Neuroprotection in Transient Focal Cerebral Ischemia by Combination Drug Therapy and Mild Hypothermia
Comparison With Customary Therapeutic Regimen

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Background and Purpose—A combined therapeutic approach has been advocated repeatedly for treatment of focal cerebral ischemia. A clinical example of combined therapy is administration of nimodipine, mannitol, dexamethasone, and barbiturates during temporary occlusion of a cerebral artery in neurovascular surgery. We have recently demonstrated outstanding neuroprotective properties of a combination therapy with magnesium (calcium antagonist and glutamate antagonist), tirilazad (antioxidant), and mild hypothermia (MTH). In this study we compared this treatment strategy with the customary treatment options in a rat model of transient focal cerebral ischemia.

Methods—Sprague-Dawley rats (n=1100; 120 per group) were subjected to 90 minutes of middle cerebral artery occlusion by an intraluminal filament (n=10 per group). In experiment 1, the customary treatment options (nimodipine, mannitol, dexamethasone, methohexital) were evaluated as monotherapy and in combination. In experiment 2, the customary and the new combination therapy (MTH) were compared. Mild hypothermia (33°C) was maintained for 2 hours. Neurological examinations were performed daily. Infarct size was assessed histologically after 7 days.

Results—in experiment 1, infarct volume was attenuated by 34% at maximum, with mannitol and methohexital being the most effective drugs given as monotherapy. In experiment 2, combined administration of the customary treatment options had no additive effect (infarct volume ~36%). Combination therapy with MTH reduced total infarction by 73% and almost completely abolished cortical infarction (~91%). None of the animals of this group had any residual neurological deficit at the end of the observation period (P<0.05 versus all other groups).

Conclusions—The efficacy of drugs (monotherapy or in combination) most commonly used for neuroprotection during neurovascular surgery is limited. The newly proposed combination therapy (magnesium, tirilazad, and mild hypothermia), which is based on pathophysiological considerations, seems to be a promising alternative for neuroprotection in cerebrovascular surgery. (Stroke. 2003;34:1526-1532.)

Key Words: cerebral ischemia, focal ■ drug therapy, combination ■ hypothermia ■ laser-Doppler flowmetry ■ neuroprotection

The molecular events that mediate ischemic brain damage are logical targets for pharmacological intervention and include glutamate accumulation, aberrant calcium fluxes, free radical formation, and lipid peroxidation. Although many therapeutic agents showed potential promise in animal models, the results of most single-agent clinical trials were sobering. Consequently, various authors advocated studies to evaluate the efficacy of combined therapeutic approaches.1,2

A clinical example of a combined drug approach to limit ischemic injury is administration of nimodipine (calcium channel blocker), mannitol, dexamethasone (corticosteroid), and barbiturates in burst-suppressive doses before temporary occlusion of a brain-supplying artery during certain neurosurgical procedures, eg, clipping of cerebrovascular aneurysms.3 We recently developed a pathophysiological oriented and highly effective combined treatment strategy in an animal model of transient focal ischemia. This approach primarily included magnesium (calcium antagonist and glutamate antagonist) and tirilazad (antioxidant), resulting in marked neuroprotection.4 In a second step we demonstrated that the neuroprotective potential of mild hypothermia (33°C) is increased by this drug combination.5 Magnesium+tirilazad, hypothermia, and magnesium+tirilazad+hypothermia (MTH) reduced total infarct volume by 56%, 63%, and 77%, respectively. The pathophysiological and therapeutic implications of these treatment modalities alone and in combination have been discussed in detail.4,5

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The objective of this study was to determine the neuroprotective efficacy of the clinically utilized treatment options, alone and in combination, and of the newly developed treatment strategy (MTH) for examination of the following questions: (1) How effective are the currently clinically utilized treatment options alone and combined under standardized experimental conditions? (2) How effective is the combined treatment strategy (MTH) in comparison with the current treatment options?

Materials and Methods

One hundred twenty male Sprague-Dawley rats (270 to 300 g body wt) were studied. Animals were purchased from Charles River Laboratory (Sulzfeld, Germany) and cared for at all stages of the study in compliance with institutional guidelines and regulations of the government of Bavaria.

Animal Preparation and Monitoring

Animals were orally intubated and mechanically ventilated with 0.8% halothane in 70% N2O and 30% O2, as previously described. A feedback-controlled heating lamp and pad were used to maintain temporalis muscle and rectal temperature at 37.0°C in normothermic animals. The tail artery and left femoral vein were cannulated for blood sampling, monitoring of arterial blood pressure, and administration of fluids and drugs. Blood gases, glucose, and hematocrit were measured before, during, and after ischemia. Laser-Doppler flowmetry (LDF) (MBF3D, Moor Instruments Ltd) was used to monitor local cortical blood flow (LCBF) of an area of each hemisphere supplied by the middle cerebral artery (MCA). A burr hole (1 mm in diameter) was drilled bilaterally 5 mm lateral and 1 mm posterior from the bregma. A rectangularly bent LDF probe was positioned above the surface of each hemisphere. LCBF was measured before, during, and after ischemia. Laser-Doppler data by 2-way ANOVA for repeated measures, and the point and the infarct volumes were analyzed by 1-way ANOVA, the Software (Jandel Scientific). The physiological data of each time point was applied. Differences were considered indicated, Dunnett test or Student-Newman-Keuls test for neurological function scores was applied. Differences were considered significant at the P<0.05 level.

MCA Occlusion

All animals were subjected to 90 minutes of MCA occlusion by a silicone-coated 4-0 nylon monofilament inserted via the external carotid artery as previously described. Briefly, the filament was advanced until LDF showed a sharp decrease of the ipsilateral LCBF to approximately 20% of baseline, indicating adequate occlusion of the MCA. Ten animals were excluded and replaced because LDF of the contralateral hemisphere also showed a sharp drop on filament insertion, indicating vessel perforation leading to subarachnoid hemorrhage.

Drug Administration and Treatment Arms

Experiment 1: Customary Treatment Options

Experiment 1A: Nimodipine/Mannitol/Dexamethasone
Throughout the complete study, all animals were randomly assigned to groups of 10 animals each. Animals received either (1) vehicle, (2) nimodipine, (3) mannitol, (4) dexamethasone, or (5) nimodipine + mannitol + dexamethasone (N+M+D).

Control animals received saline 3.0 mL/kg IV as constant infusion. Nimodipine 30 μg/kg per hour (Nimotop S, Bayer AG) was administered from 30 minutes before induction of ischemia until 60 minutes after reperfusion. Mannitol (2×1 g/kg) (mannitol 20%, Köhler GmbH) and dexamethasone (2×1 mg/kg) (Decadron, MSD AG) were applied intravenously as short infusion for 15 minutes, with the first dose given 30 minutes before induction of ischemia and the second at 15 minutes before reperfusion.

Experiment 1B: Methohexital/Nimodipine/Mannitol/Dexamethasone
Animals received either (1) saline, (2) methohexital in a dose causing burst suppression, or (3) methohexital and N+M+D.

In the groups subjected to methohexital, anesthesia was introduced by halothane and followed by methohexital (Brevimyl, Lilly) before ischemia. This required an initial infusion rate of 1.0 to 1.5 mg/kg per minute until a burst suppression pattern was reached and a maintenance level of 0.4 to 0.6 mg/kg per minute. Thirty minutes after onset of reperfusion, methohexital was discontinued; halothane was given again to allow extubation. In group 3, burst suppression was induced by methohexital together with administration of N+M+D as described above.

Experiment 2: Methohexital and N+M+D Versus MTH
Animals received either (1) vehicle infusion in normothermia, (2) methohexital and N+M+D, or (3) MTH.

In the normothermic control group, anesthesia was maintained by halothane. Of the vehicle-treated group, 5 animals received 3.0 mL/kg of 0.9% saline, and another 5 animals received 3.0 mL/kg of 0.02 mol/L citric acid (drug vehicle of tirilazad). Group 2 received methohexital and N+M+D as described above. In group 3, MgCl2 (2×1 mmol/kg) (Sigma-Aldrich) and tirilazad mesylate (2×3 mg/kg) (Fredoxy, Upjohn) were administered intravenously over 15 minutes. The first dose was given before ischemia and the second at reperfusion. Whole-body hypothermia was induced by ice packs until a rectal and temporalis muscle temperature of 33°C was reached and maintained. Before ischemia, an interval of 20 minutes was allowed for stabilization. Rewarming to normothermia (1°C per 10 minutes) was started 30 minutes after onset of reperfusion. A study diagram is given in Figure 1.

Neurological Evaluation

Neurological deficits and infarct volumes were assessed by a person blinded to the animals’ treatment. Postoperatively, the neurological function of all animals was evaluated daily by using a 6-point scale, as follows: 5 points, no deficit; 4 points, contralateral forelimb flexion; 3 points, lowered resistance to lateral push; 2 points, circling if pulled by tail; 1 point, spontaneous circling; 0 points, no spontaneous activity.

Quantification of Ischemic Damage

Seven days after ischemia, animals were anesthetized and perfused transcardially with isotonic heparinized saline, followed by 2% paraformaldehyde. The brains were removed, embedded in paraffin, cut into 24 coronal sections at 400-μm intervals, and stained with hematoxylin and eosin. The infarct areas were assessed planimetrically (OPTIMAS 5.1, BioScan Inc). Total infarct volume (IT) was calculated as the sum of the area of infarct in sections obtained at 2, 3.6, 5.2, 6.8, and 8.4 mm from the frontal pole. Cortical and basal ganglia were determined according to a stereotactic atlas of rat brain.

Statistical Analysis

Statistical analysis was performed by using SigmaStat 2.0 Statistical Software (Jandel Scientific). The physiological data of each time point and the infarct volumes were analyzed by 1-way ANOVA, the laser-Doppler data by 2-way ANOVA for repeated measures, and the neurological function scores were compared by Kruskal-Wallis ANOVA on ranks for each of the 7 postoperative days. If multiple comparisons were indicated, Dunnett’s test or multiple.Json results test for neurological function scores was applied. Differences were considered significant at the P<0.05 level. Results are presented as mean ±SD.

Results

Physiological Variables

Except for 10 animals suffering from subarachnoid hemorrhage, there was no additional mortality. There were no...
significant differences concerning all physiological parameters between normothermic animals receiving saline or the citric acid solution (vehicle). Therefore, the data of all rats receiving vehicle were included in a single control group. The experimental groups did not differ with respect to preischemic, intraischemic, or postischemic blood pressure, blood gases, or hematocrit. Blood glucose levels significantly increased after drug administration in hypothermic rats receiving MgCl₂. Blood pressure dropped by 10 to 15 mm Hg during infusion of nimodipine, methohexital, and MgCl₂, but this had no significant influence on the mean values (Tables 1 and 2).

Laser-Doppler Flowmetry

**Experiment 1A**

In all groups, MCA occlusion resulted in an immediate reduction of ipsilateral LCBF to approximately 20% of baseline, while contralateral blood flow remained unchanged throughout the experiment. A short period of postischemic hyperemia evolved on reperfusion followed by a decrease in ipsilateral LCBF to approximately 70% of baseline. Animals subjected to treatment with nimodipine, mannitol, dexamethasone, or the combination exhibited a shortened or no postischemic hyperperfusion (P<0.05); those receiving mannitol had a tendency toward a higher LCBF during ischemia.

**Experiment 1B**

The animals of the control group showed the same LCBF pattern as described above. In the methohexital-treated animals with and without N+M+D, bilateral LCBF significantly decreased to approximately 75% of baseline within 10 minutes after start of drug infusion. Contralateral LCBF remained stable at approximately 75% of baseline, recovering to baseline values after termination of the methohexital infusion. There were no significant differences between both treatment groups and of their ipsilateral flow compared with vehicle-treated controls during ischemia and reperfusion.

**Experiment 2**

The animals of the control group and of the group with methohexital and N+M+D showed the same LCBF pattern as described above. In animals treated with MTH, the ipsilateral and contralateral LCBF decreased significantly to

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**TABLE 1. Physiological Variables (Experiment 1A)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before Ischemia</th>
<th>During Ischemia</th>
<th>After Ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Nimodipine</td>
<td>Mannitol</td>
</tr>
<tr>
<td>Paco₂, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>36.2±6.5</td>
<td>34.5±4.8</td>
<td>31.7±4.5</td>
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<tr>
<td>Paco₂, mm Hg</td>
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<td>104.9±26.2</td>
<td>112.3±24.4</td>
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<td>pH</td>
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<td>7.45±0.06</td>
<td>7.47±0.04</td>
</tr>
<tr>
<td>Hc</td>
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<td>0.47±0.00</td>
<td>0.47±0.03</td>
</tr>
<tr>
<td>MABP, mm Hg</td>
<td>104±15</td>
<td>93±19</td>
<td>98±16</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>82.5±14.5</td>
<td>83.1±13.7</td>
<td>83.5±10.6</td>
</tr>
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<td>Temp, °C</td>
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<td>37.0±0.2</td>
<td>37.0±0.1</td>
</tr>
<tr>
<td>Temp, °C</td>
<td>37.0±0.1</td>
<td>37.0±0.1</td>
<td>37.0±0.01</td>
</tr>
</tbody>
</table>

MABP indicates mean arterial blood pressure.
approximately 80% during cooling, with further decrease of ipsilateral flow during occlusion to approximately 20% of baseline, while the contralateral LCBF remained stable at approximately 80% of baseline. Reperfusion led to a significantly prolonged period of postischemic hyperemia compared with the normothermic animals, followed by a gradual decrease of ipsilateral blood flow to approximately 70% of baseline (Figure 2).

**Functional Outcome**

**Experiment 1A**

Only the animals treated with mannitol had a significant improvement of their functional performance on day 2 (P < 0.05).

**Experiment 1B**

Animals treated with methohexital alone had significantly fewer neurological deficits on postischemic days 5 to 7 compared with controls, while the animals treated with methohexital in combination with N + M + D had fewer neurological deficits at days 3 to 7 (P < 0.05).

**Experiment 2**

Animals treated with methohexital and N + M + D had significantly fewer neurological deficits from day 1 to 7 compared with the vehicle-treated controls. Animals treated with MTH had significantly fewer neurological deficits on postischemic days 1 to 7 compared with both vehicle-treated controls and the standard treatment group. None of the MTH-treated animals showed any residual functional deficit at the end of the observation period (Figure 3).

**Infarct Volume**

There was no difference in brain size between the groups and between the ipsilateral and contralateral hemispheric volumes of each individual animal on postoperative day 7. Therefore, indirect measurements of infarct volumes to correct for brain size or edema were not necessary.19 The lesions mainly involved the frontoparietal cortex, thalamic region, lateral caudoputamen, and internal capsule.

**Experiment 1A**

Total infarct volume was 110.6 ± 37.5 mm³ in controls, 99.2 ± 36.8 mm³ (−10%) in animals treated with nimodipine, 73.7 ± 32.1 mm³ (−33%) in animals treated with mannitol, 112.7 ± 51.0 mm³ (−2%) in animals treated with dexamethasone, and 76.0 ± 41.0 mm³ (−31%) in animals treated with N + M + D (P < 0.05).

Mannitol and N + M + D significantly (P = 0.048) attenuated cortical infarction, while reduction in rats with nimodipine or dexamethasone did not reach a statistically significant level. Neither monotherapy nor N + M + D resulted in a significant reduction of the infarct volume of the basal ganglia.

**Experiment 1B**

Total infarct volume was 113.1 ± 25.2 mm³ in controls, 75.7 ± 39.9 mm³ (−33%) in animals treated with methohexital alone, and 74.9 ± 37.2 mm³ (−34%) in animals treated with methohexital and N + M + D. In both groups the total infarct volumes were significantly (P = 0.028) smaller than in the control group.

Administration of methohexital alone or in combination with N + M + D significantly (P = 0.048) attenuated cortical infarction. Treatment with methohexital combined with N + M + D significantly reduced the infarct volume in the basal ganglia (P = 0.006), whereas monotherapy with methohexital narrowly missed a statistically significant level.

**Experiment 2**

Total infarct volume was 112.8 ± 19.5 mm³ in normothermic controls, 75.1 ± 30.1 mm³ (−36%) in animals subjected to methohexital and N + M + D, and 30.1 ± 15.0 mm³ (−73%) in animals treated with MTH. Both treatment modalities significantly (P < 0.01) reduced the total infarct volume compared with controls (Figure 4).

This treatment efficacy was confirmed when we analyzed the infarct volumes separately in cortical tissue and basal ganglia (P = 0.01 in both groups). Cortical infarction was reduced by −38% in animals treated with methohexital and N + M + D, while it was reduced by −91% in animals treated with MTH. Similarly, methohexital and N + M + D (−32%) and MTH (−50%) significantly reduced infarction of the basal ganglia.

**Discussion**

The findings demonstrate that the currently used protective measures in neurosurgical patients subjected to transitory
artery occlusion (nimodipine, mannitol, dexamethasone, barbiturates), whether given alone or in combination, are only moderately neuroprotective under standardized experimental conditions. Furthermore, this study confirms the results of our previous studies: animals treated with MTH had the smallest infarct volumes and no residual functional deficits at the end of the observation period.

**Customary Treatment Options**

Neither nimodipine (-10%), mannitol (-33%), dexamethasone (+2%), nor their combination (-31%) was found to significantly attenuate total infarction. As mechanisms of nimodipine, an increase of cerebral blood flow by vasodilation and inhibition of calcium influx into cells by blocking voltage-dependent calcium channels have been discussed. However, there is a lack of convincing results in acute ischemic stroke. Even worsening of outcome with an increased mortality has been reported, probably as a result of arterial hypotension from nimodipine. Various properties of mannitol may afford protection: (1) improvement of the cerebral microcirculation and (2) scavenging of free radicals. Indeed, administration of mannitol was found to be the most effective monotherapy among the customary options. Mannitol attenuated cortical infarction and, as a trend, af-
forded a higher intraischemic blood flow, in agreement with findings of Rickels and collaborators. Animals treated with dexamethasone had a trend toward formation of larger infarcts. Dexamethasone seems to be particularly harmful by enhancing the extracellular accumulation of glutamate and thereby the cellular calcium overload. The combined administration of nimodipine, mannitol, and dexamethasone did not result in any additional benefit. It is conceivable that only mannitol provided some protection among the combination of drugs, which possibly was attenuated by dexamethasone. This is in accordance with findings of Sutherland et al, who compared nimodipine, mannitol, and anti-inflammatory indomethacin in rats with forebrain ischemia. When the agents were combined, the effect was not greater than that achieved with mannitol alone. In most of the earlier studies on barbiturate protection, investigators were unaware of the importance of brain temperature. Although recent studies with adequate temperature control confirmed a certain protective efficacy of barbiturates, the magnitude of protection was modest in comparison with the former results. Another explanation of overestimating neuroprotection by barbiturates in former experiments could be that untreated awake animals subjected to cerebral ischemia were studied as control. In such control animals, cerebral hyperthermia might have evolved in the postischemic period with high levels of catecholamines, enhancing development of infarction. Monotherapy with methohexital only narrowly missed significance in reducing infarction within the basal ganglia (−28%), and mannitol (−18%) correspondingly exhibited the strongest effect there in experiment IA, while combined treatment significantly reduced basal ganglia infarction (−33%). This was confirmed by the improved neurological recovery in this group. Methohexital significantly decreased cerebral blood flow, making it conceivable that mannitol-induced cerebral blood flow elevation contributed to significant tissue salvage.

In considering the results of experiment 1, we found a limited additive effect of the most effective monotherapies with methohexital (its most cited major protective mechanism is a dose-dependent diminution of CMRO_2) and antiedematous mannitol, while the calcium channel blocker nimodipine and the corticosteroid dexamethasone failed to exhibit beneficial effects. More promising approaches for combination therapies seem to be concurrent administration of N-methyl-D-aspartate antagonists and radical scavengers or caspase inhibitors, which putatively block apoptotic cell death and inhibit cytokine production.

Customary Treatment Options Versus MTH

One might counter that the objective of the neurosurgical standard regimen is not to protect the brain from the sequelae of transient focal ischemia. Nimodipine is given to prevent the development of delayed ischemic neurological deficits from vasospasm, mannitol to decrease intracranial pressure, and dexamethasone to protect the blood-brain barrier function. Nevertheless, these drugs, including barbiturate-induced burst suppression, represent the most commonly used drugs for neuroprotection in patients. Barbiturates in particular are still considered the gold standard of neuroprotection during temporary occlusion of a brain-supplying artery. Considering their limited beneficial effects and serious side effects, such as (1) respiratory and myocardial depression with the need for prolonged postoperative artificial respiration, (2) immunosuppression, (3) inhibition of glutamate uptake and increased tissue depletion of ATP, and (4) impeding early postoperative neurological assessment of patients because of persisting central depression, the intraoperative application of mild hypothermia seems to be a well-tolerable alternative.

Since mild to moderate hypothermia is increasingly used for neuroprotection in humans, additional pharmacological agents should be studied that may act synergistically. In a recent study we did not observe any additional neuroprotective effect of barbiturates under mild hypothermic conditions. All components of the new combination therapy are clinically licensed, and side effects seem to be limited and tolerable: side effects of mild hypothermia include increased cardiac afterload and decreased ventricular contractility, coagulation abnormalities, impaired metabolism of drugs, and...
immunologic depression. However, these effects were predominantly observed in connection with long hypothermic periods $>24$ hours or deep hypothermia $<33^\circ$C.27 In placebo-controlled studies, magnesium and tirilazad were usually well tolerated.28,29

In conclusion, the new combination of magnesium, tirilazad, and mild hypothermia (MTH) has a high neuroprotective potential and is superior to agents currently used for neuroprotection in situations requiring temporary occlusion of a brain-supplying artery, eg, during aneurysm surgery. Because all components of the suggested combination therapy are approved for use in humans, clinical evaluation may be performed without tedious approval procedures. A randomized clinical trial has been approved by our local ethics committee and is currently being conducted at the Department of Neurosurgery, Ludwig-Maximilians University, Munich, Germany.

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