, hypothalamic infarction.
The macrosphere MCAO technique was used. Hypothalamic infarction can be explained by an obstruction of the hypothalamic artery that originates from the distal portion of the ICA since this segment of the vessel is occluded by the suture (group I) or the macrospheres (group III), but this is not the case in group II, in which only a limited number of macrospheres were embolized.2,3

**Hypothalamic Infarction and Hyperthermia**

Mean body temperature significantly increased in groups I and III but not in group II, in which hypothalamic damage was avoided. Hyperthermia was highly correlated with the presence of hypothalamic injury. This finding is in agreement with several previous observations, indicating that hyperthermia is caused by an ischemic lesion of the hypothalamus, a region considered the most important center of thermoregulation in mammals.2,6,15,16

**Influence of Hyperthermia on Neuroprotection**

Compared with placebo, MK-801 treatment did not significantly reduce lesion volume in groups I and III but provided a highly neuroprotective effect in group II. These findings suggest that hypothalamic infarction with subsequent hyperthermia confounds the effect of neuroprotective drugs, leading to false-negative results.

Increased body temperature results in a multitude of detrimental effects during cerebral ischemia.17 The impact of body temperature on the preclinical evaluation of neuroprotective drugs was initially described by Memezawa et al,11 who reported that MK-801 failed to reduce infarct size in a rat stroke model in which the suture technique was used. In this study body temperature rose spontaneously to 39.0°C to 39.5°C after transient occlusion of the MCA for 2 hours. In a second study the animals were cooled for the first 6 hours. In this group MK-801 provided a significant reduction of lesion size.11 These findings are therefore in accordance with the present study, indicating that artificial hyperthermia reduces the effect of neuroprotective compounds. Cooling the animals, however, seems not practical for neuroprotective drug studies, since this is technically difficult to perform for the total duration of experiments (ie, ≥24 hours) and might cause additional complications.

The present study is in contrast to a previous observation that indicated MK-801 provided a significant 48% reduction of total lesion size 24 hours after permanent suture MCAO.18 In this study, however, animals were kept under chloral hydrate anesthesia for 90 minutes after MCAO. Anesthesia, particularly in small animals, leads to hypothermia and requires feedback-controlled external heating. Furthermore, animals typically need 30 to 60 minutes to recover from anesthesia that is brought about by repeated injections of chloral hydrate and require external heating for this time interval. It can therefore be assumed that animals in this study were kept normothermic for ≥2 hours and artificial hyperthermia did not occur during the hyperacute phase of ischemia in this experimental setting. In the present study we sought to minimize side effects of anesthesia using isoflurane. With this technique, animals were awake early after induction

<table>
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<th>TABLE 2. Clinical and Histopathological Findings</th>
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<td>Clinical Score</td>
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<tr>
<td>Suture MCAO</td>
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<td>Placebo</td>
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<td>Group I</td>
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<td>Suture MCAO</td>
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<td>MK-801</td>
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<td>Macrosphere H−</td>
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<td>Placebo</td>
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<td>Group II</td>
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<td>Placebo</td>
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<td>Group III</td>
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<tr>
<td>Macrosphere H−</td>
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<td>MK-801</td>
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Fifteen animals died between the 6th and the 24th hour and thus were scored 5. Animals treated with MK-801 demonstrated severe ataxia in all groups at 3 and 6 hours and were therefore scored 3 in most cases. MK-801 provided a significant reduction in lesion volume only in group II, where hypothalamic damage and hyperthermia were avoided by using the macrosphere model.

Clinical scores were expressed as median range, lesion volumes as mean [percent of hemisphere] ±SD.

ns indicates not statistically significant.
of ischemia, and body temperature was therefore not affected thereafter. The fast elimination of isoflurane early after MCAO furthermore minimizes potential neuroprotective effects of the anesthetic drug itself, which also can confound stroke studies.

Relevance for Future Preclinical Studies

The present findings are relevant for preclinical testing of neuroprotective drugs. Clinical studies demonstrate highly inhomogeneous vascular findings in patients suffering from acute territorial stroke, ranging from early recanalization and delayed or drug-induced recanalization to permanent vessel occlusion. Since the underlying pathophysiology of brain damage therefore is likewise heterogeneous, depending on the presence (and time point) of additional reperfusion injury, it has been suggested that neuroprotective therapies should be evaluated in both transient and permanent vessel occlusion models.

In the suture MCAO model, hypothalamic damage and hyperthermia seem to occur only if reperfusion is performed later than 90 minutes after MCAO. Thus, the suture technique is highly recommended to study neuroprotective drugs with transient MCAO. The duration of MCAO, however, should be limited to 90 minutes to avoid confounding hyperthermia.

The present study suggests that the suture MCAO model may be recommended to a lesser degree for neuroprotective compounds to be studied with permanent MCAO because hypothalamic damage and subsequent hyperthermia can confound the results. This appears to be the situation for the NMDA antagonist MK-801, but it seems reasonable to assume that other neuroprotective drugs may not work or may appear less effective if artificial hyperthermia is not avoided. Alternatively, intra-arterial injections of preformed blood clots can also be used to occlude the MCA. These “clot models” are of increasing interest to study thrombolytic drugs but are less highly recommended to simulate permanent MCAO because spontaneous clot lysis may lead to inadvertent reperfusion. Furthermore, clot models can also cause an occlusion of the hypothalamic artery, depending on clot size and injection technique.

Conclusion

The recently developed macrosphere model avoids hypothalamic infarction and hyperthermia, which are typical side effects of the suture technique for permanent MCAO. The present study indicates that hyperthermia can obscure neuroprotective effects and lead to false-negative results in preclinical drug studies. Thus, the macrosphere model appears to be a practical alternative to the suture model for permanent MCAO in experimental stroke research and may improve the transferability of results derived from animal studies to human stroke.

Acknowledgment

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