Combination of a Free Radical Scavenger and Heparin Reduces Cerebral Hemorrhage After Heparin Treatment in a Rabbit Middle Cerebral Artery Occlusion Model

Bing-Qiao Zhao, MD; Yasuhiro Suzuki, MS; Kazunao Kondo, MD, PhD; Yasuhiko Ikeda, MD, PhD; Kazuo Umemura, MD, PhD

Background and Purpose—We sought to investigate the effects of EPC-K1, a free radical scavenger, on reducing heparin-produced cerebral hemorrhage in a rabbit model of middle cerebral artery (MCA) photothrombosis and to investigate whether the combination of EPC-K1 and heparin enhances neuroprotection from cerebral ischemic damage.

Methods—In the heparin-alone group (n=8), heparin was administered intravenously for 24 hours, starting from 3 hours after MCA occlusion. In the EPC-K1-alone group (n=8), EPC-K1 was administered as a bolus injection (10 mg/kg) twice at 3 and 6 hours after MCA occlusion. In the combination group (n=8), EPC-K1 and heparin both were administered as in the single-drug procedures. In the vehicle group (n=10), saline were infused for 24 hours.

Results—Heparin prolonged activated partial thromboplastin time by ~3 times that of control animals. In the heparin-treated animals, the hemorrhage size was significantly increased (P<0.0001) and neurological symptoms were significantly worse (P<0.01) than in control animals at 48 hours. The combination of EPC-K1 and heparin dramatically reduced heparin-produced cerebral hemorrhage (P<0.0001), with a significant reduction in infarct volume (reduction by 63.2% and 57.2% of heparin-alone and control animals, respectively, P<0.0001) and a significant improvement in neurological symptoms (P<0.01 versus heparin-alone and control animals, respectively).

Conclusions—These data indicate that free radical formation may play a key role in intracerebral hemorrhage exacerbated by heparin treatment and that the combination of a free radical scavenger and heparin augmented neuroprotection from acute brain ischemia. The results of the present study may suggest a potential clinical approach for the treatment of acute stroke. (Stroke. 2001;32:2157-2163.)

Key Words: cerebral ischemia ■ free radicals ■ heparin ■ intracerebral hemorrhage ■ middle cerebral artery occlusion ■ rabbits

Intracerebral hemorrhagic transformations are feared events that may follow therapy with antithrombotic and thrombolytic agents in acute stroke, so the high-dose use of these agents has been limited.1,2 The merit of antithrombotic agents in clinical use depends on a balance between the benefits of a reduction in infarct volume and the risks of cerebral hemorrhage.

Published evidence indicates that matrix metalloproteinases (MMPs) increase in primates with hemorrhagic transformation after cerebral ischemia-reperfusion injury,3 and the activated forms of MMPs appeared after the blood-brain barrier opening4 and intracerebral hemorrhage.5 On the other hand, overexpression of superoxide dismutase in transgenic mice has been observed to reduce MMP expression after brain trauma.6 Recently, 2 spin-trap agents and metalloproteinase inhibition have been found to reduce thrombolysis-induced hemorrhage after thromboembolic stroke.7,8 These results together suggest a possible association of free radicals with intracerebral hemorrhage after heparin treatment.

Based on photothrombotic technique, we developed a new model of intracerebral hemorrhage induced by antithrombotic agents after middle cerebral artery (MCA) thrombotic occlusion in rabbits.9,10 In this model, spontaneous reperfusion of the MCA after the thrombotic occlusion following cyclic flow reductions was important.10,11 The rabbit was chosen because it is often used for various models of intracranial hemorrhage.12

In the present study, therefore, we wanted to use the cerebral hemorrhage model produced with heparin to investigate whether the addition of EPC-K1, a free radical scavenger, reduces the hemorrhage by heparin and, if so, whether such combination treatment enhances the neuroprotective efficacy regarding cerebral ischemic damage. EPC-K1 is a hydroxyl radical scavenger, and its chemical structure is combined with vitamins C and E by phosphate.13
Materials and Methods

The experimental protocol was approved by the Hamamatsu University School of Medicine Committee on Ethics of Animal Experimentation, and extra care was taken to avoid causing animal suffering.

Animal Preparation

Male Japanese White rabbits weighting 2.0 to 3.0 kg were anesthe-
tized with 1% to 2% isoflurane (Dainihon Pharmaceutical Co) in a
mixture of 30% O₂ and 70% N₂O with the use of a face mask. The
animals were restrained in the right lateral position with spontaneous
respiration, and body temperatures were maintained at 38°C with a
heating pad (K-module model K-20; American Pharmaseal) during
the operation. A plastic catheter was placed in the right femoral vein
for blood sampling and connected to a pressure transducer (Nihon Konden); arterial blood pressure and heart rate
femoral artery for blood sampling and connected to a pressure
transducer (Nihon Konden); arterial blood pressure and heart rate
were measured.

MCA Thrombotic Occlusion

The photothrombotic occlusion of the MCA was performed in a
similar manner, as described previously by Umemura and col-
leagues.9,14 In brief, after removal of the eyeball and the temporalis
muscle with a bipolar electric coagulator (model 80-1160; Valley
Forge Scientific Corp) and a thermoknife, an 8-mm-diameter oval
window was opened with a dental drill (model PAL-7; Morita). Un-
der the operating microscopy, the main trunk of the MCA was
observed without cutting the dura mater. Photoirradiation with green
light (wavelength 540 nm) was achieved by using a xenon lamp
(model L4887; Hamamatsu Photonics) with a heat-absorbing filter
and a green filter, whereas Watson and colleagues used laser
irradiation with either a 514.5-nm argon beam15 or a 562-nm argon
laser–activated dye laser beam.16 The machine of Watson and
colleagues is more efficient in causing a photochemical reaction
between rose bengal and light irradiation than is our machine. To
occlude a longer length of the rabbit MCA, a 3-mm-diameter optic
fiber mounted on a micromanipulator was used. The surrounding
tissue was shielded with shims to irradiate only the MCA. The head
of the optic fiber was placed on the origin of the MCA passing over
the olfactory tract, providing an irradiation dose of 0.170 W/cm². The
probe of a pulsed Doppler flowmeter (PVD-20; Crystal Biotech) was
positioned on the MCA segment distal to the irradiated area to
measure the MCA blood flow. When steady cerebral blood flow was
obtained, rose bengal (10 mg/kg body wt for 3 minutes) was injected
through a peripheral ear vein. At the same time, photoirradiation was
started for 60 minutes. The local blood flow in MCA was continu-
ously monitored for 1 hour after rose bengal injection. After the
surgical wounds were closed, the animals were allowed to recover from anesthesia.

The following parameters were measured for the evaluation of
MCA blood flow: (1) occlusion time, defined as the time from the
start of the rose bengal injection to the cessation of blood (as
indicated by the flow monitor) in the MCA, (2) total reperfusion
time, represented as the amount of time when the vessel was open
from the start of the rose bengal injection until 1 hour after the
start of photoirradiation, and (3) first reperfusion time, defined as
the time when the vessel was first reopened after the thrombotic
occlusion.

In a separate experiment with 2 rabbits (occlusion of the MCA
was performed as described), plasma rose bengal concentrations
were determined spectrophotometrically (Ultraspex-2001; Hitachi
Co) at a 550-nm wavelength after appropriate dilution with water.
The rose bengal blood concentrations in the 2 rabbits were 193.1
mol/L at 60 minutes after the rose bengal injection (10 mg/kg).

TABLE 1. Physiological Variables Before and 1 Hour After the Start of Photoirradiation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>pH</th>
<th>PaO₂, mm Hg</th>
<th>PaCO₂, mm Hg</th>
<th>MABP, mm Hg</th>
<th>HR, bpm</th>
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<tbody>
<tr>
<td>Vehicle group</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Before photoirradiation</td>
<td>10</td>
<td>7.44±0.04</td>
<td>108±17</td>
<td>38.7±5.5</td>
<td>73.7±6.7</td>
<td>316±26</td>
</tr>
<tr>
<td>1 h after photoirradiation</td>
<td>10</td>
<td>7.46±0.04</td>
<td>108±10</td>
<td>42.5±4.4</td>
<td>75.6±11.1</td>
<td>313±32</td>
</tr>
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<td>Heparin group</td>
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<td></td>
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<tr>
<td>Before photoirradiation</td>
<td>8</td>
<td>7.45±0.02</td>
<td>112±13</td>
<td>42.8±5.0</td>
<td>74.4±8.8</td>
<td>300±20</td>
</tr>
<tr>
<td>1 h after photoirradiation</td>
<td>8</td>
<td>7.43±0.06</td>
<td>114±21</td>
<td>42.0±6.6</td>
<td>79.2±7.8</td>
<td>298±20</td>
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<tr>
<td>Before photoirradiation</td>
<td>8</td>
<td>7.46±0.03</td>
<td>109±20</td>
<td>41.2±5.9</td>
<td>72.8±6.8</td>
<td>306±20</td>
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<tr>
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<td>7.44±0.05</td>
<td>106±18</td>
<td>41.3±6.6</td>
<td>75.5±7.6</td>
<td>301±17</td>
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<td>EPC-K1+heparin group</td>
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<tr>
<td>Before photoirradiation</td>
<td>8</td>
<td>7.47±0.03</td>
<td>122±17</td>
<td>38.3±3.4</td>
<td>74.2±6.9</td>
<td>313±23</td>
</tr>
<tr>
<td>1 h after photoirradiation</td>
<td>8</td>
<td>7.47±0.04</td>
<td>113±19</td>
<td>38.0±5.1</td>
<td>78.8±9.1</td>
<td>308±25</td>
</tr>
</tbody>
</table>

MABP indicates mean arterial blood pressure; HR, heart rate. Values are mean±SD.
Measurement of Temperature at the Site of the Irradiated Tissue Near the MCA

To verify that irradiation or the photochemical reaction did not heat the irradiated tissue, in 3 animals the temperature at the site of the irradiated tissue was measured continuously with an implanted digital thermometer probe (0.5 mm diameter; Inter Medical). The digital thermometer probe was touched directly to the MCA and was at the irradiation center (it is impossible to measure the temperature inside the rabbit’s MCA).

Administration of EPC-K1 and Heparin

EPC-K1 and heparin were diluted with saline. All bolus injections were made at a volume of 1 mL/kg, and heparin infusion was made at a delivery rate of 0.5 mL · kg⁻¹ · h⁻¹. In the heparin-alone group, heparin was administered as an intravenous bolus injection of 100 IU/kg followed by a 75 IU · kg⁻¹ · h⁻¹ continuous infusion for 24 hours, beginning 3 hours after the start of photoirradiation in 8 animals. In EPC-K1-alone group (n=8), EPC-K1 was administered intravenously as a bolus injection of 10 mg/kg twice at 3 and 6 hours after the start of photoirradiation. In the combination of EPC-K1 and heparin group (n=8), EPC-K1 and heparin were administered as in the single-drug procedures. In the vehicle-treated group (control), 10 animals were infused continuously with saline beginning 3 hours for 24 hours.

Drugs

Heparin (1000 IU/mL) was purchased from Hoechst Pharmaceutical Co. Rose bengal was purchased from Wako Chemical Co. EPC-K1 (potassium (5(S)-5-[(1S)-1,2-dihydroxyethyl]-4-hydroxy-2-oxo-2,5-dihydro-3-furanyl]oxy)oxy)[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2H-chromen-6-yl]oxy]phosphoranolate) was obtained from Toyama Chemical Co.

Analysis of Activated Partial Thromboplastin Time

Analysis of activated partial thromboplastin time (aPTT) was performed using an automatic Coagulometer KC4A (Heinrich Ame-lung) as previously described. In all groups, aPTT was measured before photoirradiation and 24 hours after drug administration.

Examination of Neurological Symptoms

Neurological symptoms of each animal were examined blindly 48 hours after photothrombosis. In the wry-neck test, the animal was placed in a cage and the torsion of the neck was observed. Behavior was scored with 0 indicating normal and 1 indicating twisting of the neck. In the righting reflex test, the animal was placed on its back, and scored as follows: 0, righted within 1 second; 1, righted within 5 seconds; or 2, did not right within 5 seconds. Regarding paw dysfunction, the forepaw or hindpaw was pulled toward the body. The time to reextend the paw was scored as follows: 0, achieved within 1 second; 1, achieved within 5 seconds; or 2, not achieved within 5 seconds. In the postural reflex test, the animal was pushed in the contralateral direction and scored as follows: 0, normal; 1, reduced resistance to lateral push; or 2, fell down on the contralateral side. In the circling test, the animal was placed on the ground; 1 point was given when the animal circled with touching the body, and 2 points were given when the animal circled without touching the body. Each score was summed and represented the neurological score (0 to 11). In the wry-neck test, shoulder adduction was observed. In paw dysfunction, mild wrist flexion or full flexion of the wrist and elbow was observed. In the righting reflex test, the postural reflex test, and the circling test, hemiparesis and obtundation of animals were observed.

Determination of Cerebral Hemorrhage Volume

After measurement of the other variables at 48 hours after photothrombosis, animals were intravenously injected with pentobarbital sodium and then immediately perfused transcardially with normal saline. Their cerebrums were rapidly isolated. Twelve consecutive coronal sections that were 1 mm thick were cut from each of cerebrums using a slicing apparatus (RBM-7000C; Activational Systems Inc). All coronal sections were immediately photographed. The red and brown areas in the aspects of the sections were defined as hemorrhage. The size of cerebral hemorrhage was measured using a computerized image analysis system (NIH Image 1.62 Program; Internet).

Determination of Cerebral Infarct Volume

The brain slices were immersed for 30 minutes in a 1% solution of triphenyltetrazolium chloride at 37°C and then fixed with buffered formaldehyde (pH 7.2) for 24 hours. Photographs of each section were taken again after staining with triphenyltetrazolium chloride, and the size of cerebral infarction was measured with the computerized image analysis system NIH Image. The unstained and white softening areas on each section were considered to be the areas of cerebral ischemic damage.
Statistical Analysis
Data are represented as mean±SD. Physiological variables, parameters of cerebral blood flow, aPTT, size of cerebral hemorrhage, and area of cerebral damage were analyzed statistically by 1-way ANOVA and Fisher’s PLSD test. Scores in neurological deficits were analyzed by Kruskal-Wallis test followed by the Mann-Whitney U test. P<0.05 was considered statistically significant.

Results

Temperature at the Site of the Irradiated Tissue Near the MCA
During the low-intensity irradiation period, the temperature at the site of the irradiated tissue near the MCA was not increased (Figure 1).

Physiological Parameters
Table 1 summarizes the values of physiological variables obtained at pretreatment and 1 hour after the start of photoirradiation in each group. No significant differences were noted among the 4 groups in these parameters after the surgical procedure and the photothrombotic MCA occlusion.

Blood Flow in the MCA
The blood flow in the MCA was occluded completely by the formation of platelet-rich thrombus at 10.8±2.2 minutes after the rose bengal injection. There was no significant difference in the time to occlusion after the start of photoirradiation among the 4 groups, and this revealed that the degree of damage to the MCA was the same (Table 2). Figure 2 shows a typical recording of cyclic flow reductions in the MCA. Spontaneous reperfusion of the occluded MCA after cyclic flow reductions was observed within 1 hour in 85.3% of animals (29 of 34). Reperfusion and reocclusion in the MCA, the first reperfusion time, and the total vascular patency time during the 1-hour observation period did not show significant differences among the 4 groups.

aPTT Values
The baseline aPTT values were similar among the 4 groups (Table 2). The administration of saline or EPC-K1 did not affect aPTT values. In the heparin-alone group or the combination of EPC-K1 and heparin group, aPTT was markedly prolonged at 24 hours after the infusion of heparin by ≥3 times compared with the control group. EPC-K1 did not affect aPTT prolonged with heparin.

Neurological Symptoms
The median neurological score at 48 hours was 4.5 in the vehicle group (Figure 3). The administration of heparin, beginning 3 hours after photothrombosis, significantly worsened neurological symptoms (P<0.01) compared with the control group. On the contrary, the combination treatment of EPC-K1 and heparin significantly improved the neurological score to 0.5 (P<0.01 versus heparin alone and control, respectively). There was no significant difference in neurological scores between the EPC-K1–alone animals and the vehicle-treated animals.

Cerebral Hemorrhage Size and Cerebral Infarct Volume
Figure 4 shows typical photographs of intracerebral hemorrhage and cerebral infarction in each group. In most of the animals, hemorrhage was mainly observed in the basal ganglia. The administration of heparin significantly increased the size of cerebral hemorrhage (79.4±43.2 versus 23.4±17.3 mm³ in the control group, P<0.0001) (Figures 4 and 5). The total infarct volume in the heparin-alone animals also tended to be larger than that of control group (304.5±64.9 versus 261.8±40.1 mm³ in the control group, P=0.06) (Figure 6). EPC-K1 alone did not affect the hemorrhagic size (P=0.39 versus control) and significantly decreased the infarct volumes of total and basal ganglia by 29.1% (P<0.01 versus control) and 31.3% (P<0.01 versus control), respectively (Figure 6). The combination treatment with EPC-K1 and heparin dramatically reduced heparin-produced hemorrhage (P<0.0001 versus heparin alone) (Figures 4 and 5). Consequently, the infarct volumes of the cortex (by 66.5% of heparin alone, P<0.01; by 60.0% of control, P<0.01) and of the basal ganglia (by 62.2% of heparin alone, P<0.0001; by 56.3% of control, P<0.0001) and the total volume (by 63.2% of heparin alone, P<0.0001; by 57.2% of control, P<0.0001) were significantly reduced (Figure 6). Infarct volume in the combination of EPC-K1 and heparin group was significantly decreased by 39.6% (P<0.01) (Figure 6) compared with the EPC-K1–alone group.

Discussion
In the present study, the formation of a platelet- and fibrin-rich thrombus in the MCA was induced photochemically with rose bengal injection and green light from a xenon lamp irradiation system. The thrombotic occlusion of MCA caused by endothelial injury through singlet oxygen resulted in ischemic lesions in the brain tissues. The composition of the thrombus is different than the pure platelet thrombus induced by the laser irradiation. With use of the photothrombotic model in the rabbit MCA, the present study demonstrated that the administration of heparin initiated 3
hours after thrombotic occlusion of the MCA increased the size of intracerebral hemorrhage and resulted in worse neurological symptoms. EPC-K1, a hydroxyl radical scavenger, reduced the heparin-produced hemorrhage and thus decreased infarct volume and improved neurological symptoms. The results suggest that free radicals may play a key role in the development of intracerebral hemorrhage via heparin treatment in ischemic brain damage.

We recently developed and reported a model of cyclic flow reductions in the guinea pig and in the rabbit, in which a 3-mm-diameter optic fiber was used. Up to now, we have used only a 3-mm-diameter optic fiber, and therefore, it is presently unclear to us whether the occlusion of a 3-mm segment of the MCA is the most appropriate length for the observation of cyclic flow reductions. We used 10 minutes of irradiation before the injection of rose bengal in our earlier study. In our recent reports, rose bengal injection and photoirradiation were started simultaneously. We have seen no difference in the formation of thrombus in the MCA and the cerebral infarct volume between the 2 approaches.

Sixty percent of patients recover MCA blood flow in the early phase of ischemic stroke. In our recent reports, animals that had more cyclic flow reductions had a significantly higher cerebral hemorrhage volume. Therefore, we sought to establish an MCA thrombotic occlusion model in which continued cyclic flow reductions are observed after reperfusion of the occluded MCA. This model is thought to readily induce cerebral hemorrhage by antithrombotic agents. In the preliminary studies, we examined various experimental conditions, including various doses of rose bengal and intensities of irradiation, and we measured the corresponding infarct volumes together with neurological deficits. For example, for rose bengal injection at 10 mg/kg with an irradiation intensity of 0.170 W/cm² for 30 minutes, although the
Clinical trials in patients with acute stroke showed that higher doses and delayed treatment of heparin were associated with increased intracerebral hemorrhage rates,\textsuperscript{1,2} leading to masking of its benefit. The dose limitations of heparin may also reduce its efficacy. The mechanisms of hemorrhage by heparin are still unclear. In the present study, heparin significantly increased intracerebral hemorrhage when administrated beginning 3 hours after MCA occlusion. Further, heparin induced hemorrhagic infarction, resulting in slightly, but not significantly, increased infarct volume. In the present study, heparin prolonged aPTT by \textasciitilde 3 times compared with the vehicle group, which is close to the dose used clinically.\textsuperscript{22} However, heparin at the same dose, which was administered beginning 30 minutes after MCA occlusion, could reduce infarct volume without enhancement of cerebral hemorrhage.\textsuperscript{9} This suggests that the risk of hemorrhage with heparin is associated with the time from the onset of ischemia to the initiation of treatment, and cerebral hemorrhage due to heparin limits its neuroprotective effects regarding cerebral ischemic damage in this model. Previous reports showed that reperfusion injury may be associated with hemorrhage after brain ischemia.\textsuperscript{23} Early reperfusion was found in most of animals in this study, but the time of the first reperfusion and the total patency time of the MCA did not differ between heparin-treated animals and control animals during the 1-hour observation period. These results suggest that heparin-produced hemorrhage in this model may not be caused by early recanalization of the occluded MCA. The possible explanation for heparin-produced hemorrhage is that heparin increases plasmin activity,\textsuperscript{24} which directly degrades extracellular matrix components such as laminin and fibronectin,\textsuperscript{25} resulting in impaired vascular integrity and induction of hemorrhage. Dietrich et al\textsuperscript{26} reported that the photochemical approach accentuates downstream blood-brain barrier dysfunction; this is also a likely exacerbative factor that contributes to hemorrhage.

Some studies have shown that free radical scavengers such as the spin-trap agents and others reduce infarct volume after ischemia or ischemia-reperfusion; although the improvements in neurological scores were not reported,\textsuperscript{27,28} \textalpha-phenyl-\textnu-tert-butyl-nitrone and oxypurinol were reported to be effective in improving neurological scores.\textsuperscript{29,30} In the previous study, we demonstrated neuroprotective effects of EPC-K1 in focal cerebral ischemia in rats.\textsuperscript{31} However, in the present study, EPC-K1 reduced infarct volume but did not significantly improve neurological scores. This result may be attributable to mild reduction in infarct volume. This suggests that reduction in infarct volume by the free radical scavenger is unlikely to achieve an optimal protection of brain. EPC-K1 did not prevent hemorrhage in the vehicle-treated group but reduced markedly heparin-produced hemorrhage. Lapchak et al\textsuperscript{32} also reported that 2 spin-trap agents (\textnu-N-butyl-phenylnitrone and 2,2,6,6-tetramethylpiperidine-\textnu-oxyl) failed to reduce the rate and volume of hemorrhage when administrated alone. The reason for hemorrhage in the control rabbits is unclear. Because EPC-K1 is a scavenger of hydroxyl radicals,\textsuperscript{32} it is possible that this hemorrhage was caused by superoxide radicals or other radicals. Further study should be undertaken to evaluate the mechanism of hemorrhage that occurred naturally after stroke.
In the present study, combination therapy with EPC-K1, which was administered twice, at 3 and 6 hours after ischemia, had a marked effect on reduction in hemorrhage due to heparin, with significantly improved infarct volume and neurological symptoms. The reduction in infarct volume with the combination of EPC-K1 and heparin was significantly greater than that with EPC-K1 alone. The results demonstrate that when the vasculature is pharmacologically protected, the beneficial effects of heparin on reduction in infarct volume are uncovered. This result is consistent with recent studies that demonstrated the spin-trap agents reduced thrombolyis-induced hemorrhage. These findings suggest that free radicals may be the major mediator in neuronal cell death and that the hydroxyl radical may be the most likely candidate for vascular wall damage. The exact molecular mechanisms involved here are not determined; it is possible that EPC-K1, which inhibits hydroxyl radicals, reduces the expression of MMPs and ameliorates the aggravation of basal lamina damage after cerebral ischemia.

In conclusion, the delayed administration of heparin initiated 3 hours after photothrombosis increased intracerebral hemorrhage, which limited its efficacy and resulted in deteriorating neurological symptoms in rabbits. Reduction in heparin-produced hemorrhage and improvement of neurological symptoms by the free radical scavenger suggest that free radical formation may play a key role in intracerebral hemorrhage produced by heparin. This may provide some implications for the treatment of acute stroke in humans.

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References
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