Different Degrees of Hypothermia After Experimental Stroke
Short- and Long-Term Outcome

Rainer Kollmar, MD; Tobias Blank; Junliang L. Han, MD; Dimitrios Georgiadis, MD; Stefan Schwab, MD, PhD

Background and Purpose—The neuroprotective role of mild therapeutic hypothermia was established in animal models of cerebral ischemia. Still, several issues, including optimal target temperature, remain unclear. The optimal depth of hypothermia in a rat model of focal cerebral ischemia was investigated.

Methods—Eighty-four male Wistar rats (n = 84) were subjected to filament occlusion of the middle cerebral artery for 90 minutes. Sixty animals were equally split into 6 groups kept at core temperatures of 37°C, 36°C, 35°C, 34°C, 33°C, and 32°C over a period of 4 hours starting 90 minutes after middle cerebral artery occlusion. Twenty-four hours later, after performing a neuroscore, animals were killed and brains examined for infarct size, edema, and invasion of leukocytes. In the second part, 24 animals (8 per group) were kept at 33°C, 34°C, and 37°C for 4 hours, allowed to survive for 5 days, and underwent additional investigation of transferase dUTP nick-end labeling.

Results—In the first part, one animal in each treatment group and 2 animals in group 37°C died. The infarct size and edema were smaller for 34°C and 33°C compared with all other groups (P<0.05) over 24 hours. These animals also had better functional outcome (P<0.05) with an advantage for 34°C versus 33°C (P<0.05). Leukocyte count was lower for 34°C and 33°C as compared with the 37°C group. Similar results were obtained in the second part of the study with an advantage for 34°C versus 33°C.

Conclusion—Our results suggest that the optimal depth of therapeutic hypothermia in temporary middle cerebral artery occlusion is 34°C. (Stroke. 2007;38:1585-1589.)

Key Words: brain edema ■ hypothermia ■ rat ■ stroke

Therapeutic hypothermia has been shown to be neuroprotective in different models of focal cerebral ischemia.1–5 Still, some issues concerning its appropriate use remain unsolved. The optimal depth is probably the most important factor. Surprisingly, there are no animal investigations comparing different degrees of therapeutic hypothermia in a stepwise manner, which would be a prerequisite for the use of pharmacological agents in patients. Most experimental studies compare normothermia with one or 2 different degrees of hypothermia.1,2,4 However, results are inconsistent. Although cooling to 34°C reduced infarct size by 60%, there was no infarct visible at 29°C.1 Huh et al showed 59% infarct reduction for 33°C but less reduction at 27°C.4 The question of the optimal depth of therapeutic hypothermia has major clinical relevance, because side effects of hypothermia go in parallel with its degree.6 Moreover, moderate therapeutic hypothermia requires mechanical ventilation because of patient discomfort and shivering. The intensive care treatment and mechanical ventilation exclude the majority of patients with stroke from hypothermic treatment and probably prevents a large trial on hypothermia in acute patients with stroke.

The present study addresses the question how different body temperatures influence infarct size, brain edema, and survival. Moreover, the invasion of leukocytes was assessed as a marker of postischemic inflammation. In contrast to previous publications, different temperatures are compared in a detailed, stepwise manner and analyzed over 5 days in subgroups to describe possible transient effects.

Materials and Methods

Experimental Procedures

Experimental protocols were approved by the local ethics committee. Rats had free access to food and water before the experiments. Animals were anesthetized using a mixture of halothane (Halocarbon Laboratories), oxygen (30%), and N₂O (70%). Minimum alveolar concentrations were corrected for the actual body temper-
perature. In accordance, halothane concentrations ranged between 0.45% and 1%. The right femoral arterial blood was cannulated by a polyethylene catheter (PE-50; NeoLab, Heidelberg, Germany) for continuous monitoring of blood pressure, heart frequency, and blood gases during the experiment. Rectal temperature was monitored by a thermostatically controlled heating pad (Foehr Medical Instruments). A rectal probe was inserted 4 cm into the rectum to measure the actual body core temperature. Prior experimental data indicated that the body core temperature correlates to intracranial and pericranial temperature during normothermia and therapeutic hypothermia.\(^5\)\(^6\)\(^7\) Transient focal cerebral ischemia for 90 minutes was induced using the suture occlusion model.\(^8\) Cooling of the animals was performed immediately after middle cerebral artery occlusion (MCAO) of 90 minutes by using ice packs and temperature was maintained by adjusting the heating pad to the target temperature.\(^9\) After the hypothermic period, rewarming was initiated by readjusting the temperature pad to 37°C. This goal was reached after 20 to 30 minutes subjected to the depth of the target temperature.

Twenty-four hours or 5 days after MCAO, rats were killed by an overdose of ketamine (10%) and xylazine hydrochloride (100 mg/kg body weight). After decapitation, brains were rapidly removed and frozen in isopentane at −20°C and stored until use at −80°C.

**Short-Term Experiment**

Sixty male Wistar rats (Charles River, Sulzfeld) weighing 280 to 330 g were used and randomly assigned to six treatment groups: All animals were subjected to transient MCAO of 90 minutes. For the control group, the rectal temperature was maintained at 37°C for 4 hours after reperfusion. In the other groups, rectal temperature was lowered directly after reperfusion at 90 minutes to 36°C, 35°C, 34°C, 33°C, and 32°C, respectively. Temperature was maintained for 4 hours in each group before rewarming. The target temperature was reached after 10 to 20 minutes.

**Long-Term Experiment**

An additional 24 male Wistar rats (Charles River, Sulzfeld) weighing 280 to 330 g were randomly assigned to three treatment groups. All procedures were the same as in the 24-hour group. However, groups were limited to 37°C, 34°C, and 33°C (n=8 per group) according to the results from the short-term experiment and in respect to animal welfare. Animals were intended to survive for 5 days after MCAO.

**Silver Infarct Staining and Brain Edema**

Silver Infarct Staining Method

Silver Infarct Staining was used to measure the infarct size.\(^10\) With this method, ischemic brain tissue can be reliably distinguished from nonischemic white and gray matter of rat brain cryosections as soon as 2 hours after MCAO.\(^10\) Frozen brains were dissected into 14-μm sections on 5 coronal levels 2 mm apart from each other: (1) level 1, 2.2 mm off bregma; (2) level 2, 0.2 to −0.26 mm off bregma; (3) level 3, −1.4 to −1.8 mm off bregma; (4) level 4, −3.3 to −5.6 mm off bregma; and (5) level 5, −5.2 to −5.3 mm off bregma.

A modified version of the semiautomated method\(^11\) was used to measure the cerebral infarct volume. Briefly, the lowest optical density of the noninfarcted hemisphere was calculated using an image processing system (MCID 4; Imaging Research) and taken as the threshold value. The brain area with an optical density equal to or higher than this threshold was considered to be nonischemic, whereas areas with values below threshold values were considered to represent infarcted brain tissue. In addition, the size of the ischemic and nonischemic hemisphere was measured. The areas of the nonischemic hemisphere, ischemic hemisphere, and infarction were multiplied by the slice thickness. The following formula was used to calculate the infarct volume corrected for the cerebral edema:\(^5\)\(^6\)\(^7\): corrected infarct volume = volume of the left, nonischemic hemisphere − (volume of the right, ischemic hemisphere − infarct volume). The volume of the brain edema has been calculated with the following formula: volume of the left, nonischemic hemisphere − volume of the right, ischemic hemisphere. The brain sections were analyzed by an investigator who was blind to the treatment of each group. The code was uncovers for statistical analysis of the data. This was the case for immunostaining procedures as well.

**Neuroscore**

All animals were tested daily for neurological outcome using the neuroscore according to Menzies\(^12\): 0 = no apparent deficit, 1 = contralateral forelimb flexion; 2 = decreased grip of contralateral forelimb grip while tail pulled; 3 = spontaneous movement in all directions, contralateral circling only if pull by tail; 4 = spontaneous contralateral circling. The testing was performed by a coworker (J.L.H.) who was blind to the earlier treatment regimen.

**Immunohistochemistry**

Frozen sections of 14 μm thickness out of the third level −1.4 to −1.8 mm to bregma were used for immunohistochemical analysis. Studies were performed with antisera against myeloperoxidase (MPO; DAKO).

Sections were fixed in acetone for 10 minutes and blocked for endogenous peroxidases with MeOH containing 0.33% H\(_2\)O\(_2\). Sections were then incubated in normal swine serum (NSS; DAKO; 5% in phosphate-buffered saline [PBS]) for 30 minutes followed by the primary antibody (1:500) for 1 hour at room temperature. Immunoreactivity was visualized by the avidin biotin complex method (Vectastatin; Vector Laboratories). When the primary antibody was omitted, no immunostaining was produced (not shown).

MPO-positive cells were quantitatively measured counting MPO-positive cells per infarcted hemisphere of the normothermic group in the 33°C group and the 34°C group. In the nonischemic hemisphere, no MPO staining occurred. Cells were counted for the whole brain using a counting grid and a Leica DMR upright microscope at 100× magnification. Photomicrographs were done using a Leica DMR upright microscope, a Leica DCM 500 digital camera (Leica, Bensheim, Germany), and Irfanview imaging software (www.irfanview.com).

**Long-Term Study**

**Immunohistochemistry**

For paraffin sections, rats were perfused transcardially with 4% paraformaldehyde and brains were removed carefully. Brains were stored in 0.5% paraformaldehyde until cutting. Paraffin sections of 1μm thickness were deparaffinized in 3 steps of xylene after hydration in a decreasing alcohol series. To quench endogenous peroxidases, specimens were incubated in 3% hydrogen peroxide for 15 minutes at room temperature and rinsed in distilled water. For antigen unmasking, slices were heated for 20 minutes in a 10 mmol/L citrate buffer using a microwave. After cooling for 60 minutes, sections were rinsed in PBS and transferred to a humidified chamber after incubation with PBS-0.1% Triton-X 100. After blocking for 30 minutes in 5%, NSS sections were incubated overnight at 4°C primary antibody. Primary antibodies were diluted 1:600 (MPO: DAKO) and 1:200 (MAP-2, Sigma, Hamburg, Germany). Immunoreactivity was visualized by the ABC method (Vectastatin; Vector Laboratories) as described for the frozen sections.

Cell counting of MPO-positive cells was performed identical to the frozen sections of the short-term study. Slices stained with MAP-2 antibody were scanned and used for calculation of infarct volume. Calculation of infarct volume was performed as described for silver infarct staining.

**Transf erase dUTP Nick-End Labeling Staining**

Transf erase dUTP nick-end labeling (TUNEL) staining was performed with the ApopTag Peroxidase in situ Apoptosis detection Kit (MP Biomedicals, Heidelberg, Germany). Tissue sections were deparaffinized in xylene and hydrated in a sequence of ethanol washes followed by a final wash in PBS for 5 minutes. Nuclei of specimen were stripped of proteins by incubation with Proteinase K (20 μg/mL) for 15 minutes at room temperature. Slices were then washed twice in distilled water and incubated in 3% hydrogen peroxide dissolved in PBS to remove endogenous peroxidases followed by 2 washing steps in PBS. After equilibration for 10 seconds, slices were incubated at 37°C for 1 hour in a humidified
Infarct size in the different groups is displayed in Figure 1. The infarct volume in the 34°C and 33°C groups were smaller than in all other groups (P<0.05; ANOVA). No significant differences in infarct volume were noted when comparing the 34°C and 33°C groups.

Brain Edema
Brain edema is displayed in Figure 1. Extent of brain edema among the various groups was essentially the same as that of brain infarct.

Neuroscore
Analysis of the Menzies score showed significantly better results for the groups treated with 34°C and 33°C as compared with all other groups (P<0.05, ANOVA). There was no difference between the other groups, including 32°C. Results of the neuroscore are displayed in Figure 2.

Immunohistochemistry
There were significantly more MPO-positive cells in the infarcted hemisphere in the 37°C group as compared with the 34°C and 33°C groups (both P<0.05, ANOVA). No MPO-positive cells were found in the noninfarcted hemisphere in any group. MPO-positive cells were prominently found in the piriform cortex and the caudate putamen. Results of MPO count are shown in Figure 3.

Results

Physiological Parameters Before and During Hypothermia*

<table>
<thead>
<tr>
<th>Group</th>
<th>Temperature, °C</th>
<th>pH</th>
<th>Pco₂, mm Hg</th>
<th>P₂O₃, mm Hg</th>
<th>Base Excess</th>
<th>Mean Arterial Blood Pressure, mm Hg</th>
<th>Heart Rate, beats/min</th>
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<tr>
<td>During MCAO</td>
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<td>7.44±0.04</td>
<td>42±5</td>
<td>132±17</td>
<td>4±3</td>
<td>93±2</td>
<td>446±40</td>
</tr>
<tr>
<td>36</td>
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<td>7.4±0.01</td>
<td>44±3</td>
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<tr>
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<td>135±19</td>
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<tr>
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<td>1.78±3</td>
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<tr>
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<td>46±10</td>
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<td>96±9</td>
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<td>51±9</td>
<td>107±11</td>
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<td>115±45</td>
<td>0.6±2</td>
<td>105±7</td>
<td>395±10</td>
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</tbody>
</table>

*With the exception of the intended goal temperature, there were no significant differences.
Long-Term Study

Physiological Variables
There were no significant differences between the groups except for body temperature (data not shown).

Survival
One animal died in the 33°C group after 72 hours. In group 34°C, one animal died after 48 hours. Finally, in the control group, two animals died during a 48-hour period after stroke. Animals that died within the 5-day periods were examined and TTC staining showed that they all experienced complete infarcts in the territory of the MCA.

Infarct Size
A significant difference in infarct size was noted between the 37°C and the 34°C and 33°C groups (203 ± 30, 119 ± 19, and 114 ± 26 mm³, respectively, both P < 0.05, ANOVA). Differences between the 33°C and 34°C groups were not significant.

Brain Edema
After 5 days, there was no brain edema detectable in any group (data not shown).

Neuroscore
Analysis of the Menzies score showed significantly better results for the group treated with 34°C and 33°C as compared with the 37°C group (P < 0.05; ANOVA) after 5 days. This effect was observed at each day after MCAO for 34°C and 33°C. Data of neuroscore after 24 hours and 5 days are shown in Figure 2.

Immunohistochemistry
Lower invasion of neutrophils into the ischemic hemisphere was observed in the 33°C and 34°C as compared with the 37°C group; this difference was only significant for the 34°C group. No significant differences were observed between the 33°C and 34°C groups (Figure 3).

Transferase dUTP Nick-End Labeling
TUNEL staining exhibited significantly higher counts of apoptotic cells in the 37°C as compared with the 33°C and 34°C groups. Five days after MCAO, TUNEL staining was 42% less in the 34°C group and 40% in the 33°C group compared with 37°C. Although TUNEL-stained nuclei were found scattered throughout the ischemic area, they tended to concentrate in the boundaries of the striatum in the 33°C and 34°C groups. The cortex of animals treated by 37°C showed many TUNEL-stained nuclei, whereas they were almost absent in all other groups.

Discussion
The recent study showed that treatment of 34°C and 33°C in the reperfusion period of experimental focal cerebral ischemia was superior to all other applied temperatures. There was a U-shaped curve of effectiveness on cerebral infarct and neurological performance during the first 24 hours after stroke onset. This effect was stable over a period of 5 days after stroke. Moreover, neurological outcome was superior for animals treated by 34°C compared with 33°C. Calculation of invasion of MPO-positive leukocytes suggested that anti-inflammatory effects might be a cofactor for these results as well as apoptotic mechanisms shown in TUNEL staining.

Experimentally, therapeutic hypothermia is accepted to be neuroprotective in the acute phase of focal cerebral ischemia. However, the optimal depth has not been identified yet. Most experiments in acute cerebral ischemia compare a single degree of therapeutic hypothermia to normothermia. There is only the study of Huh et al.4 in which 2 different levels of hypothermia (32°C versus 27°C) are investigated at the same time. Although a body temperature of 32°C led to a reduction of the total infarct volume by 69% compared with normothermia, postischemic cooling to 27°C resulted in a nonsignificant less reduction by 49%. So far, there was no stepwise investigation of the optimal treatment temperature for experimental focal cerebral ischemia. Therefore, our study compared for the first time different levels of mild and moderate therapeutic hypothermia in the relevant postischemic period.

The results of our study indicate that therapeutic hypothermia of 34°C and 33°C are superior to higher temperatures and to 32°C in terms of infarct size, edema, and neurological outcome as assessed by the Menzies neuroscore. Our study suggests that 34°C is superior to 33°C and effects are sustained over a period of 5 days. This long-term effect is
important because therapeutic hypothermia might lead to transient effects depending on its onset and duration. In general, the neuroprotective effect of therapeutic hypothermia can be explained by a decrease of reperfusion-associated injury and secondary pathological mechanisms appearing in the subacute phase of cerebral ischemia. Different experimental studies indicated that inflammation contributes significantly to cerebral injury after ischemia and that mild hypothermia in part attenuates this inflammatory response. Within ischemic brain tissue, leukocytes contribute to secondary injury releasing reactive oxygen species, activating thrombosis, disrupting the blood–brain barrier, increasing cerebral edema, and plugging the cerebral microvasculature. Various studies show that blocking leukocytes infiltration decreases ischemic brain injury and mild hypothermia decreases their accumulation after focal cerebral brain ischemia. In accordance to this, we measured the effect of different levels of postischemic hypothermia on polymorphonuclear leukocyte accumulation after transient focal ischemia by counting MPO-positive cells in the brain as a correlating factor for inflammatory response. We found a significant reduction of MPO-positive cells after 5 days for 34°C and 33°C. This finding demonstrates that these levels of therapeutic hypothermia attenuate the inflammatory response to transient focal ischemia permanently. Moreover, even mild decrease of body core temperature resulted in lower numbers of TUNEL-positive cells and suggest efficacy of 36°C and 35°C. They are in accordance to the results of Toyada et al in a period of 24 hours and Wang over a period of 7 days after experimental stroke. However, each study investigated only one level of therapeutic hypothermia. Besides, Wang et al showed that mild hypothermia was associated with decreased endothelial intercellular adhesion molecule-1 expression and microglial activation as measures for inflammatory response.

The recent experimental finding supports the thesis that target temperatures other than 33°C might be beneficial for patients with stroke. However, most clinical studies used 33°C as the target temperature so far. Because side effects of hypothermia increase gradually by lowering the body temperature, only a moderate decrease might be sufficient. Our data even suggests that 36°C or 35°C could have been helpful, because there was a trend toward infarct reduction and smaller numbers of TUNEL-positive cells. A combination with antishivering drugs might help to treat acute stroke by hypothermia without mechanically ventilation. However, a target temperature of 33°C requires sedation and ventilation so far.

In conclusion, our study shows that postischemic hypothermia of 34°C and 33°C is superior other treatment temperatures in experimental stroke. A temperature of 34°C might be superior to 33°C and there was a nonsignificant trend for 35°C and 36°C as well. These results are important for further clinical studies, because there is no clear evidence to cool patients to 33°C to get effectiveness in stroke. Other goal temperatures might be as effective without known side effects and requirement of artificial ventilation.

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Disclosures
None.

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