Enoxaparin in Experimental Stroke
Neuroprotection and Therapeutic Window of Opportunity

Véronique Mary, PharmD; Florence Wahl, PhD; André Uzan, PharmD; Jean-Marie Stutzmann, PhD

Background and Purpose—Heparin and heparinoids have long been proposed for stroke treatment. This study investigates the effect of enoxaparin (Lovenox, Clexane), a low-molecular-weight heparin, on functional outcome (neuroscore) and lesion size in stroke models with reversible and irreversible cerebral ischemia using middle cerebral artery occlusion (MCAO) in the rat.

Methods—Ischemia was induced in rats by transient occlusion for 2 hours or by permanent electrocoagulation of the left MCA. Forty-eight hours after ischemia, neurological deficit was evaluated by scoring sensorimotor functions and ischemic damage was quantified by histological evaluation of lesion volumes.

Results—After transient MCAO, enoxaparin at 2.3 1.5 mg/kg IV (2 and 24 hours after insult) significantly reduced lesion size by 30% (P < 0.05) and improved neuroscore (P < 0.01). This significant effect on lesion size and neuroscore was still evident when treatment was started 5 hours after insult. Administered under the same protocol with a 5 hours delay post permanent MCAO, enoxaparin reduced lesion size by 49% (P < 0.05) and improved neuroscore (P < 0.01).

Conclusions—This study indicates that standard nonhemorrhagic doses of enoxaparin reduce ischemic damage with a wide therapeutic window. In addition to its anticoagulant properties, other properties of enoxaparin could act in synergy to explain its neuroprotective profile in ischemia. Thus clinical application of enoxaparin treatment in stroke warrants serious consideration. (Stroke. 2001;32:993-999.)

Key Words: cerebral ischemia, focal ■ heparin ■ neuroprotection ■ thrombolytic therapy ■ rats

Stroke is the third most common cause of death and the most common single cause of severe disability among western populations.1 Because a majority of strokes are caused by cerebral embolism, the anticoagulant properties of heparin have long been proposed as a strategy for stroke therapy.2 A recent large clinical trial3 showed that patients allocated to heparin had significantly fewer recurrent ischemic strokes within 14 days; this, however, was offset by a similar-sized increase in hemorrhagic events, and neither heparin regimen offered any clinical advantage at 6 months.3-5 The high risk of bleeding thus limits the use of unfractionated heparin in the treatment of cerebral ischemia.

Low-molecular-weight heparins (LMWH), such as enoxaparin (Lovenox, Clexane), were designed to reduce risk of hemorrhage by modifying the relative activities on key coagulation factors.6,7 Various LMWH and heparinoids have been tested in treatment of acute ischemic stroke. The first nadroparin trial (Fraxiparin International Stroke Study [FISS]) suggested a long-term benefit with LMWH treatment8,9; however, in the FISS bis study that followed, nadroparin treatment did not demonstrate an overall benefit on functional recovery or death rate, and bleeding episodes and intracranial hemorrhage increased after treatment.9 More recently, in the Heparin in Acute Embolic Stroke Trial (HAEST), dalteparin did not demonstrate an overall benefit on functional recovery and death rate.10 Finally, the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) indicated that despite an apparent positive response to treatment at 7 days, emergent administration of the antithrombotic agent, ORG 10172, was not associated with an improvement in favorable outcome at 3 months.11 These clinical results do not encourage the use of LMWH for the treatment of stroke, but studies were planned only on the basis of the anticoagulant properties of heparin or LMWH preparation.

Nevertheless, Yanaka et al12,13 have demonstrated that heparin can inhibit leukocyte accumulation in ischemic tissue, reduce the size of infarction, and improve neurological outcome after experimental stroke in rats. That study demonstrates that beyond its anticoagulant activity, non-anticoagulant actions of heparin, including inhibition of leukocyte accumulation (ie, anti-inflammatory effects) could be of interest for stroke treatment. By analogy with the anti-inflammatory effects of heparin, LMWH such as enoxaparin may be of interest in stroke treatment. In particular, in a canine model of myocardial ischemia, enoxaparin inhibited leukocyte infiltration and reduced infarction size.14 More-
over, Pratt et al\textsuperscript{15} have demonstrated that enoxaparin reduces brain edema provoked by a photothrombosis-induced ischemia in rats, without hemorrhagic side effects. Recently, in a rat model of traumatic brain injury, enoxaparin reduced the cerebral edema and the lesion size and improved the neurological score and the cognitive recovery.\textsuperscript{16}

The aim of this study was to explore the effects of enoxaparin in 2 physiopathologically different models of focal cerebral ischemia in the rat.

Materials and Methods

Surgery

Male Sprague-Dawley rats (230 to 250 g; Iffa Credo, France) were allowed food and water ad libitum and maintained on a 12-hour light/dark cycle. Halothane anesthesia (1.4\% in a mixture of 70\% nitrogen and 30\% oxygen) was used. During anesthesia, normothermia was kept by placing the rat under a heating pad controlled by rectal feedback (Harvard). Brain temperature was measured by a thermocouple (Physistemp) inserted into the temporalis muscle, and normothermia was maintained during surgery. All the procedures were performed following approval of Aventis Animal Care and Use Committee and in accordance with standards of the guide for the care and use of laboratory animals (National Research Council ILAR) and with respect to French and European Community rules.

Transient Middle Cerebral Occlusion (MCAO)

The left common carotid artery (CCA) was isolated through a ventral midline neck incision and a loose ligature was placed around it. The left middle cerebral artery (MCA) was exposed via a temporal craniectomy.\textsuperscript{17} A microsurgical clip (Yasargil, Nemco) was carefully applied to the MCA proximally to the medial border of the olfactory tract with microbipolar coagulation and its lenticulostriatal branch were occluded proximally to the entrance of the anterior cerebral artery. Restoration of blood flow in both cerebral circulation was reestablished by removing the microclip and use of laboratory animals (National Research Council ILAR) and with respect to French and European Community rules.

Drug Treatment

Doses and regimen of administration were chosen according to the studies of Yanaka et al.\textsuperscript{12,13} In all experiments drugs (dissolved in saline) were administered by intravenous route (5 mL/kg). Enoxaparin and heparin were prepared by Aventis laboratory.

Transient MCAO

In the first study (study I), we compared the effect of both enoxaparin and heparin. Drugs were administered at 1.5 mg/kg 2 and 24 hours after the onset of ischemia. Control rats received vehicle according to the same protocol.

In a second study (study II), the therapeutic window of opportunity of enoxaparin was explored. Treatment started at 5 hours after ischemia onset, followed by a second administration at 24 hours, as in the first study. This study consisted of a dose-effect of enoxaparin on cerebral lesions. The doses studied were 0.5, 1, and 1.5 mg/kg. Control rats received vehicle according to the same protocol.

To complete the previous study, in a third experiment (study III) we studied the effect of enoxaparin administered at 1.5 mg/kg 5 and 24 hours after the onset of ischemia on both functional outcome (neuroscore) and cerebral lesions. Control rats received vehicle according to the same protocol.

Permanent MCAO

In a fourth study (study IV), the neuroprotective effect of enoxaparin was studied in a model of permanent ischemia. The same protocol as in transient MCAO was chosen, with 1.5 mg/kg IV 5 hours and 24 hours after ischemic insult. Control rats received vehicle according to the same protocol.

Effect of Enoxaparin on Coagulation

To evaluate the bleeding risk after enoxaparin treatment (study V), APTT was measured in ischemic rats, after treatment by enoxaparin at the higher dose used: 1.5 mg/kg, 5 and 24 hours after ischemia onset. APTT was evaluated 10 minutes or 1 hour after the last administration of enoxaparin. Control rats received saline vehicle according to the same protocol. Simultaneously, APTT was evaluated in 3 groups of nonischemic rats receiving enoxaparin or saline according to the same protocol. Finally, APTT was evaluated in a seventh group of normal rats.

Materials and Methods

Surgery

Male Sprague-Dawley rats (230 to 250 g; Iffa Credo, France) were allowed food and water ad libitum and maintained on a 12-hour light/dark cycle. Halothane anesthesia (1.4\% in a mixture of 70\% nitrogen and 30\% oxygen) was used. During anesthesia, normothermia was kept by placing the rat under a heating pad controlled by rectal feedback (Harvard). Brain temperature was measured by a thermocouple (Physistemp) inserted into the temporalis muscle, and normothermia was maintained during surgery. All the procedures were performed following approval of Aventis Animal Care and Use Committee and in accordance with standards of the guide for the care and use of laboratory animals (National Research Council ILAR) and with respect to French and European Community rules.

Activated Partial Thromboplastin Time (APTT) Measurement

With the rats under halothane anesthesia, arterial aortic blood samples were collected in a citrated tube and centrifuged at 3000 rpm for 15 minutes. The measurement of APTT was carried out with an

| TABLE 1. Neurological Examination Grading Scale for Rats |
|----------------|-------------|-------------|
| Item           | Normal      | Deficit     |
| Grasping reflex, right forepaw | 1 | 0 |
| Placing reaction, leg hanging    | 1 | 0 |
| Right forepaw                   | 1 | 0 |
| Right hindpaw                   | 1 | 0 |
| Righting reflex, head tilted    | 1 | 0 |
| Right side                      | 1 | 0 |
| Left side                       | 1 | 0 |
| Abnormal posture                | 7 | 0 |
| Forelimb flexion                | 1 | 0 |
| Body twisting                   | 1 | 0 |

Adapted from Wahl et al.\textsuperscript{18}

ACL Futura photometer on plasma after activation by ellagic acid and cephalin. Coagulation was triggered by addition of calcium chloride. The analyzer detected clot formation by variation in absorbance.
Expression of Results and Data Analysis

Values were expressed as mean±SEM. ANOVA followed by the Student-Newman-Keuls test for comparison between groups was used in study I. Kruskal-Wallis’ test for nonparametric ANOVA followed by the Dunn test for comparison between groups was used in studies II and V. The Mann-Whitney test for comparison of 2 groups was used in studies III and IV. Nonparametric tests were preferred when variance was not homogenous. Nevertheless, normal distribution was always confirmed by a normality test.

Results

In all studies, MCA occlusion in rats provoked a significant deficit in neurological score and a hemispheric lesion involving both cortical and striatal tissues (see Table 2).

Study I: Heparin Versus Enoxaparin

Administered at 2×1.5 mg/kg IV, enoxaparin significantly improved the neuroscore 48 hours after ischemia (P<0.01). In addition, enoxaparin significantly reduced the cortical lesion by 30% (P<0.05). Enoxaparin had no effect on striatal lesion volume (Figure 1). At 2×1.5 mg/kg IV, heparin had no significant effect on the neurological score. Nevertheless, the effect on cortical lesion was similar to that of enoxaparin (~30%) but nonsignificant (Figure 1). Five of 13 rats treated by heparin died 24 hours after ischemia, and 2 of the remaining 8 animals had notable evidence of hemorrhage and therefore were excluded from the final results. No mortality was observed in saline- and enoxaparin-treated groups, and only 1 of 13 rats died in the enoxaparin-treated group.

Studies II and III: Therapeutic Window and Dose-Effect of Enoxaparin in Transient MCAO

When administered 5 hours after ischemia onset, at 2×0.5 mg/kg IV, enoxaparin reduced the volume of the cortical lesion by 19% but this effect is not statistically significant. Administered at higher doses (2×1 mg/kg IV and 2×1.5 mg/kg IV), enoxaparin significantly reduced the cortical lesion volume by 30% and 36%, respectively (P<0.05 in both cases). At each

<table>
<thead>
<tr>
<th>Study/Group (N/n)</th>
<th>Cortical Lesion, mm³</th>
<th>Striatal Lesion, mm³</th>
<th>Neuroscore (7-Point Scale)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transient MCAO</strong></td>
<td></td>
<td></td>
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<tr>
<td>Study I (2 h after injury)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (10/10)</td>
<td>186±18</td>
<td>49±3</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>Heparin (6/13)</td>
<td>130±17</td>
<td>42±4</td>
<td>2.3±0.4</td>
</tr>
<tr>
<td>Enoxaparin (12/13)</td>
<td>131±13*</td>
<td>44±2</td>
<td>3.1±0.2†</td>
</tr>
<tr>
<td>Study II (5 h after injury)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (11/11)</td>
<td>203±12</td>
<td>53±1</td>
<td>...</td>
</tr>
<tr>
<td>Enoxaparin 0.5 (9/9)</td>
<td>164±15</td>
<td>51±2</td>
<td>...</td>
</tr>
<tr>
<td>Enoxaparin 1 (10/10)</td>
<td>142±24*</td>
<td>47±3</td>
<td>...</td>
</tr>
<tr>
<td>Enoxaparin 1.5 (9/10)</td>
<td>129±17*</td>
<td>48±3</td>
<td>...</td>
</tr>
<tr>
<td>Study III (5 h after injury)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (13/13)</td>
<td>195±12</td>
<td>53±2</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>Enoxaparin (10/10)</td>
<td>129±16†</td>
<td>45±4</td>
<td>3.4±0.3‡</td>
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<tr>
<td><strong>Permanent MCAO</strong></td>
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<tr>
<td>Study V (5 h after injury)</td>
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<td></td>
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</tr>
<tr>
<td>Control (11/11)</td>
<td>137±23</td>
<td>35±6</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>Enoxaparin (13/13)</td>
<td>71±3*</td>
<td>32±4</td>
<td>2.9±0.3†</td>
</tr>
</tbody>
</table>

Values in parentheses are numbers of animals: N indicates number of rats included in results; n, total of rats used. Differences are due to mortality, hemorrhage, or exclusion in case of absence of cortical lesion.

*P<0.05, †P<0.01, ‡P<0.001 vs control group.

Figure 1. Effect of heparin and enoxaparin (1.5 mg/kg IV 24 hours after insult) on volume of left cortical lesion (A) and neuroscore deficit (B), induced by 2 hours of MCAO followed by 48 hours of reperfusion in rats (n=10 to 13 rats per group). *P<0.05, **P<0.01 vs control group.
dose, enoxaparin had no effect on the striatal lesion volume. Administered at 2×1.5 mg/kg IV (treatment starting 5 hours after ischemia), enoxaparin significantly improved the neuroscore compared with the control group ($P<0.001$). In addition, enoxaparin was still able to reduce significantly the cortical lesion volume by 34% ($P<0.01$; Figure 2).

**Study IV: Effect of Enoxaparin in Permanent Focal Ischemia**

Administered at 2×1.5 mg/kg IV (treatment starting 5 hours after ischemia onset), enoxaparin significantly improved the neuroscore compared with the saline-treated group ($P<0.01$) and significantly reduced the cortical lesion volume by 49% ($P<0.05$) (Figure 3).

**Study V: Effect of Enoxaparin on Coagulation Time**

Average APTT times in normal rat plasma samples was 18.8±1.1 seconds (see Table 3). No significant differences in APTT were seen between normal rats and nonischemic saline-treated rats or lesioned ischemic saline-treated rats. Nevertheless, whatever the treatment, average APTT values are prolonged by approximately 30% in ischemic rats versus nonischemic rats. Concerning the effect of enoxaparin, we observed that (1) in animals sampled 10 minutes after enoxaparin treatment (1.5 mg/kg IV), APTT values were prolonged by 95% ($P<0.01$) and 79% ($P<0.01$) compared with the saline group for both nonischemic and ischemic rats, respectively; and (2) in animals sampled 1 hour after enoxaparin treatment, APTT values were not significantly different from values for corresponding saline-treated groups for both nonischemic and ischemic rats.

**Discussion**

The studies presented above show that intravenous administration of enoxaparin significantly reduces infarct size and improves neuroscore after acute brain insult induced by both permanent and transient focal ischemia in rats. Moreover, enoxaparin treatment confers a large therapeutic window of at least 5 hours and is devoid of major hemorrhagic risk.

Study I indicates that heparin and enoxaparin administered 2 hours after ischemia onset have the same beneficial effect on cortical lesion size (reduction of 30%). Nevertheless, heparin treatment induces notable mortality and cerebral hemorrhage, which resulted in half of the heparin-treated rats being excluded. This could explain why the effect of heparin did not reach statistical significance. In all studies, enoxaparin did not induce major cerebral hemorrhage. To confirm safety, APTT was measured after treatment with enoxaparin. Small changes in this parameter did not result in any increase in bleed potential. The slight and reversible (in 1 hour) elevation of APTT, in addition to the fact that no macroscopic hemorrhage after enoxaparin treatment was observed in our model, suggest that enoxaparin is neuroprotective at nonhemorrhagic doses. At the same dose, heparin induced such a considerable prolongation of APTT that it could not be measured.

One of the most important and obvious factors to bear in mind when considering acute treatment of stroke is that patients are taken in charge by medical care some hours after they have had their stroke. Thus, studies II and III are important in demonstrating the dose-response to enoxaparin within a 5-hour therapeutic window.

Stroke is a highly variable clinical condition, and in particular, the delay in spontaneous reperfusion is a variant
(from hours to days). Therefore, we also evaluated the effect of enoxaparin in a model of permanent ischemia. This study indicates that enoxaparin is still active in a model of ischemia without reperfusion and therefore that this effect is not dependent on reperfusion time, which reinforces the interest of enoxaparin in stroke.

Another important point for public health is that in a large proportion of surviving patients, stroke induces important disability and dependence because of functional impairment. In clinical studies, functional outcome after an ischemic episode is one of the most useful parameters. To provide a more sensitive measure of neuroprotection, we associated evaluation of functional outcome (neuroscore) with lesion size. In all cases, enoxaparin provided both infarct reduction and functional improvement. These complementary effects provide another strong argument for a potential activity of enoxaparin in the clinic.

Heparin as well as LMWH have been successfully tested previously in models of cerebral ischemia. Our study indicates that enoxaparin is also neuroprotective in 2 different models of focal ischemia and, moreover, at a lower dosage (approximately 100 USP total dose) than those used in previous studies. In addition, the effect is fully maintained with a first administration at 5 hours after ischemia. This supports the hypothesis that enoxaparin has a better efficacy with a better safety margin than some other LMWHs. Why should LMWHs have different biological profiles, since they have some similarities in molecular weight? Chemical studies and investigations, such as NMR spectroscopy, reveal that LMWHs differ in molecular weight mean and distribution, sulfation patterns, and types and quantities of minor residues (for review, see References 21 and 22). Moreover, each LMWH has a specific, assay-dependent pharmacokinetic profile and a unique biochemical profile, which reinforces the concept that each LMWH is a distinct, noninterchangeable pharmaceutical product. These differences may all confer a different pharmacological profile on these LMWHs and therefore modify their biological activities.

The most well-known activity of enoxaparin is the antithrombotic one. Since in tMCAO model occlusion and reperfusion are mechanically induced and in pMCAO model occlusion is definitive, antithrombotic activity alone cannot explain the neuroprotective effect. Nevertheless, reperfusion of a major feeding artery after transient obstruction has been accompanied by downstream microvascular defects. This no-reflow phenomenon may be responsible in part for secondary damage. After experimental ischemia-reperfusion in baboons, microvascular fibrin deposition accumulates in a time-dependent manner during the reperfusion period, and elements of a prethrombotic state are present in patients with cerebral ischemia. These postreperfusion occlusions may be reduced by preischemia exposure to heparin and ticlopidine in nonhuman primate models of MCA occlusion-reperfusion. Previous studies have shown that enoxaparin inhibits thrombin generation. This suggests that in the brain-injured area, enoxaparin could prevent thrombus formation and restrict thrombus extension. By limiting the fibrin formation, enoxaparin could reduce the secondary microvascular ischemia and thus the associated lesion.

Nevertheless, the neuroprotective effect of enoxaparin seen in our models of permanent and transient cerebral ischemia cannot be exclusively related to its activity on coagulation parameters. Acute neurodegenerative diseases such as stroke are physiopathological events that initiate a deleterious cascade, which includes, at the least, excitotoxicity, edema, inflammatory processes, oxidative stress, and finally, neuronal cell death, including necrosis and apoptosis (from hours to weeks in duration; for review, see Reference 32). Recent studies indicate that enoxaparin could interfere with this deleterious cascade at various levels.

Excitotoxicity has been widely documented in ischemia and is a relatively transient phenomenon associated with excessive neuronal depolarization and ionic homeostasis failure, leading to an increase in intracellular Ca2+. In vitro, heparin reduces intracellular Ca2+ release induced by inositol triphosphate system stimulation. Treatment of Purkinje cells in guinea pig cerebellar slices by heparin or enoxaparin blocks the glutamate-induced intracellular Ca2+ release. Therefore, in our models a possible interaction of enoxaparin with Ca2+ internal stores cannot be excluded.

Inflammatory reaction has also been widely described in experimental stroke. In particular, leukocyte infiltration contributes to tissue injury by inducing lipid peroxidation and release of cytotoxic products (for review, see Reference 35). Adherent neutrophils may also exacerbate cerebral ischemia by participating in the noreflow phenomenon. Factor Xa could act as a proinflammatory agent (for review, see Reference 37). Enoxaparin induces a significant and persistent plasma anti-Xa activity. In other hand, P- and L-selectins appear to play a primordial role in ischemia-reperfusion injury by participating in leukocyte infiltration. Recent studies show that enoxaparin decreases P-selectin mediating platelet-neutrophil adhesion in plasma in vitro and MPO

<table>
<thead>
<tr>
<th>TABLE 3. Effect of Enoxaparin on APTT</th>
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<tr>
<td><strong>Treatment</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>None, n=12</td>
</tr>
<tr>
<td>Saline, n=5</td>
</tr>
<tr>
<td>10 min, n=6</td>
</tr>
<tr>
<td>1 h, n=5</td>
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</table>
*P<0.01 vs corresponding saline group.
activity (reflecting neutrophil infiltration) in vivo. Finally, the complement system has also been implicated in stroke. Complement inhibition provides neuroprotection in several stroke models. Both heparin and enoxaparin inhibit complement pathways of activation (for review, see Reference 44) in vitro, but this effect was not reproduced in any in vivo model. All these data support the hypothesis that in our models, enoxaparin could be neuroprotective in part by interfering with the inflammatory process at various levels (eg, neutrophil infiltration, complement activation). Free radical production that may damage cells has been widely reported in ischemia-reperfusion injury. Antioxidant strategies have been found to be active in experimental models of stroke, and heparin interferes with reactive oxygen species production (for review, see Reference 44) via reduction of superoxide production, and increase of plasma superoxide dismutase in vivo activity. Another reactive species, nitric oxide (NO), is thought to be a key actor in the pathophysiology of CNS injuries such as cerebral ischemia. Recently, heparin was found to attenuate inducible NO synthase (iNOS) expression and NO release after cytokine activation in rat brain microvascular endothelial cells, whereas iNOS knockout mice are less susceptible than wild-type mice to ischemic insult. Although no results with enoxaparin have been reported, the hypothesis of an interaction between enoxaparin and free radicals could also be considered.

Finally, heparin and heparinoids interact with the heparin-binding growth factor (HBGF) family. These proteins have a high affinity for heparin and heparan sulfate, and it is well known that these growth factors are stabilized by heparin and heparan sulfate. Because it has been suggested that some HBGF could play an endogenous neuroprotective function, heparin and heparan sulfate may reinforce this endogenous effect. Nevertheless, this effect has not yet been clearly demonstrated in vivo and thus remains a hypothesis.

All these considerations suggest that neuroprotective activity of enoxaparin may be the result of various biological properties acting in synergy.

In conclusion, we found that enoxaparin treatment reduces both cerebral lesions and functional deficit induced by focal cerebral ischemia. We demonstrated that this effect is reproducible in different models of stroke with or without reperfusion and occurs within a large therapeutic window and at nonhemorrhagic dosage. These results, in addition to previous results obtained in photocoagulation and TBI, strongly suggest that enoxaparin has neuroprotective properties and could be a future treatment for stroke.

Acknowledgments

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References

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