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Delayed matrix metalloproteinase inhibition reduces intracerebral hemorrhage after embolic stroke in rats

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Abstract

Hemorrhagic transformation (HT) and brain edema are life-threatening complications of recombinant tissue plasminogen activator (rt-PA)-induced reperfusion after ischemic stroke. The risk of HT limits the therapeutic window for reperfusion to 3 hours after stroke onset. Pre-treatment with matrix metalloproteinase (MMP) inhibitors reduces HT and cerebral edema in experimental stroke. However, whether a delayed therapeutic intervention would be beneficial is unknown. In this study, 215 male Sprague-Dawley rats were subjected to embolic stroke and 75 rats were included in the final analysis. The animals were treated with the MMP inhibitor p-aminobenzoyl-gly-pro-D-leu-D-ala-hydroxamate before or after 3 or 6 hours of ischemia. Animals were monitored for reperfusion and received rt-PA 6 hours after ischemia onset. The results at 24 hours showed that MMP inhibition 3 hours after ischemia significantly decreased the degree of brain edema (17% of hemispheric enlargement in the treated group versus 24% in controls, $P=0.018$), reduced the risk (OR=0.163; 95% CI: 0.029 to 0.953) and gravity (0.09 versus 0.19 mg of parenchymal hemoglobin, $P=0.02$) of intracerebral hemorrhage, and improved neurological outcome (20% of the treated animals had a slight deficit; all of the controls had a bad outcome, $P<0.05$). Delaying MMP inhibition to 6 hours after ischemia restricted the beneficial role of the treatment to a reduction in the risk of parenchymal hemorrhage (OR=0.242; 95% CI: 0.060 to 0.989). Our results confirm the involvement of MMPs in HT and support the possibility of extending the therapeutic window for thrombolysis in stroke by administering a broad-spectrum MMP inhibitor after the onset of ischemia.

Keywords

blood-brain barrier; ischemia; matrix metalloproteinase; tissue plasminogen activator; thrombolysis

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INTRODUCTION

Early thrombolytic treatment with recombinant tissue plasminogen activator (rt-PA) within 3 hours of the onset of ischemic stroke, leads to improvement of the clinical outcome despite an increase in the incidence of intracerebral hemorrhage (Hacke, et al., 2004, NINDS-group, 1995). Unfortunately, a majority of stroke patients are not treated with rt-PA because they do not reach the hospital in time to benefit from safe thrombolysis (Barber, et al., 2001, Schellinger, et al., 2001).

Numerous laboratories have developed different animal models of thrombo-embolic stroke to study the pathophysiological consequences of thrombolysis with rt-PA (Busch, et al., 1997, Lapchak, et al., 2000, Niessen, et al., 2003, Sumii and Lo, 2002, Wang, et al., 2001, Zhang, et al., 1997). Special attention has been paid to type-9 matrix metalloproteinase (MMP-9) because of its involvement in blood-brain barrier (BBB) disruption and brain edema (Asahi, et al., 2001, Gasche, et al., 2001, Gidday, et al., 2005, Rosenberg, et al., 1998). MMP-9 is increased during experimental stroke (Gasche, et al., 1999, Gidday, et al., 2005, Kelly, et al., 2006, Romanic, et al., 1998, Rosenberg, et al., 2001). In humans as well as non-human primates, high plasma and tissue levels of MMP-9 are correlated with hemorrhagic transformation (HT) (Castellanos, et al., 2004, Castellanos, et al., 2007, Heo, et al., 1999, Latour, et al., 2004, Montaner, et al., 2003, Neumann-Haefelin, et al., 2002). Moreover, BBB disruption is associated with the progression of HT in both animals subjected to embolic middle cerebral artery occlusion (MCAo) and stroke patients (Castellanos, et al., 2004, Hamann, et al., 1995, Neumann-Haefelin, et al., 2002).

Early inhibition of MMPs before or five minutes after the onset of embolic stroke reduced cerebral hemorrhage in spontaneously hypertensive rats and in New Zealand white rabbits (Lapchak, et al., 2000, Sumii and Lo, 2002), but to date, no experimental studies of delayed MMP inhibition have been conducted at a more clinically relevant time point after MCAo. Therefore, using a large group of Sprague-Dawley rats subjected to thrombo-embolic MCAo, we investigated the effects of 3 to 6 hours of delayed MMP-inhibitor treatment on the development of brain edema, HT, and neurological impairment. Additionally, we looked at the effect of leucopenia on the aforementioned complications, on the basis of our previous report that showed the important role played by leukocytic MMP-9 in BBB disruption and brain edema after focal cerebral ischemia (Gidday, et al., 2005).

MATERIAL AND METHODS

Surgical Procedures and Physiological Monitoring

All animal experiments were performed in accordance with the regulations of the Swiss Federal Veterinary Office and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats, weighing 275 to 350 g ($n=215$), were anesthetized with 3% isoflurane and maintained during surgery at a level of 1% to 2% isoflurane, 70% N₂O, and 30% O₂ under spontaneous respiration. Rectal temperature was maintained at 37 ± 0.5 °C with a thermostat-controlled heating blanket. The femoral artery was cannulated with a PE-10 catheter for blood pressure and blood gas measurements. After a midline skin incision, the left common, internal, and external carotid arteries were exposed. Two 6.0 silk sutures were tied at the proximal part of the external carotid artery, which was then sectioned between the sutures. The common and internal carotid arteries and pterygopalatine artery were temporarily clipped. A PE-50 catheter containing 12 1.5-mm pieces of clot was inserted into the external carotid artery through a small puncture. The catheter was advanced into the internal carotid artery and the clots were launched with a small volume of phosphate-buffered saline (Busch, et al., 1997). The catheter was removed 10 minutes after injection. MCAo was confirmed by laser Doppler flowmetry (Perimed AB, Stockholm,

Sweden). For placement of the laser Doppler probe, a burr hole 2 mm in diameter was created in the left parietal bone (2 mm posterior and 5 mm lateral to bregma). Regional cerebral blood flow in the middle cerebral artery territory was measured 0, 3, 6, and 24 hours after occlusion and expressed in perfusion units. The laser Doppler probe was carefully placed with a micromanipulator in contact with the surface of the brain and was removed after each reading. Between periods of regional cerebral blood flow measurements, the animals were allowed to wake up and have full access to food and water.

Preparation of Clots

The day before surgery, 0.6 ml of arterial blood from a donor rat were mixed with 0.1 ml of thrombin (30 U/ml, Sigma, Fluka, Buchs, Switzerland) and immediately injected into a PE-60 catheter. The blood was incubated at 37 C for 2 hours and subsequently at 5 C overnight. The clots were then removed from the catheters, rinsed in phosphate-buffered saline to remove blood cells and inspected under a microscope for selection of fibrin-rich sections. These sections were cut into small pieces 1.5 mm in length (Busch, et al., 1997).

Thrombolysis

Six hours after occlusion, 10 mg/kg of rt-PA (Actilyse, Boehringer Ingelheim, Basel, Switzerland), dissolved at a concentration of 2 mg/ml, were injected in the jugular vein, 10% of the dose in bolus and the remaining over 30 minutes. The efficiency of reperfusion was checked by laser Doppler flowmetry and by looking at the disappearance of clots within the cerebral arteries upon sacrifice. Reperfusion was considered to have occurred if the regional cerebral blood flow returned to more than 50% of the basal value at a checkpoint.

Macroscopic Hemorrhage Evaluation

Twenty-four hour after ischemia, the rats were anesthetized with a lethal dose of isoflurane and perfused through the heart with 10 U/ml heparin in 0.9% saline. Coronal brain sections (2 mm thick) were scanned and then stored at -20 C for further analysis. Macroscopic hemorrhages were assessed on digital images and were classified in three groups showing no hemorrhage (NH), hemorrhagic infarction (HI), or parenchymal hemorrhage (PH). HI was defined as small or more confluent petechia within the infarct area without space-occupying effect; PH was defined as blood clots with some slight or substantial space-occupying effect (del Zoppo, et al., 1992, von Kummer and Hacke, 1992).

Parenchymal Hemoglobin Measurement

Brain sections were thawed and homogenized in 10 volumes of phosphate-buffered saline (w/v) in a Potter-Elvehjem homogenizer with a Teflon pestle and centrifuged at 20000 g for 30 minutes. Hemoglobin was measured in triplicate in the supernatants by spectrophotometric absorption at 600 nm after 20 minutes of incubation at room temperature with 0.15% hydrogen peroxide and 2.5 mg/ml of 3,3',5,5'-tetramethylbenzidine (Standefer and Vanderjagt, 1977). The quantity of hemoglobin in the ipsilateral fraction, Hb_{ipsi} , was determined based on the optical densities of the contralateral and ipsilateral fractions, OD_{contra} and OD_{ipsi} , and the optical densities OD_i' obtained after addition of known quantities Hb_i' of hemoglobin in the ipsilateral fraction, by the formula:

$$Hb_{ipsi} = Hb_i' \times (OD_{ipsi} - OD_{contra}) / (OD_i' - OD_{ipsi})$$

MMP Extraction and Zymography

Brain homogenates in phosphate-buffered saline were equilibrated with a binding buffer at a final concentration of 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% BRIJ-35, and 1% Triton X-100 and centrifuged at 20000 g for 30 minutes. The supernatants were incubated for 60 minutes with gelatin-sepharose 4B (GE Healthcare Bio-Sciences AB,

Uppsala, Sweden) and centrifuged. Gelatinases were eluted from the pellets with 10% dimethyl sulfoxide and MMP activity was detected by gelatin zymography as previously described (Gasche, et al., 1999). Human pro-MMP-9 and human pro-MMP-2 (Oncogene Research, Boston, MA, USA) were loaded onto each gel as standards. MMP-9 activity was assessed on inverted digital images by measuring the optical density of the bands with NIH image software version 1.63. Data were expressed in arbitrary units.

Brain Edema Measurement

Brain edema was assessed on digital images by measuring the hemispheric surfaces on serial 2-mm fresh sections with Image J version 1.3. Brain edema was expressed as a percent of hemispheric enlargement by comparison with the contralateral sides.

Infarct Volume Measurement

Edema-corrected infarct volume was assessed on digital images by measuring the total cross-sectional area and the unstained portion of serial 2-mm coronal brain sections stained with 2% 2,3-triphenyltetrazolium chloride at 37 C for 20 minutes as previously described (Gidday, et al., 2005).

MMP Inhibitor Treatment

The MMP inhibitor, p-aminobenzoyl-gly-pro-D-leu-D-ala-hydroxamate (AHA), was purchased from MP Biomedicals, Heidelberg, Germany, and dissolved in phosphate-buffered saline at a concentration of 10 mg/ml. The drug inhibits interstitial collagenases, gelatinases, and stromelysin with IC₅₀ values of 1 μ M, 30 μ M, and 150 μ M, respectively (Otake, et al., 1994). The drug was successfully used in many different studies, both *in vivo* and *ex vivo* (Falo, et al., 2006, Gasche, et al., 2001, Lehoux, et al., 2004, Reeves, et al., 2003). Rats were treated with 30 mg/kg of AHA by intraperitoneal injection immediately before surgery or at 3 or 6 hours after occlusion. Control animals were injected with phosphate-buffered saline.

In Situ Zymography

In situ zymography was performed to detect MMP activity in brain sections as previously described with slight modifications (Gasche, et al., 2001). Frozen coronal brain sections (30 μ m thick) from ischemic rats were brought to room temperature and incubated with 40 μ g/ml FITC-labeled DQ-gelatin (Molecular Probes, Eugene, OR, USA) in 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, and 0.2 mM NaN₃, pH 7.6 overnight at room temperature in a humidified chamber. The *in situ* gelatinolysis was revealed by the appearance of fluorescent brain constituents.

Cyclophosphamide Treatment

Leukopenia was induced in rats by an intraperitoneal injection of 75 mg/kg of cyclophosphamide (Sigma) 4 to 6 days before surgery. A daily blood cell count was performed with an automated analyzer (CBC analyzer, F-520; Sysmex Digitana, Horgen, Switzerland). Thrombo-embolic occlusion was performed only on animals with a minimal 80% drop in neutrophils on the day of surgery.

Neurological Evaluation

Twenty-four hours after MCAo, the rats were assessed with a four-point neurological deficit scale according to Bederson et al. (1986): grade 0, no observable deficit; grade 1, difficulty in extending the right forelimb while held gently by the tail; grade 2, circling toward the paretic side when allowed to move freely; grade 3, no spontaneous movements; grade 4, death. Good

outcome was considered when the animals showed grade 0 or 1 symptoms, bad outcome was considered when the animals showed grade 2, 3, or 4 symptoms.

Statistics

Macroscopic hemorrhage, parenchymal hemoglobin, and hemispheric enlargement were assessed in all animals, including the animals that died during the period of reperfusion. Statistical analysis was conducted using SPSS release 13 for the Macintosh. Risks of mortality and risk of parenchymal hemorrhage are reported as percentages and were compared by logistic regression. Normally distributed data, as shown with the Shapiro-Wilk test, are reported as means $\pm$ SD. Non-parametric data are expressed as medians [interquartile ranges]. Multiple group comparisons were done by one-way analysis of variance followed by a Fisher PLSD test or by Kruskal-Wallis analysis of variance followed by a Mann-Whitney test, when appropriate. $P < 0.05$ was considered significant.

RESULTS

Animal Use

A total of 215 rats were subjected to embolic stroke. Fifty-two animals (24%) were discarded because of no significant decrease in regional cerebral blood flow. One hundred sixty-three animals (76%) were properly occluded and showed a significant drop in regional cerebral blood flow within the middle cerebral artery territory immediately after occlusion (49 ± 27 perfusion units) compared with pre-ischemic values (255 ± 89 perfusion units). Among them, 70 animals (43%) were spontaneously reperfused within 6 hours of occlusion and 93 animals (57%) remained occluded for at least 6 hours. In the latter group, 12 animals (13%) were not reperfused after rt-PA injection and were discarded. Six other animals were discarded because of technical problems such as massive neck bleeding or respiratory arrest. The rate of spontaneous reperfusion was identical in all groups. Blood pressure and blood physiological parameters remained in the normal range. Only animals occluded for 6 hours and properly reperfused after rt-PA injection were included in the study since we found that the risk and gravity of HT were dependent on the duration of MCAo (data not shown).

Effect of MMP Inhibition

Twenty-four hours after the onset of ischemia, all rats exhibited neurological deficits with mild or more severe symptoms consisting of difficulty in extending the right forelimb (grade 1 of the neurological scale, Table 1), circling toward the paretic side (grade 2), absence of spontaneous movements (grade 3), or death (grade 4). Intraperitoneal injection of AHA, a broad-spectrum MMP inhibitor, before MCAo (T0) or 3 hours after the onset of ischemia (T3), significantly reduced the risk of unfavorable neurological outcome. Late post-treatment 6 hours after occlusion (T6) did not improve the neurological score. The mortality rate was similar in all groups (Table 1). Infarct volume was not statistically different between the vehicle-treated animals (237 ± 48 mm³, $n=11$) and animals treated with AHA immediately before (223 ± 81 mm³, $n=9$) or after 3 hours (221 ± 43 mm³, $n=6$) or 6 hours (191 ± 57 mm³, $n=8$) of ischemia. Microvascular staining, by *in situ* zymography, was reduced in the MCA territory of the ischemic animals 24 hours after intraperitoneal injection of the MMP inhibitor at a dose of 30 mg/kg (Figure 1).

The risk of severe HT (Figure 2) was statistically reduced by injection of the MMP inhibitor before MCAo (Table 2). Only 13% of the pre-treated animals was identified with parenchymal hemorrhage in comparison with 60% of the vehicle group. The number of animals that severely bled remained at a low level of 20% or 27%, respectively, by postponing MMP inhibition for 3 or 6 hours after MCAo.

Despite an increase in the number of animals showing hemorrhagic infarctions after MCAo and MMP inhibition, the quantity of parenchymal hemoglobin in the HI groups was statistically decreased by 69% ($P=0.001$) and 50% ($P=0.02$), respectively, after pre-treatment and treatment 3 hours after the onset of ischemia compared with the vehicle-treated group. This quantity was higher when MMP inhibition was postponed for 6 hours and was not significantly different from that in the vehicle-treated animals (Figure 3). Within the PH group, the quantity of hemoglobin was decreased by approximately 40% after early treatment (0.396 mg/hemisphere [0.322, 0.475], $n=4$) compared with the vehicle-treated animals (0.649 mg/hemisphere [0.401, 1.271], $n=15$), but remained high when MMP inhibition was postponed for 6 hours (0.806 mg/hemisphere [0.375, 0.880], $n=4$).

In the vehicle-treated rats, 6 hours of ischemia induced brain swelling characterized by a hemispheric enlargement of 23.9% [16.6, 30.9] 24 hours after MCAo (Figure 4). The MMP inhibitor, injected immediately before or 3 hours after onset of ischemia, significantly reduced cerebral edema ($P=0.002$ and $P=0.018$, respectively). But delaying MMP inhibition for 6 hours after MCAo did not decrease cerebral edema compared with control animals.

Effect of Leukocyte Depletion

Cyclophosphamide treatment resulted in a progressive depletion of circulating leukocytes (including neutrophils) within 4 to 5 days after injection (data not shown). When the concentration of neutrophils dropped below 300 cells/ μ l, the leukopenic rats were subjected to 6 hours of ischemia. Cyclophosphamide-induced leukopenia statistically reduced the risk of HT after thrombolysis (Table 2). In the HI group, the quantity of hemoglobin measured in the leukocyte-depleted rats (0.100 mg/hemisphere [0.035, 0.151], $n=6$) was 46% lower than in the vehicle-treated rats (0.185 mg/hemisphere [0.137, 0.297], $n=9$, $P=0.03$). Leukopenia also significantly reduced the unfavorable neurological outcome due to embolic stroke (Table 1).

Zymography analysis showed a 10-fold increase in the optical density of the MMP-9 band from the ipsilateral hemispheres of the control animals 24 hours after ischemia compared with the values obtained from the contralateral sides (Figure 5). In contrast, cyclophosphamide treatment totally prevented a MMP-9 increase after MCAo.

DISCUSSION

In this study, we demonstrated for the first time that the injection of an MMP inhibitor 3 hours after the onset of cerebral embolic stroke in Sprague-Dawley rats significantly decreased the degree of brain edema and reduced the risk and gravity of HT due to thrombolysis with rt-PA. Delaying MMP inhibition to 6 hours after stroke restricted the beneficial role of the treatment only to a significant reduction in PH. The loss of treatment efficacy as early as 6 hours after stroke is consistent with rapid BBB proteolytic damage within 3 hours after MCAo in rats (Rosenberg, et al., 1998). Among the proteases involved in this process, we showed previously that MMP-9 is overexpressed and activated as soon as 2 hours after focal cerebral ischemia (Gasche, et al., 1999). Nevertheless, our demonstration of an efficient reduction in HT in a model of 6-hour MCAo by a treatment initiated 3 hours after the onset of ischemia provides a working strategy for extending the therapeutic window for thrombolysis beyond the current 3-hour limit.

In our study, MMP inhibition initiated immediately before ischemia did not significantly reduce mortality, which remained around 40% 24 hours after ischemia. This contrasted with a significant decrease reported in spontaneously hypertensive rats treated with another MMP inhibitor within 5 minutes of ischemia onset (Sumii and Lo, 2002). However in the latter study, the authors did not find any differences in neurological deficits, whereas we found a significant

reduction in the incidence of unfavorable neurological outcome. These discrepancies might be due to differences in animal species or treatment protocols, since the MMP inhibitor was injected three times during the total period of embolic stroke in the hypertensive rats while we administered only a single injection at a chosen time point.

In a related, albeit non-embolic, model of 6 hours of MCAo using an intraluminal thread, MMP inhibition initiated 2 hours after ischemia dramatically reduced mortality in ischemic rats. However in this study, middle cerebral artery recanalization never induced intracerebral bleeding (Pfefferkorn and Rosenberg, 2003). The lack of a hemorrhagic component highlights the significant differences between the thread and embolic models of cerebral ischemia, which do not allow direct comparisons of the animal outcomes. In the thread model, the authors suggested that the reduction in mortality was related to a reduction in rt-PA brain penetration and toxicity due to the preservation of the BBB by the MMP inhibitor (Pfefferkorn and Rosenberg, 2003). In our model, we consistently observed BBB protection after pre-ischemic and 3-hour delayed MMP inhibition as shown by a reduction in brain edema and HT. The absence of reduction in mortality could be related to the early assessment of clinical outcome 24 hours after ischemia. Later outcome evaluations could potentially show a larger difference in mortality between treated and control animals, because of the evolving consequences of cerebral edema and HT on survival. Long-term neurological evaluations after 3 to 4 weeks could also increase confidence in possible neurological protection as a whole provided by inhibition of MMP.

The lack of reduction of the cerebral infarct by injection of the MMP inhibitor before or after ischemic onset suggests that neurodegeneration after embolic stroke is mainly unrelated to early MMP activation. Our results are consistent with results obtained in spontaneously hypertensive rats in which pre-treatment with an MMP inhibitor did not reduce the infarct volume 24 hours after 6 hours of ischemia (Sumii and Lo, 2002). However, we cannot rule out that MMP inhibition could reduce cerebral infarction in an embolic stroke model featuring shorter MCAo.

Additionally in the present study, we showed that leukocyte depletion with cyclophosphamide before embolic stroke blocked MMP-9 increase in the ischemic brain and decreased the intensity of HT leading to an improvement in the neurological outcome. Justicia et al. (2003) obtained similar results on MMP-9 in rats after intraluminal MCAo by injecting an antibody against ICAM-1 that prevented neutrophil infiltration. Moreover, it was shown in postmortem fresh brain tissue from ischemic stroke patients, that MMP-9 was mainly located around blood vessels within the infarct core, associated with neutrophil infiltration (Rosell, et al., 2006). Overall, these results suggest that leukocyte-derived MMP-9 plays a role in early BBB disruption and HT after thrombolysis with rt-PA. However, they do not rule out a later role for leukocytes in the inflammatory phenomena that occur after the formation of the brain infarct. It must be noted that to date, the chronology of leukocyte participation in stroke is debated (Emerich, et al., 2002).

Many experimental approaches aiming at extending the therapeutic window for thrombolysis have been reported (Lapchak, 2007, Zhang, et al., 2004, Zhang, et al., 2001, Zhang, et al., 2002). These studies targeted free radicals, inflammatory cascades, platelet activation, or the extravascular neurotoxic effect of rt-PA. It has also been suggested that the use of magnetic resonance imaging techniques could help to identify patients who might be treated with rt-PA beyond 3 hours after stroke onset (Ribo, et al., 2005). None of these approaches are exclusive and future therapeutic developments may consider the combination of different neuroprotective strategies together with reperfusion to convincingly improve stroke prognosis. There is no doubt that a 20% risk of PH, which is the lowest value we obtained by delaying MMP inhibition to 3 hours after onset of ischemia, compared to 60% in the vehicle group,

remains too high for clinical practice. In the ECASS II trial, the risk of PH was not higher than 11% when thrombolysis with rt-PA was performed within 6 hours of ischemia (Hacke, et al., 1998). Despite the high risk of HT in our model, delayed MMP inhibition clearly reduced the volume of blood released in the brain parenchyma, which is a very promising point *per se*.

In conclusion, our study shows for the first time in a rat embolic model of stroke, where thrombolysis with rt-PA is induced 6 hours after MCAo, that the severity of HT and the risk of neurological impairments can be reduced by injecting a broad spectrum MMP inhibitor 3 hours after the onset of ischemia.

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Figure 1. Representative photographs of the *in situ* gelatinolytic activity in animals treated with the MMP inhibitor or a vehicle. Twenty-four hours after ischemia, MMP activity was assessed *in situ* at the level of the MCA territory in vehicle-treated animals (A) and animals treated with AHA immediately before ischemia (B). Insets show contralateral sides. Scale bars are 100 μ m.

Figure 2.

Representative photographs of the different hemorrhagic groups. Animals were classified in three groups showing no macroscopic hemorrhage (NH), hemorrhagic infarction (HI), or parenchymal hemorrhage (PH).

Figure 3.

Intensity of hemorrhagic infarction after the onset of embolic stroke. Twenty-four hours after ischemia, the quantity of parenchymal hemoglobin was measured in the vehicle-treated group ($n=9$) and the groups treated with AHA immediately before (T0, $n=8$), or 3 hours (T3, $n=6$) and 6 hours (T6, $n=7$) after onset of MCAo. The graph shows the values in the HI groups. * Statistically different from the vehicle-treated group. † Statistically different from the T6 group.

Figure 4.

Degree of hemispheric enlargement after the onset of embolic stroke. Twenty-four hours after ischemia, the degree of hemispheric enlargement was measured in the vehicle-treated group ($n=20$) and the groups treated with AHA immediately before (T0, $n=10$), or 3 hours (T3, $n=10$) and 6 hours (T6, $n=15$) after onset of MCAo. * Statistically different from the vehicle-treated group. † Statistically different from the T6 group.

Figure 5.

Effect of leukopenia on MMP-9 expression. Twenty-four hours after ischemia, the quantity of MMP-9 was assessed by zymography in the ipsilateral (Ipsi) and contralateral (Contra) hemispheres of control ($n=7$) and cyclophosphamide-treated animals ($n=6$). A) Inverted photograph of a representative zymogram. B) Densitometric analysis. * Statistically different from the contralateral side. † Statistically different from the cyclophosphamide-treated group.

TABLE 1
Neurological evaluation 24 hours after the onset of embolic stroke

Group	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Good outcome	Bad outcome
Vehicle, n=25	0	0	36	20	44	0	100
T0, n=15	0	33	27	0	40	33*	67
T3, n=10	0	20	30	10	40	20*	80
T6, n=15	0	7	20	27	47	7	93
Cyclo, n=10	0	30	30	0	40	30*	70

Neurological scores are reported as percent of the total number (n) of animals per group.

Good outcome was considered when animals showed grade 0 or 1 symptoms at 24 h.

* denotes a significant improvement compared with the vehicle group.

TABLE 2
Risk of parenchymal hemorrhage due to 6 hours of embolic stroke

Group	Macroscopic hemorrhage (%)			Risk of PH (%) [*]	
	NH	HI	PH	OR (95% CI)	P value
Vehicle, n=25	4	36	60		
T0, n=15	33	53	13	0.103 (0.019-0.556)	0.008
T3, n=10	29	60	20	0.167 (0.029-0.953)	0.044
T6, n=15	27	47	27	0.242 (0.060-0.980)	0.047
Cyclo, n=10	30	60	10	0.074 (0.008-0.679)	0.021

^{*} Comparison with the vehicle group.