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Neuroprotective Profile of Enoxaparin, a Low Molecular Weight Heparin, in *In Vivo* Models of Cerebral Ischemia or Traumatic Brain Injury in Rats: a Review

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ABSTRACT

The development of treatments for acute neurodegenerative diseases (stroke and brain trauma) has focused on (i) re-establishing blood flow to ischemic areas as quickly as possible (i.e. mainly antithrombotics or thrombolytics for stroke therapy) and (ii) on protecting neurons from cytotoxic events (i.e. neuroprotective therapies such as anti-excitotoxic or anti-inflammatory agents for stroke and neurotrauma therapies). This paper reviews the preclinical data for enoxaparin in *in vivo* models of ischemia and brain trauma in rats. Following a photothrombotic lesion in the rat, enoxaparin significantly reduced edema at 24 h after lesion when the treatment was started up to 18 h after insult. Enoxaparin was also tested after an ischemic insult using the transient middle cerebral artery occlusion (tMCAO) model in the rat. Enoxaparin, 2 × 1.5 mg/kg i.v., significantly reduced the lesion size and improved the neuroscore when the treatment was started up to 5 h after ischemia. Enoxaparin, administered at 5h after insult, reduced cortical lesion size in a dose-dependent manner. In permanent MCAO, enoxaparin (5 and 24 h after insult) significantly reduced lesion size and improved neuroscore. A slight and reversible elevation of activated partial thromboplastin time (APTT) suggests that enoxaparin is neuroprotective at a non-hemorrhagic dose. Traumatic brain injury (TBI) is often accompanied by secondary ischemia due in part to edema-induced compression of blood vessels. When

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enoxaparin, at 0.5 mg/kg i.v. + 4 × 1 mg/kg s.c., was administered later than 30h after TBI, it significantly reduced edema in hippocampus and parietal cortex. At one week after TBI the lesion size was significantly reduced and the neurological deficit significantly improved in enoxaparin treated animals. Finally, the cognitive impairment was significantly improved by enoxaparin at 48 h to 2 weeks after TBI. The anticoagulant properties of unfractionated heparin and specifically enoxaparin can explain their anti-ischemic effects in experimental models. Furthermore, unfractionated heparin and specifically enoxaparin, have, in addition to anticoagulant, many other pharmacological effects (i.e. reduction of intracellular Ca^{2+} release; antioxidant effect; anti-inflammatory or neurotrophic effects) that could act in synergy to explain the neuroprotective activity of enoxaparin in acute neurodegenerative diseases. Finally, we demonstrated, that in different *in vivo* models of acute neurodegenerative diseases, enoxaparin reduces brain edema and lesion size and improves motor and cognitive functional recovery with a large therapeutic window of opportunity (compatible with a clinical application). Taking into account these experimental data in models of ischemia and brain trauma, the clinical use of enoxaparin in acute neurodegenerative diseases warrants serious consideration.

INTRODUCTION

Acute damage to the brain, either as the result of a mechanical trauma or subsequent to interruption of the blood supply, engenders a cascade of pathological events that are only slowly being unraveled. Evidence is accumulating to suggest that this cascade of events follows, in variable degree, a large number of seemingly diverse insults to the head or brain, including thromboembolic stroke, cerebral hemorrhage, cardiac arrest, strangulation, mountain sickness and traumatic brain injury. It also appears that the brain damage progresses markedly during the hours and days following the initial insult. The resultant brain injuries cause an immeasurable amount of human misery and are of immense cost to the community. Stroke, for instance, is the third leading cause of death in the Western world (stroke incidence: in USA is 374/100,000) and is the leading cause of disability (81). The death rate from traumatic brain injury (TBI) is more than one million per year, and world hospital admissions represent around 9.5 million people (brain trauma incidence in USA is 200/100,000). Furthermore, TBI is most frequent cause of death in young people (41).

Currently there are few effective drug treatments for these pathologies. The cerebral insults, despite their varied causes, seem to have common mechanisms leading to pathological events that are frequently interconnected due to the structure of the brain.

First, the brain is protected or separated from its vascular supply by a selectively permeable barrier, called the blood-brain barrier, which is made up of vascular endothelial cells joined by tight junctions. The maintenance of this barrier is of importance to the brain as it controls the access of body fluids, including trophic factors, signaling and other molecules as well as water and immune cells, that migrate to the neurons and glia that make up the brain. This barrier of endothelial cells is sensitive to mechanical stretching or blockade of blood vessels following ischemic insults. Leakage allows the passage of plasma elements out of the vascular system and into the parenchyma, where they will

come into contact with neurons normally protected from them, and leakage increases parenchymal volume. The endothelial cells play also an important role in attracting monocytes that leave circulation and invade brain parenchyma.

Secondly the brain is held within a rigid box, so that the increase in volume of any component of the brain must result in an increase in intracranial pressure and a decrease in the other compressible components. Thus intracranial hemorrhage, extracellular and intracellular edema may all provoke secondary ischemia.

Thirdly, neurones are metabolically active cells, which need to maintain their ionic homeostasis and control their water content. They have low reserves of glycogen and are dependent upon the continuous supply of glucose from the blood stream. Likewise, the nerve's capacity for anaerobic metabolism is low and a fall in oxygen tension or glucose availability, such as during an ischemic insult, results in depolarization of neurons and opening of voltage-operated ion channels. Passage of cations through these channels will activate enzymatic reactions as well as encourage the entry of water and thus increase the cells' volume.

Finally, the brain is a mass of intercommunicating cells, which pass messages along neurons via depolarization and from one to another via depolarization-initiated release of excitatory and inhibitory transmitter molecules, such as glutamate, into synapses. In many cases these transmitter molecules open cation channels in the post-synaptic target neuron. Such activity is energy intensive as the transmitter molecule must be removed from the synapse and the ions involved in the signal must be removed from the target neuron. If there is an energetic deficit, the signal molecules will remain in the synapse and the cell will remain partially depolarized.

Thus there is a propagation of activity throughout the brain from the insult's focus to other previously unaffected areas. These signals may result in waves of inactivation, such as are seen during spreading depression, and may initiate processes such as apoptosis.

Neuronal hyperdepolarization activates various enzyme systems that produce excessive quantities of damaging free radicals, rapid oxidation of glucose, accumulation of lactic acid and a concomitant lowering of local pH which further inhibits mitochondrial oxidative phosphorylation and consequently triggers inflammatory responses and cell death.

Thus, we can view the ischemic or the traumatic processes as initial energetic or mechanical insults, which generate excitotoxic and edematous reactions, that last for hours after injury. Subsequently inflammatory mechanisms enter into play leading to either necrotic or apoptotic cell death. The whole process may take hours, days or weeks (25).

In TBI, the ischemic insult *per se* is not the initial event, but is a frequent secondary injury. In this case, edema and hemorrhage within and around the damaged tissue causes an increase of intracranial pressure that, in turn, provokes compression of cerebral blood vessels, leading in turn to reduced blood flow and ischemia (33). Formation of microthrombi has recently been reported to occur in head trauma patients (44), and may also contribute to the secondary ischemic insult.

Besides the primary injuries (such as epidural, subdural or intracerebral hematomas) which develop at the time of a posttraumatic cerebral lesion, secondary components (such as intracranial hypertension, cerebral blood flow abnormalities or brain edema) occur in minutes, hours or days after injury. Brain edema is certainly a major component in TBI and prevention of ischemia and edema form the basis for surgical treatment of traumatic intracranial lesions.

Heparin is a straight chain anionic mucopolysaccharide that occurs intracellularly in mammalian tissues that contain mast cells. It possesses anticoagulant properties via antithrombin III which accelerates by up to 1000 fold the inactivation of several serine protease clotting factors, factor IIa, IXa, Xa, XIa and XIIa. The exact profile of this effect depends on the molecular weight of heparin.

Because the majority of strokes are due to cerebral embolism, the anticoagulation with heparin has been proposed for a long time as a strategy for stroke therapy (63). The results of heparin treatments in clinical trials of cerebral insults have been controversial. A recent clinical trial (IST: International Stroke Trial; 38) has shown a nonsignificant trend toward fewer recurrent ischemic strokes among heparin-treated patients compared with patients not treated with heparin. But the number of deaths resulting from hemorrhage stroke due to extracranial bleeding was significantly greater with the highest dose of heparin. The IST study indicates that there is no net long-term benefit with heparin treatment (24,61). But in addition to its anticoagulant effect, heparin has other pharmacological effects. Non-anticoagulant properties, such as anti-inflammatory (53,82,83) and trophic properties (71) have been attributed to unfractioned heparin. The accumulation and adherence of leukocytes to the endothelium may obstruct partially or totally microvasculature in the brain, aggravating the ischemic insult (22). Aside from its anticoagulant activity, non-anticoagulant actions of heparin, including inhibition of leukocyte accumulation (i.e. an antiinflammatory effect) could be of interest in the treatment of cerebral insults with an ischemic element. Indeed, heparin was shown to be protective in myocardial ischemia in the absence of its anticoagulant effect. In rabbits treated with N-acetyl heparin (which has no anticoagulant property) or with heparin sulfate, a protective activity was observed against the induction of global cardiac ischemia (30). Black et al. (10) demonstrated that dogs treated with these agents after a coronary artery occlusion had significantly reduced infarct size. However, the main drawback of unfractionated heparin is its very short half-life and high risk of bleeding, limiting its use in certain clinical situations. Low molecular weight heparin (LMWH) has been developed with the aim of reducing the hemorrhagic risk of heparin treatments and, incidentally, improving its bioavailability and the pharmacokinetic profile.

There is a strong theoretical and experimental basis for pursuing the possibility that a LMWH could reduce ischemic brain injury. For instance, it has been demonstrated that fibrin is deposited in cerebral microvessels and along with platelets and leukocytes contributes to the occlusion of microvessels (55) and that heparin and ticlopidine could significantly decrease the development of these occlusions (21).

In thromboembolic strokes, which account for approximately 90% of strokes, ischemia arises from several concomitant events, including compression of vessels by edema formation that contributes to blood flow reduction. In addition to blood flow reduction, the two major processes are inflammation and thrombosis. Platelet activation occurs in ischemic stroke (29). It leads to the secretion of alpha granules within which P-selectin is localized. It mediates the adhesion of platelets to leukocytes and contributes to the recruitment of leukocytes at the inflammatory site. Leukocyte activation is partially responsible for tissue injury through the release of superoxide anions. Furthermore, activated platelets contribute to occlusion of blood vessels, not only through formation of platelet aggregates, but also through stimulation of thrombin generation due to exposure of procoagulant phospholipids on their surfaces . This series of events justifies the present study. As a matter of fact, enoxaparin is an inhibitor of P-selectin mediated platelet-

neutrophil adhesion and also an inhibitor of thrombin generation potentially implicating LMWHs in the reduction of both inflammatory processes and thrombus formation.

Various LMWHs and heparinoids have been tested in the treatment of acute ischemic stroke. The first nadroparin trial suggested a long-term benefit of nadroparin since this LMWH improved the outcome in acute stroke patients at 6 months with no increase in bleeding and mortality (42,61). On the contrary, in the FISS study nadroparin produced no overall benefits in functional recovery. In addition, mortality, bleedings and intracranial hemorrhages were increased (37). More recently in the HAEST study, dalteparin did not demonstrate an overall benefit on functional recovery and death rate (7). Finally the TOAST study indicated that, despite an apparent positive response to treatment at 7 days, administration of the antithrombotic agent, ORG 10172, was not associated with an improvement in favorable outcome at 3 months (69).

These clinical results were based mainly on the anticoagulant properties of heparin and LMWHs. They are not encouraging for the use of a LMWHs for treatment of stroke. In all cases, however, these agents were applied quite late after the stroke and there were no experimental studies that preceded failed clinical trials. Nevertheless, it has been well documented in the literature that LMWHs differ from heparin and among each other in structural, chemical and biological properties. This point of view was supported by clinical evidence, at least in acute coronary syndrome trials (see 26 for a review).

Transport of heparin and enoxaparin across the blood brain barrier (BBB) has been studied in an *in vitro* model of BBB. No transport of heparin could be detected across the BBB *in vitro*, whilst under normoxic conditions enoxaparin, at high concentrations, crossed the monolayer slowly. During hypoxia, a large increase in the permeability of BBB to enoxaparin, but not to heparin, was observed. This finding indicated that under hypoxic conditions, enoxaparin can reach brain parenchyma (Cechelli, personal communication). In a canine model of myocardial ischemia-reperfusion, Libersan, et al. (47) showed that in contrast to heparin, enoxaparin, (Lovenox®, Clexane®) a LMWH, reduces myocardial platelets and neutrophil accumulation and limits infarct size by ca. 50%. The efficacy of enoxaparin has been confirmed in a clinical trial on patients with unstable angina. Moreover, LMWH have also been shown to reduce polymorphonuclear leukocytes infiltration induced by cerebral ischemia (82,83).

As compared to heparin, enoxaparin has 6 times less anti-IIa (thrombin) activity and half the anti-Xa (prothrombinase) activity. These properties may make it a safer compound for use in critical situations (12). Clinical use of enoxaparin is associated with a lower risk of bleeding (12). Enoxaparin has better bioavailability and a longer half-life with lower plasma binding and a more predictable dose-response relationship than heparin (2,84).

There are many variants of several major acute neurodegenerative models, none of which is known to be of predictive value. The ideal stroke model should be predictive of clinical efficacy. Presently, no drug (except nimodipine in subarachnoid hemorrhage) has been shown to have protective effect in stroke in humans so that there is no way of knowing whether the activity in a particular model will predict efficacy.

Nevertheless, there are certain points, which should be considered as mandatory in the design of experiments for evaluation of a putative protective agent, irrespective of the model used. This is essential, because in many cases claims for possible clinical efficacy

have been made on the basis of limited animal experimental data and clinical trials with protocols that are irrelevant to the clinical situation. These points are:

1. The choice and the variety of the models tested (i.e. photothrombosis, transient or permanent focal occlusion of the middle cerebral artery, fluid percussion model of brain trauma).
2. The outcomes measured: brain damage and functional recovery.
3. Time of treatment onset: after lesion.
4. Route of administration: intravenous.
5. Choice of the dosage: realistic to human situation.
6. Therapeutic window: the most questionable point; as large as possible.

We decided to evaluate the effect of enoxaparin on neurological and histological parameters in different models of stroke and brain trauma in rats. The reasons for this decision were: (i) enoxaparin was active in models of myocardial ischemia; (ii) LMWH reduced neutrophil infiltration in ischemic brain (82,83); (iii) secondary ischemia and neutrophil infiltration often occur after TBI (9,13,18,58,60).

MATERIALS AND METHODS

Stroke Models

Photothrombosis

Detailed protocols of lesion and treatment have been described previously (57). Briefly, male Sprague Dawley rats were anesthetized and a cold light with a 3 mm diameter beam was placed in contact with the right side of the skull forward of lambda. Rose Bengal dye at 10 mg/kg i.v. in saline, was administered and illumination of the skull started immediately and continued for 5 min. In order to study the kinetics of edema formation, animals were killed at 1, 2, 4, 24, 48, 76, and 168 h after lesion and their brains removed. In experiments involving enoxaparin treatment animals were killed at twenty-four hours after the photothrombotic lesion. Brain samples were examined macroscopically for evidence of intracranial or intraventricular hemorrhage, that occured with high doses of anticoagulants in preliminary experiments.

Water content was determined by wet weight of tissue/dry weight of core samples of brain tissue. Edema was expressed as % excess water on the core sample from the lesioned hemisphere compared with the sample from the contralateral hemisphere for each rat.

Hemoglobin content in core samples of brain was assayed by the method of Drabkin and Austin, with adaptations by Choudhri et al. (19), and using the Sigma total hemoglobin kit. Hemoglobin concentration was read off from a calibration curve prepared with rat hemoglobin (Sigma) made up in tissue samples as described above.

In the time-course experiment enoxaparin was administered at 0.5 mg/kg i.v. followed 15 min later by 2 mg/kg s.c. starting either at 2, 6, or 18 h after lesion (post lesion delay). In addition, enoxaparin, 2 mg/kg s.c., was administered at 6 h after lesion for the animals treated at 2 and at 18 h after lesion. In the dose-response studies enoxaparin was administered at 0.25, 0.5, or 1 mg/kg i.v. at 18 h after lesion, followed by additional injections of enoxaparin at 1, 2, or 4 mg/kg s.c. Hemoglobin content was assayed after enoxaparin treat-

ment, at 18 h after lesion. Control lesioned animals received physiological saline. Sham-operated animals received either saline or enoxaparin.

The major issue when heparin is administered to patients with stroke is the risk of bleeding, limiting the dose that can be injected. This is due to the anticoagulant activity and, consequently, prolongation of clotting time. In animal models, the efficacy in a stroke model is only considered acceptable when it is obtained at a dose that would not induce a risk of bleeding. It is well known that LMWHs, including enoxaparin, are less likely to cause bleeding than unfractionated heparin. Nevertheless, hypocoagulability can be induced by high doses of these drugs. It was, therefore, necessary to monitor the effects of active doses of enoxaparin on coagulation parameters.

Bleeding time was also studied in photothrombotic animals. Eighteen hours after photothrombosis rats were re-anesthetized with sodium pentobarbital (60 mg/kg i.p.) and the bleeding time was assessed in each animal from a cut made by a simplate bleeding time cutter. Enoxaparin was then administered at 0.5 mg/kg i.v. and the bleeding time re-assessed at 10 minutes after injection. Fifteen minutes after the intravenous administration rats received 2 mg/kg enoxaparin s.c. Bleeding time was again assessed at 2 h after s.c. injection.

Transient MCAO

Male Sprague-Dawley rats were used for this study as already described (49). Left common carotid artery (CCA) was isolated through a ventral midline neck incision and loose ligature placed around it under halothane anesthesia. The left middle cerebral artery (MCA) was exposed via a temporal craniectomy. A microsurgical clip was carefully applied to the MCA, followed by immediate tightening of CCA ligature. Two hours later rats were re-anesthetized and cerebral circulation was reinstated by removing the microclip and loosening the CCA ligature. Restoration of blood flow in both arteries was established under microscope. In Study I, enoxaparin and heparin were administered at 2 × 1.5 mg/kg i.v., 2 and 24 h after the onset of ischemia. In Study II, enoxaparin was administered at 2 × 0.5, 1 and 1.5 mg/kg i.v., 5 and 24 h after the onset of ischemia. In Study III, enoxaparin was administered at 2 × 1.5 mg/kg i.v., 5 and 24 h after the onset of ischemia. Forty-eight hours after the surgery a neurological examination was performed blindly by a single examiner. The grading scale used was modified from the one previously described by Wahl et al. (76) (Table 1).

After 48 h of reperfusion, serial coronal brain sections (1.5 mm thickness) were prepared and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC). Volumes of infarction were calculated by integrating the necrotic areas. Values were expressed as means ±S.E.M. Analysis of variance (ANOVA) followed by Student-Neuman-Keuls' test for comparison between groups was used in Study I. Kruskal-Wallis's test for non-parametric ANOVA followed by Dunn's test for comparison between groups was used in Study II. Student's t test for comparison of 2 groups was used in Study III.

In another set of experiments (Study IV), enoxaparin was administered at 1.5 mg/kg i.v., at 5 and 24 h after the onset of ischemia. Activated partial thromboplastin time (APTT) was evaluated 10 min ($n = 7$) or 1 h ($n = 6$) after the last administration of enoxaparin. Control rats received vehicle according to the same protocol ($n = 6$). Simultaneously non-ischemic rats received enoxaparin according to the same protocol and APTT was evaluated at 10 min ($n = 6$) and 1 h ($n = 5$) after the last administration of enoxaparin. Control

TABLE 1. Neurological Examination Grading scale for rats

Item		Normal	Deficit
Grasping reflex	Right Forepaw	1	0
Placing reaction			
Leg hanging	Right Forepaw	1	0
	Right Hindpaw	1	0
Righting reflex			
Head tilted	Right side	1	0
	Left side	1	0
Abnormal posture		Absent	Present
Forelimb flexion		1	0
Body twisting		1	0
Neurological scores		7	0

From Wahl et al. (76).

rats received vehicle according to the same protocol (n = 5). Finally APTT was evaluated in a seventh group consisting of normal rats (n = 12). For each rat 1 mL blood was taken at the level of abdominal aorta. The measurement of APTT was carried out on an ACL Futura on citrated plasma after activation by ellegic acid and cephalin. Coagulation was triggered by addition of calcium chloride. The analyzer detected clot formation by variation in absorption. In Study V, enoxaparin was administered at 1.5 mg/kg, at 5 and 24 h after the onset of ischemia. The neurological score was evaluated at 48 h, 7, 14, 21, and 28 days after the insult.

Permanent MCAO

In Study VI, the left MCA was exposed by temporal craniectomy (68). The MCA and its lenticulostriatal branch were occluded proximally to the medial border of the olfactory tract with microbipolar coagulation (Martin®). The wound was sutured and animals were returned to their home cage in a room kept at 26 to 28°C. The same protocol as in Study III was chosen, enoxaparin was administered at 1.5 mg/kg i.v. at 5 and 24 h after ischemic insult. Control rats received vehicle according to the same protocol. Neurological evaluation and histology were performed as already described for transient MCAO. Mann-Whitney's test for comparison of 2 groups was used in this study.

Traumatic Brain Injury

Male Sprague-Dawley rats were anesthetized with halothane and positioned on a stereotaxic frame. A 3mm craniotomy was prepared laterally to the right parietal cortex and a fluid percussion of moderate severity (1.6 to 1.8 bars pressure) was performed according to Wahl et al. (75,77).

Brain edema

Cerebral edema was measured using the wet weight/dry weight technique (3). At 48 h after trauma, hippocampus, temporal cortex (site of injury) and parietal cortex (adjacent

cortex) were removed from the lesioned hemisphere. To determine the wet weight fresh tissue samples were immediately weighed and kept at 100°C for 24 h. The next day, the samples were re-weighed to determine the dry weight. The results were expressed as a percentage of water content and calculated as follows: % of water: [(wet weight – dry weight)/ wet weight] × 100.

Study I: enoxaparin was administered as an i.v. bolus at 0.5 mg/kg at 15 min after TBI, additional s.c. injections of enoxaparin, 2 mg/kg, were administered at 30 min, and at 6, 24, and 30 h after TBI.

Study II**:** enoxaparin was administered at 0.5 mg/kg i.v. bolus at 2 h after TBI followed by additional injections of enoxaparin, 2 mg/kg s.c., at 2 h 15 min, 24 and 30 h after TBI, some animals received enoxaparin at the same dose also at 6 h after TBI.

Study III: enoxaparin was administered at 0.5 mg/kg i.v. bolus 2 h after TBI followed by additional injections at 0.5 or 1 mg/kg s.c.at 2 h 15 min, 6, 24, and 30 h after TBI.

The same protocol was used for administration of vehicle to injured rats or for enoxaparin in uninjured rats, used as controls.

Brain lesions

Histological evaluation of the brain lesions was carried out 1 week after TBI. Coronal cryostat sections 40 μm thick were cut from 13.7 to 0.7 mm relative to the interaural line, and stained with hematoxylin-eosin. The lesion areas were measured with an image analyzer. Rats received enoxaparin at 0.5 mg/kg i.v. bolus 2 h after TBI followed by additional injections of 1 or 2 mg/kg s.c. at 2 h 15 min, 6, 24, and 30 h. Vehicle was administered to injured rats using the same protocol.

Neurological function

One, 2, 3, and 4 weeks after TBI a neurological examination was performed blindly and separately by two examiners using the previously described grading scale (77). The contralateral sensory motor functions examined were: placing reactions (leg hanging and visual), righting reflex ("head tilted"), grasping reflex and abnormal postures (forelimb flexion and thorax twisting) were scored in rats placed on a table. Rats suspended by the tail were examined for thorax twisting and left forelimb flexion. In order to quantify the neurological deficit produced by TBI, a score was given to each item, and their sum gave a global neurological score (maximum of 9 in normal rats). Rats received vehicle or enoxaparin at 0.5 mg/kg i.v. bolus at 2 h after TBI, followed by additional injections at 1 or 2 mg/kg s.c. at 2 h 15 min, 6, 24, and 30 h after TBI. Normal rats were used as controls.

Cognitive function

Cognitive deficits were assessed using a complex maze task first described by Lashley (45) and modified by Grosjean-Piot et al. (34). This maze consisted of 4 alleys leading into each other with open doors. The aim of this test was to train rats to go from a starting point to a goal box, with a minimum number of errors (<5) and a latency time less than 60 sec, defined as the selection criteria. To this end, 2 training periods of 5 days each were performed during which rats were trained in two daily sessions (morning and afternoon) of 15 min each. During these sessions, both the number of errors and the latency time(s)

were recorded. On the day before the first training sessions, rats were deprived of food (11 g/day/rat, i.e. –60% of normal intake) with water *ad libitum*. Food deprivation was maintained for the duration of the experiment. The performance of each animal was scored as follows: an entry into a wrong direction was scored as an error; and the latency time(s) necessary to succeed, i.e. to reach the goal box was recorded. At the end of the training period, only the selected rats were kept and divided into control, sham-operated and injured groups. The sham-operated animals were submitted to the surgery except for the fluid percussion. At 48 h and then on days 6, 13, 20, and 27 following surgery, rats were re-tested in the maze. In this post-training test, all the alleys were opened, and only the goalbox was reinforced. Rats received vehicle or enoxaparin at 0.5 mg/kg i.v. bolus 2 h after TBI, followed by 1 mg/kg s.c. at 2 h 15 min, 6, 24, and 30 h after TBI. Normal rats were used as controls.

Myeloperoxidase (HPO) activity

Enoxaparin was administered at 0.5 mg/kg i.v. bolus 2 h post-TBI followed by additional injections at 1 or 2 mg/kg s.c.at 2 h 15 min, 6, 24, and 30 h after TBI. Control animals received vehicle.

Myeloperoxidase assay: At 48 h after TBI, rats were re-anesthetized with sodium pentobarbital (60 mg/kg i.p., Sanofi, France). In order to eliminate the PMN present in the brain vasculature, thoracotomy was performed, the abdominal aorta was clamped and 200 mL of isotonic saline solution (at 25°C) was perfused transcardially with a peristaltic pump. The right atria were incised to allow blood and saline to flow. Brains were removed and a 3 mm coronal section centered on the traumatized area was performed and ipsilateral temporal cortex was dissected. In previous experiments in TBI MPO activity was found to be highest in the temporal cortex. The samples were immediately frozen in dry ice and stored at –45°C until the MPO activity assay (58).

Each sample was thawed on ice, weighed and homogenized with an UltraTurrax® homogenirek (3 × 5 sec) in 1 mL of 5 mM phosphate buffer saline (PBS, pH 6 at 4°C). It was then centrifuged at 25000 g for 30 min at 4°C. Supernatant was discarded and the pellet was resuspended in 1 mL of PBS and centrifuged as described above. Samples were washed twice to remove inhibitors of MPO activity. The pellet was resuspended in a 1/10 wet weight to volume ratio of hexadecyl-trimethylammonium bromide (HTAB). HTAB is a detergent that solubilizes MPO. Three freeze-thaw cycles of 10 min each were performed between cycles, in order to disrupt the azurophilic granules containing the enzyme. After the last sonication the samples were incubated at 4°C for 20 min and centrifuged at 12,500 g for 15 min at 4°C. For the assay, 70 µL of the supernatant were added to 200 µL of *o*-dianisidine hydrochloride (167 mg/L, Sigma, USA) dissolved in PBS (50 mM, pH 6, 25°C), and H_2O_2 at 0.0005%; *o*-dianisidine is colored proportionally to the degradation of hydrogen peroxide by MPO. Change in absorbance was recorded at intervals of 30 sec for 2 min at 460 nm with a spectrophotometer. One unit of MPO activity is defined as the degradation of 1 µmole of H_2O_2/min at 25°C and is expressed per gram of brain tissue (U MPO/g tissue). All data were expressed as means ±S.E.M. All experimental protocols were approved by the company ethical committee.

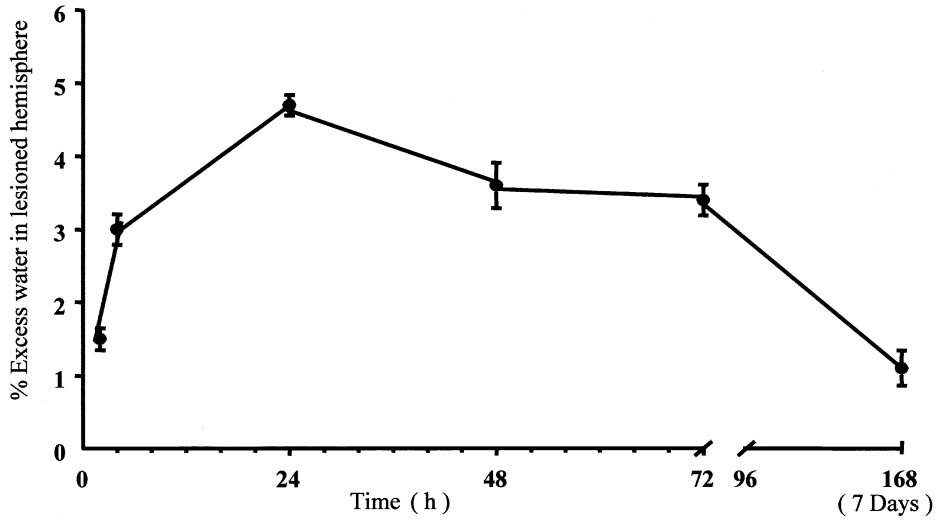

Fig. 1. The evolution of cerebral edema in rats after photothrombotic lesion. Edema reached a peak at 24 h after insult. Means ± S.E.M.

RESULTS

Stroke Models

Photothrombosis

Upon removal of the brain a visible lesion, identifiable by a whitening of the brain surface and a swelling could be observed. The preliminary kinetics experiment (Fig. 1) shows that edema developed rapidly during the first 4 h after lesion, then more slowly thereafter. The maximum edema was observed at 24 h after lesion and decreased slowly. By 7 days after lesion edema was greatly reduced, but the lesion site remained full of soft unstructured material. In subsequent experiments animals were killed at 24 h after lesion, at the time of maximal edema formation.

Treatment with enoxaparin at 0.5 mg/kg i.v. plus 2 mg/kg s.c. significantly reduced brain water content when treatment was started at 2 h after photothrombotic lesion and continued with s.c. injections to maintain enoxaparin levels. When treatment was started at 18 h after lesion a significant effect was still observed (Fig. 2). In the dose-response study for the effects of enoxaparin on edema formation the animals were treated at 18 h after lesion (Fig. 3). In sham-operated animals enoxaparin, 2.5 mg/kg total dose at 18 h after sham-operation, did not modify hemoglobin content in core samples from either hemisphere of the rat brain (Fig. 4). The photothrombotic lesion produced a significant reduction in hemoglobin concentration in the core taken from the lesioned hemisphere compared to the sample taken from the unlesioned hemisphere, or from the sham-operated rats (Fig. 4). Enoxaparin, at 2.5, 5, or 10 mg/kg total dose, did not produce any macroscopic evidence of intracranial or intraventricular bleeding or any significant increase in hemoglobin concentration in lesioned animals, indicating the absence of hemorrhage.

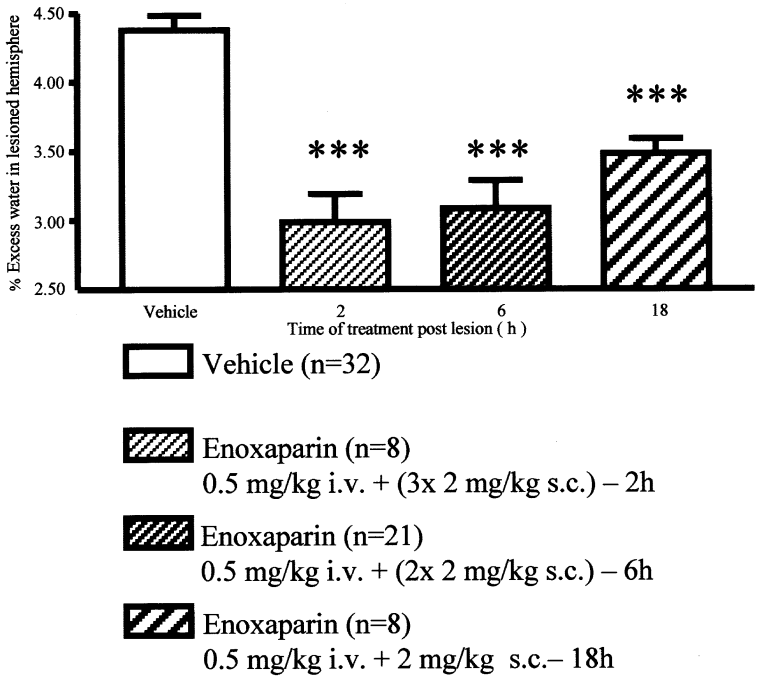

Fig. 2. Effect of enoxaparin, 0.5 mg/kg i.v followed by 2 mg/kg s.c. 15 min later, on cerebral edema induced in rats by photothrombotic lesion. The therapy was started at either 2, 6, or 18 h after injury. Additional doses of 2 mg/kg s.c. were administered at 6 and 18 h after lesion where appropriate. Means ± S.E.M., analysis of variance followed by Tukey-Kramer multiple comparison test. ***$P < 0.001$.

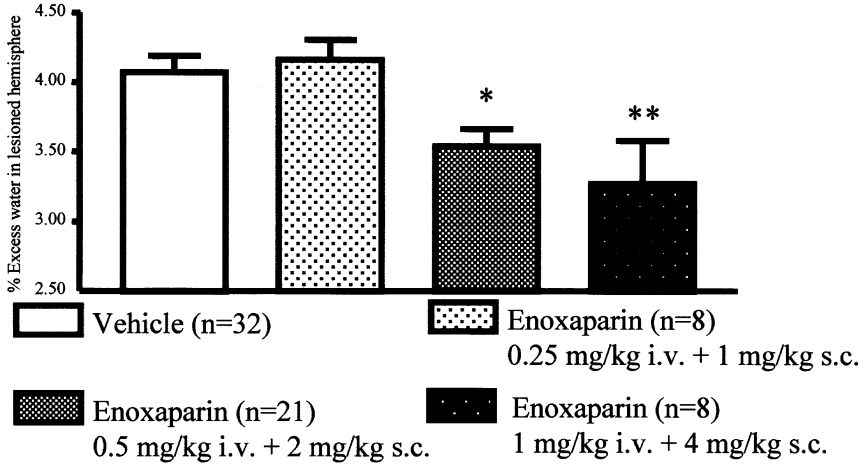

Fig. 3. Dose-response data for anti-edematous effect of enoxaparin at 24 h after lesion when treatment was started at 18 h after lesion. Enoxaparin was administered at 0.25, 0.5, or 1 mg/kg i.v. at 18 h post lesion, followed by the same drug at 1, 2, or 4 mg/kg s.c. 15 min later. Means ± S.E.M., analysis of variance followed by Tukey-Kramer multiple comparison test *$P = 0.05$, **$P < 0.01$).

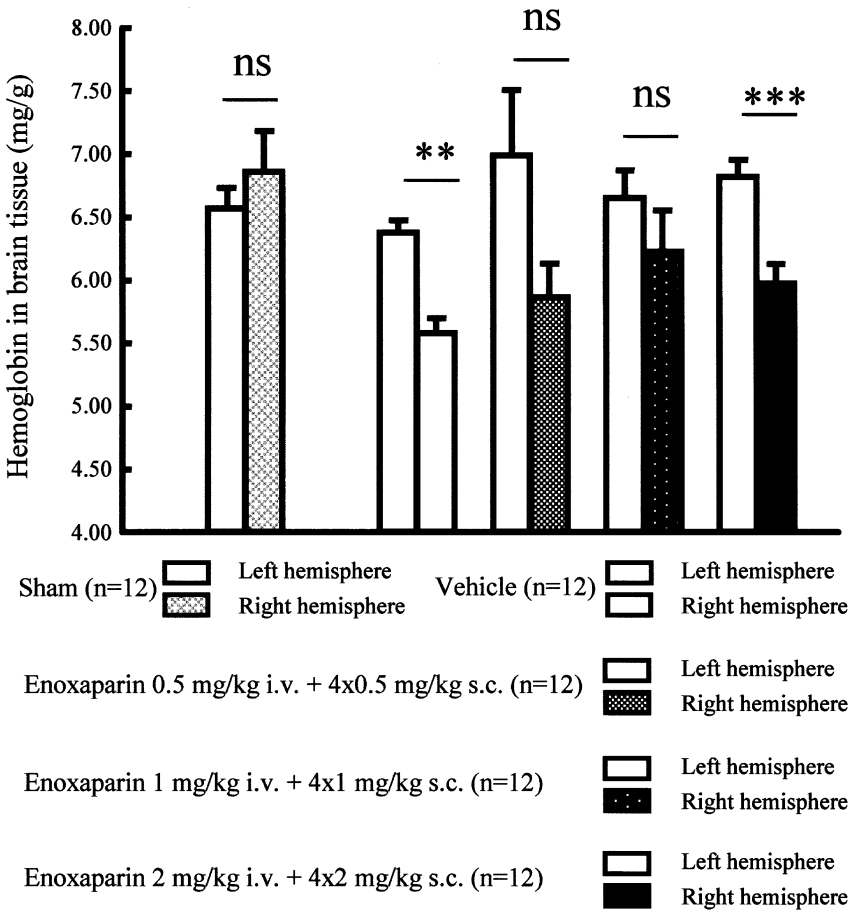

Fig. 4. Effect of enoxaparin on hemoglobin content in the core samples from sham-operated or lesioned animals. The hemoglobin content in the lesioned (right), unlesioned (left) hemispheres are compared with Student's t test. The hemoglobin content in the right (lesioned) hemisphere samples from rats treated with enoxaparin at the total dose of 2.5, 5, or 10 mg/kg (i.v. + s.c.) was not significantly increased compared with vehicle-treated animals (Tukey-Kramer multiple comparison test). Means ± S.E.M. (**$P < 0.01$, ***$P < 0.001$).

Bleeding time was also examined using the same protocol of enoxaparin administration as in the photothrombotic lesions study. At 10 min after enoxaparin, 0.5 mg/kg i.v., a small but significant increase in bleeding time of 1.1 min was observed. At 15 min after i.v. administration the animals received enoxaparin, 2 mg/kg s.c. At two h after s.c. injection the bleeding time was not significantly increased (Table 2).

Transient MCAO

Study I: Administered at 2 × 1.5 mg/kg i.v., enoxaparin improved significantly the neuroscore at 48 h after ischemia (1.7 ± 0.3 for control group *versus* 3.1 ± 0.2 for enoxaparin-treated group; $P < 0.01$). In addition enoxaparin reduced significantly the cortical

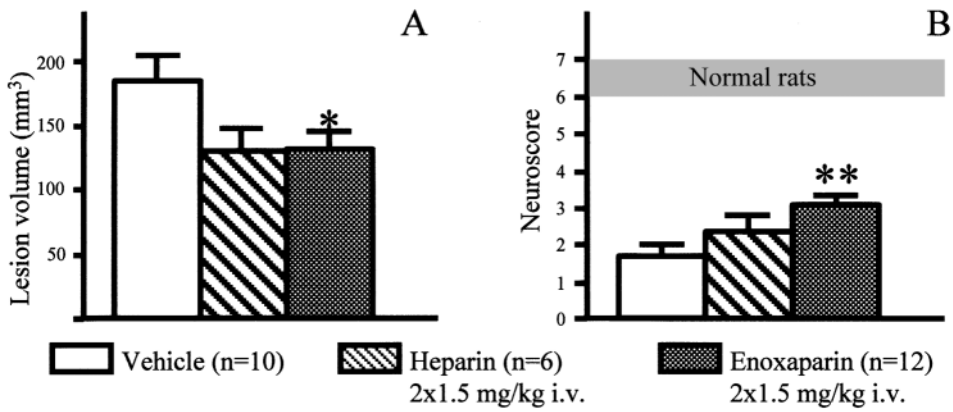

Fig. 5. Effects of heparin, enoxaparin (1.5 mg/kg i.v. at 2 and 24 h after insult) on volume of left cortical lesion (**A**), and neuroscore deficit (**B**), induced by 2 h of MCAO followed by 48 h of reperfusion, in rats. Range of neuroscore of normal rats is represented by the gray stripe. Analysis of variance (ANOVA) followed by Student-Neuman-Keuls' test for comparison between groups was used (*$P < 0.05$, **$P < 0.01$).

lesion by 30% (186 ± 18 mm^3 for control group *versus* 131 ± 13 mm^3 for enoxaparin-treated group; $P < 0.05$). This corresponded to a significant reduction of global lesion by 25% (239 ± 20 mm^3 for control group *versus* 179 ± 15 mm^3 for enoxaparin-treated group; $P < 0.05$). At 2 × 1.5 mg/kg i.v., heparin had no significant effect on neurological score (1.7 ± 0.3 for control group *versus* 2.3 ± 0.4 for heparin-treated group). It reduced by 30% the cortical lesion (186 ± 18 mm^3 for control group *versus* 130 ± 17 mm^3 for heparin-treated group). This corresponded to a reduction of global lesion by 25% (239 ± 20 mm^3 for control group *versus* 179 ± 22 mm^3 for heparin-treated group) (Fig. 5). However these effects were not significant. In addition 5 of 13 rats treated with heparin died and 2 of 13 had a major hemorrhage and were, therefore, excluded from the results. No hemorrhage was observed in saline and enoxaparin treated groups and only 1 of 13 rats died in the enoxaparin-treated group.

Study II: At 2 × 0.5 mg/kg i.v., enoxaparin reduced the cortical lesion by 19% but this effect was not statistically significant (203 ± 12 mm^3 for control group *versus* 164 ± 15 mm^3 for enoxaparin-treated group). At 2 × 1 mg/kg i.v. and at 2 × 1.5 mg/kg i.v., enoxaparin significantly reduced the cortical lesion by 30 and 36%, respectively (203 ± 12 mm^3 for control group *versus* 142 ± 24 and 129 ± 17 mm^3 for enoxaparin-treated groups, respectively; $P < 0.05$ in both cases). These effects corresponded to significant reduction of global lesion by 25 and 30%, respectively (270 ± 13 mm^3 for control group *versus* 202 ± 26 and

TABLE 2. Effect of enoxaparin on bleeding time (in minutes)

Control	At 10 min after enoxaparin, 0.5 mg/kg i.v.	At 2 h after enoxaparin, 2 mg/kg s.c.
3.35 ± 0.15	4.51 ± 0.5 *	4.32 ± 0.43

* Student's paired *t*-test, $P < 0.05$, Mean ± S.E.M. ($n = 6$).

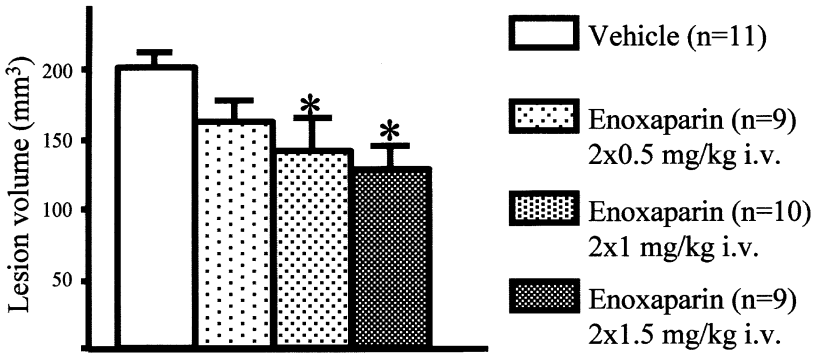

Fig. 6. Dose-response study with enoxaparin (0.5, 1, and 1.5 mg/kg i.v. at 5 and 24 h after insult) on volume of left cortical lesion induced by 2 h of MCAO followed by 48 h of reperfusion, in rats. Kruskal-Wallis's test for non-parametric ANOVA followed by Dunn's test for comparison between groups was used (*$P < 0.05$).

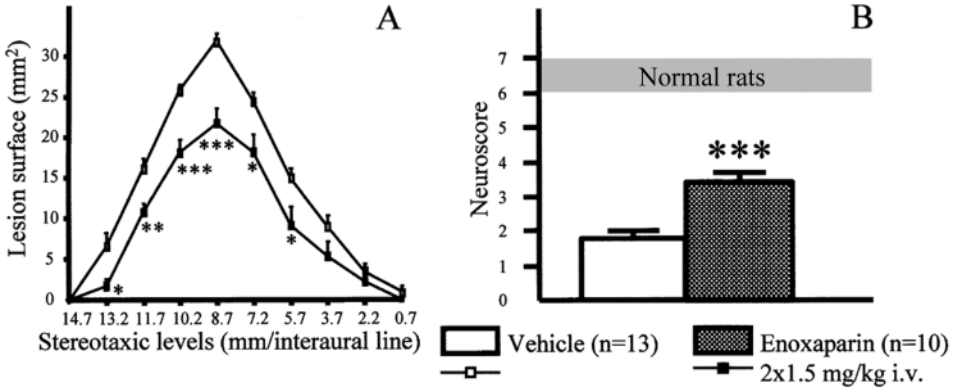

Fig. 7. Effect of enoxaparin (1.5 mg/kg i.v. 5 and 24 h post-insult) on surfaces of left cortical lesion (**A**), and neuroscore deficit (**B**), induced by 2 h of MCAO followed by 48 h of reperfusion, in rats. Range of neuroscore for normal rats is represented by the gray stripe. Student's t test for comparison of 2 groups was used (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

270 ± 13 mm^3 for control group *versus* 191 ± 19 mm^3, respectively; $P < 0.05$ in both cases) (Fig. 6). At dose tested, enoxaparin had no effect on the striatal lesion.

Study III: Administered at 2 × 1.5 mg/kg i.v. (treatment starting 5 h after ischemia onset), enoxaparin improved the neuroscore significantly, as compared to saline-treated animals (3.4 ± 0.3 *versus* 1.8 ± 0.3, $P < 0.001$). In addition, enoxaparin significantly reduced the cortical lesion by 34% (195 ± 12 mm^3 for control group versus 129 ± 16 mm^3 for enoxaparin-treated group, $P < 0.001$). This effect corresponded to a reduction of the global lesion by 23% (237 ± 15 mm^3 for control group versus 182 ± 20 mm^3 for enoxaparin-treated group, $P < 0.01$) (Fig. 7).

Study IV: The average APTT value in normal rats was 18.76 ± 1.12 sec. There was no significant difference in APTT values for normal and non-ischemic saline-treated rats (17.1 ± 0.4 sec) and ischemic saline-treated rats (23.1 ± 2 sec). Nevertheless, the average

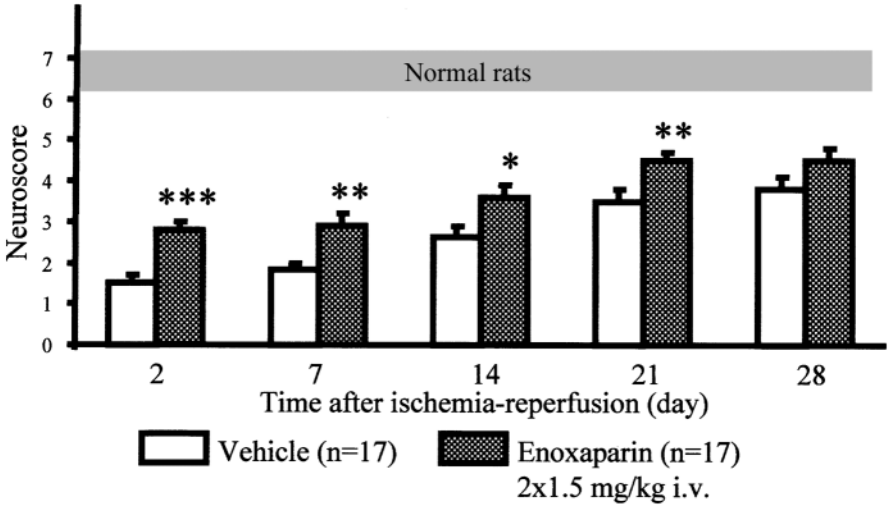

Fig. 8. Effect of enoxaparin (1.5 mg/kg i.v. 5 and 24 h after insult) on neuroscore deficit, induced by 2 h of MCAO followed by 4 weeks of reperfusion, in rats. Range of neuroscore for normal rats is represented by gray stripe. Student's *t*-test (with Welch correction) was used at each time-point (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

APTT values were prolonged in ischemic as compared to non-ischemic rats in each of the three treated groups. APTT was increased by 35% in saline-treated groups (17.1 ± 0.4 sec *versus* 23.1 ± 2 sec). At 10 min after enoxaparin APTT values were prolonged by 95% ($P < 0.01$) and 79% ($P < 0.5$) as compared to the saline group for both non-ischemic and ischemic rats, respectively. At 1 h after enoxaparin APTT values were not significantly different from values in the corresponding saline group for both non-ischemic or ischemic rats.

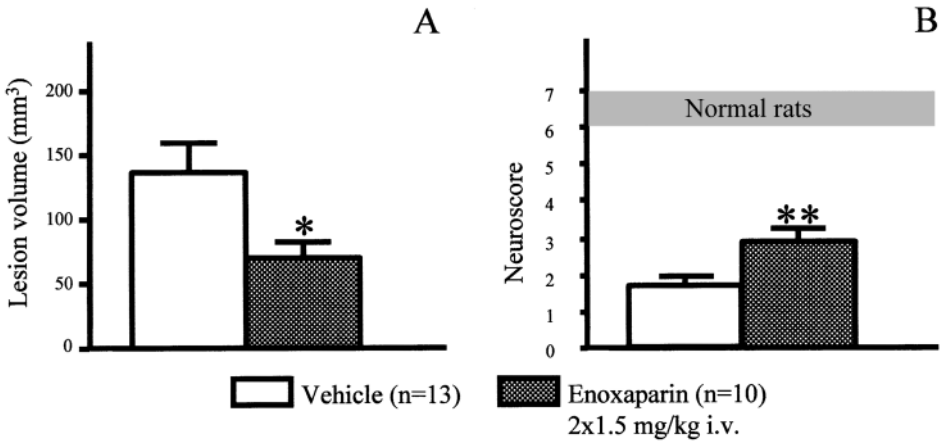

Fig. 9. Effect of enoxaparin on volume of left cortical lesion (**A**), and neuroscore deficit (**B**), induced by 48 h of permanent MCAO, in rats. Range of neuroscore for normal rats is represented by the gray stripe. Enoxaparin was administered at the dose of 1.5 mg/kg i.v. at 5 and 24 h after insult. Mann-Whitney's test for comparison of 2 groups was used in this study (*$P < 0.05$, **$P < 0.01$ *versus* control group).

Study V: As previously described, enoxaparin significantly improved the neurological score at 48 h after transient cerebral ischemia (1.5 ± 0.2 *versus* 2.8 ± 0.2; $P < 0.001$). At 7, 14, and 21 days after insult, neurological score increased in both groups, but enoxaparin group had a better neuroscore than the control group (1.8 ± 0.2 *versus* 2.9 ± 0.3, $P < 0.01$, 2.6 ± 0.3 *versus* 3.6 ± 0.3, $P < 0.05$, 3.5 ± 0.3 *versus* 4.5 ± 0.2, $P < 0.01$, respectively). This effect was not significant at 28 days after insult (3.8 ± 0.3 *versus* 4.5 ± 0.3) (Fig. 8).

Permanent MCAO

Study VI: At 2 × 1.5 mg/kg i.v. (treatment starting 5 h after ischemia onset), enoxaparin significantly improved the neuroscore compared to the saline-treated group ($P < 0.01$). In addition, enoxaparin significantly reduced the volume of the cortical lesion by 49% ($P < 0.05$) (Fig 9).

Traumatic Brain Injury

Brain edema

In all structures examined TBI produced a significant edema in the ipsilateral hemisphere (Fig. 10) with severity: hippocampus<parietal cortex<temporal cortex. No increase in the brain water content was observed in the hemisphere contralateral to the impact injury. In addition, enoxaparin did not modify the water content in the brain of normal rats (data not shown).

Study I : Enoxaparin, 0.5 mg/kg i.v. bolus at 15 min after TBI and followed by 4 × 2 mg/kg s.c. did not significantly reduce brain edema in any of the structures examined (Table 3).

Study II: Enoxaparin, 0.5 mg/kg i.v. bolus was administered at 2 h after TBI and followed by 4 × 2 mg/kg s.c. significantly reduced edema in hippocampus (69%, $P < 0.01$) and in the parietal cortex (50%, $P < 0.05$) (Fig. 10). A slight but nonsignificant reduction of edema was also observed in the temporal cortex (28%). However, if one dose of enoxaparin (2 mg/kg s.c. at 6 h) was omitted, the reduction was less pronounced in the hippocampus (–46%, $P < 0.05$) and in parietal cortex (40%, ns), while no effect was seen in the temporal cortex (Table 4). These results demonstrated that (i) initiation of treatment should be at 2 h post-TBI, (ii) to reach the maximal effect an additional injection should be administered at 6 h. Therefore, in the last study we evaluated the effects of additional lower doses of 0.5 and 1 mg/kg s.c., according to the protocol, i.e. we administered enoxaparin four times over 30 h.

TABLE 3. Effect of enoxaparin, 0.5 mg/kg i.v. at 15 min after TBI, followed by 2 mg/kg s.c. at 30 min, 6, 24, and 30 h after TBI, on the brain edema in rats measured at 48 h after TBI. Per cent of water content in various brain areas. Mean values ± S.E.M.

	Hippocampus	Parietal cortex	Temporal cortex
Control ($n = 8$)	79.61 ± 0.24	78.26 ± 0.37	79.09 ± 0.28
TBI vehicle ($n = 12$)	80.69 ± 0.22 **	80.00 ± 0.35 *	81.98 ± 0.61 ***
TBI enoxaparin($n = 12$)	80.47 ± 0.26 (ns)	79.99 ± 0.47 (ns)	81.74 ± 0.58 (ns)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$: *vs.* control. (ns) : non significant : *vs.* TBI vehicle.

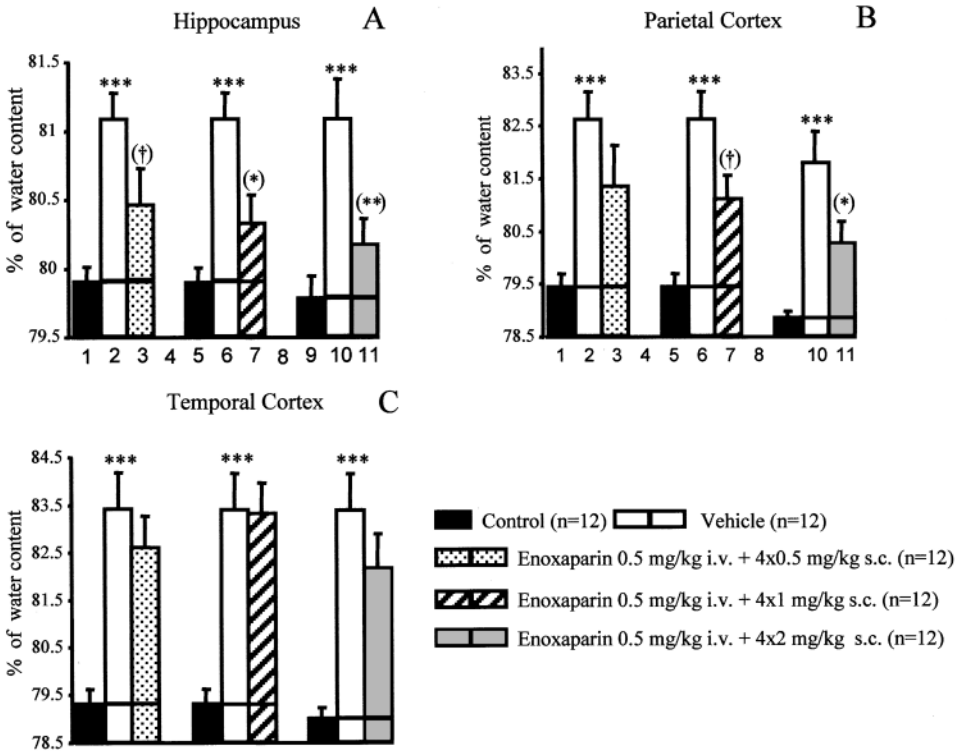

Fig. 10. Effect of enoxaparin on brain edema at 48 h after TBI, in the hippocampus (**A**), the parietal cortex (**B**), and the temporal cortex (**C**). Enoxaparin was administered at 0.5 mg/kg i.v. at 2 h after TBI followed by 0.5, 1, or 2 mg/kg s.c. at 2 h 15 min, 6, 24, and 30 h after TBI. Vehicle was administered under the same conditions. The hatched parts of histograms represent the excess water (i.e. edema) determined by comparison to the water content of control rats. Kruskal-Wallis's test for non-parametric ANOVA followed by Dunn's test for comparison between groups was used (***$P < 0.001$: *vs.* control. (†) : $0.05 < P < 0.10$, *$P < 0.05$, **$P < 0.01$: *vs.* TBI vehicle).

Study III: treatment with enoxaparin initiated 2 h after TBI at 0.5 + 4 × 0.5 mg/kg reduced the edema in the hippocampus by 53% ($P < 0.07$) and by –39% in the parietal cortex (ns). At the higher dose, 0.5 + 4 × 1 mg/kg (Fig. 10), the effect was more significant in the hippocampus (–63%, $P < 0.05$) and in the parietal cortex (–47%, $P < 0.06$), whereas no significant effect could be demonstrated in the temporal cortex.

TABLE 4. Effect of enoxaparin, at 0.5 mg/kg i.v. at 2 h, followed by 2 mg/kg s.c. at 2 h 15 min, 24 and 30 h after TBI, on the brain edema in rats as measured at 48 h after TBI. Per cent of water content in various brain areas. Means ± S.E.M.

	Hippocampus	Parietal cortex	Temporal cortex
Control ($n = 8$)	79.79 ± 0.15	78.88 ± 0.90	79.04 ± 0.19
TBI vehicle ($n = 12$)	81.10 ± 0.27 ***	81.87 ± 0.57 ***	83.47 ± 0.74 ***
TBI enoxaparin ($n = 12$)	80.49 ± 0.26 *	80.67 ± 0.62 (ns)	83.30 ± 0.70 (ns)

***$P < 0.001$: *vs.* control. *$P < 0.05$, (ns) not significant: *vs.* TBI vehicle.

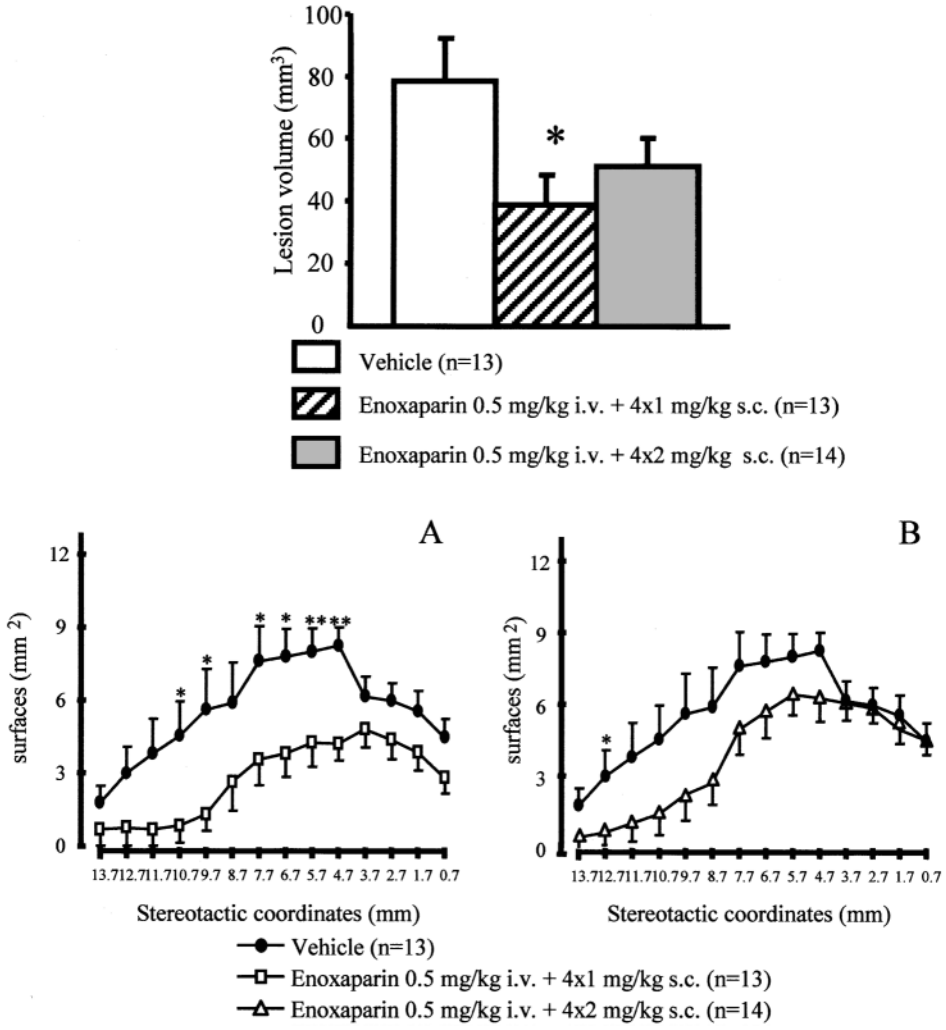

Fig. 11. Effect of enoxaparin on the lesion volume at 1 after TBI. Enoxaparin was administered at 0.5 mg/kg i.v. at 2 h post-TBI, then at 1 or 2 mg/kg s.c. at 2 h 15 min, 6, 24, and 30 h after TBI. Vehicle was administered following the same protocol to injured rats. Effect of enoxaparin on the lesion surfaces was measured at various coronal levels, at 1 week after TBI. Enoxaparin was administered at 0.5 mg/kg i.v. at 2 h after TBI, then at 1 (**A**) or 2 (**B**) mg/kg s.c. at 2 h 15 min, 6, 24, and 30 h after TBI. Vehicle was administered in accordance with the same protocol to injured rats ($n = 13$). Kruskal-Wallis's test for non-parametric ANOVA followed by Dunn's test for comparison between groups was used (*$P < 0.05$, **$P < 0.01$: *vs.* vehicle).

Brain Lesions

TBI provoked lesions to the cortex which extended around the impact site. Treatment with enoxaparin at 0.5 mg/kg + 4 × 1 mg/kg significantly reduced (by 50%, $P < 0.05$) the lesion volume (Fig. 11). This neuroprotection was significant on the lesion areas at several coronal levels**.** In contrast, when the dose was increased to 4 × 2 mg/kg the reduction of lesion size (35%) was not significant statistically.

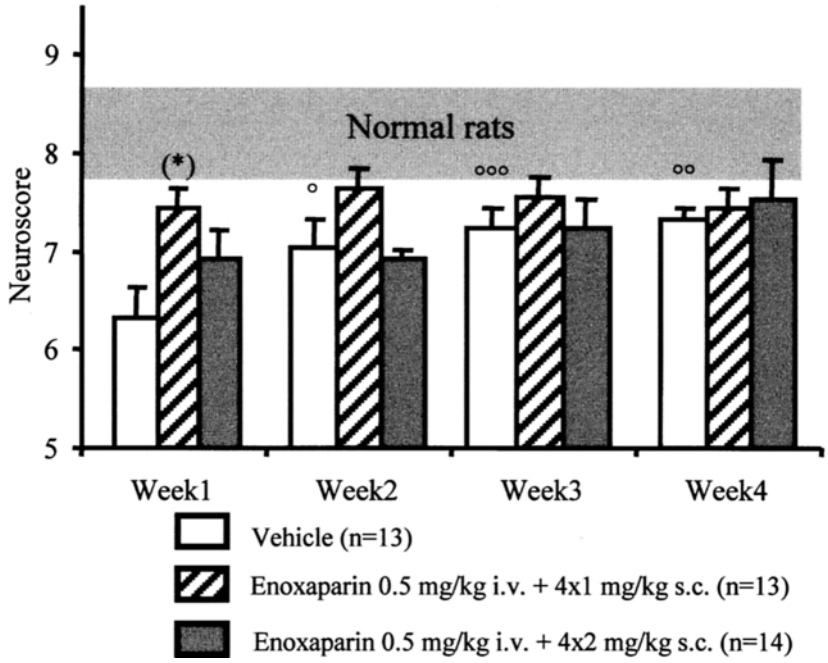

Fig. 12. Effect of enoxaparin on neurological function at 1, 2, 3, and 4 weeks after TBI. Enoxaparin was administered at 0.5 mg/kg i.v. bolus at 2 h after TBI followed by additional injections at 1 or 2 mg/kg s.c. at 2 h 15 min, 6, 24, and 30 h after TBI. Vehicle was administered to injured rats in accordance with the same protocol. Normal rats were used as controls ($n = 16$). Range of neuroscore for normal rats is represented by the gray stripe. Kruskal-Wallis's test for non-parametric ANOVA followed by Dunn's test for comparison between groups was used. $^{*}P < 0.05$: *vs.* TBI vehicle at the same time point. $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.001$: *vs.* neuroscore at 1 week).

Neurological function

Control rats had a normal neuroscore over the 4 weeks examination (Fig. 12). In contrast, compared to normal rats, TBI produced a neurological deficit that remained significant from week 1 ($P < 0.01$) up to weeks 2 ($P < 0.01$), 3 ($P < 0.001$), and 4 ($P < 0.01$). TBI produced neurological deficits in most rats. These deficits included moderate to severe disturbances of visual placing reaction; leg hanging placing reaction of anterior and posterior left paws; grasping reflex of both paws; "head tilted" placing reaction as well as abnormal postures when suspended by the tail, i.e. thorax twisting and forelimb flexion. None of the scores for any individual item was significantly lower than that of control rats, but there was a significant global neurological deficit on the summed scores. Compared to untreated rats subjected to TBI, animals treated with enoxaparin, 0.5 mg/kg i.v. + 4 × 1 mg/kg s.c. (but not at higher doses) significantly improved neuroscore at 1 week post-TBI ($P < 0.05$). Subsequently, the recovery rate of rats treated with enoxaparin was not significantly different from that in non-treated animals. However, there was no difference in the rate of recovery between treated and non-treated injured rats after one week because all injured rats substantially recovered from TBI after one week. At the higher dose of enoxaparin (0.5 mg/kg + 4 × 2 mg/kg), the neuroscore was not improved at any time point examined.

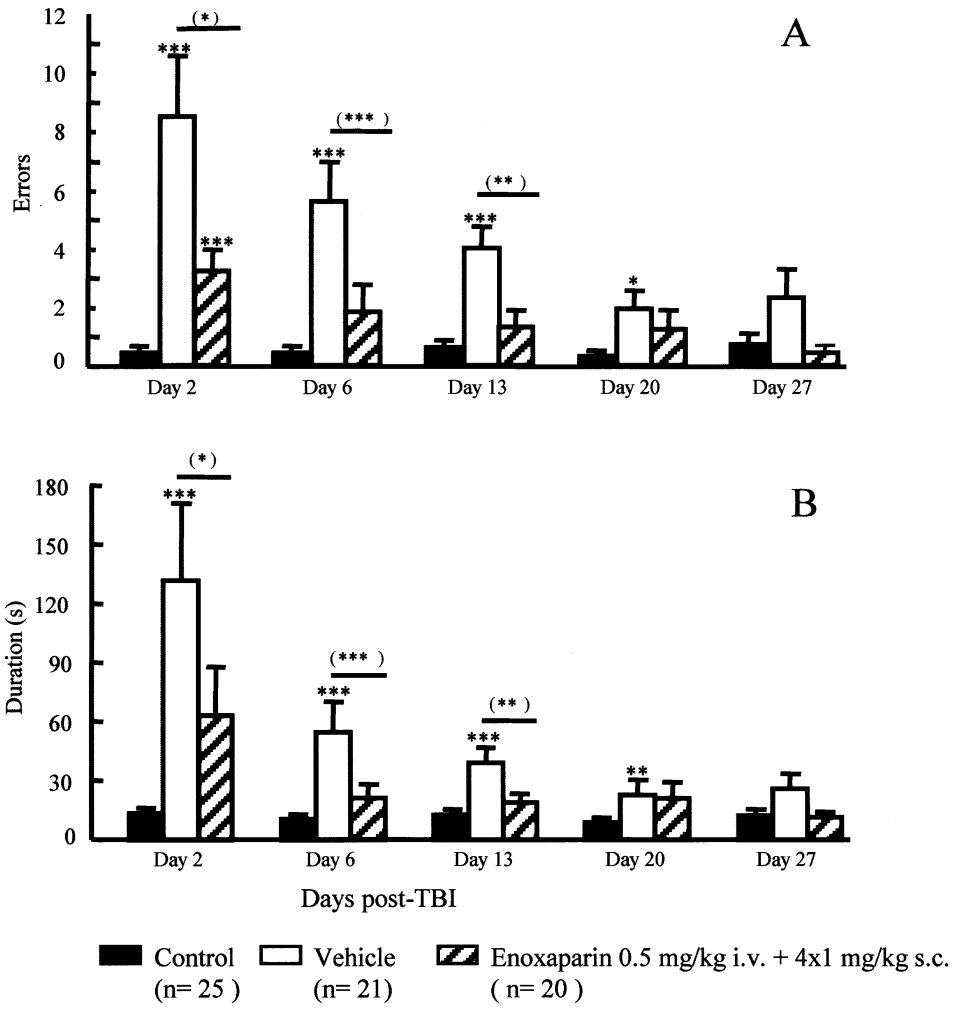

Fig. 13. Effect of enoxaparin on cognitive function evaluated in the Lashley maze. Number of errors (**A**) and duration to reach the goal box (**B**) were measured from days 2 up to 27 post-TBI. Enoxaparin was administered at 0.5 mg/kg i.v. bolus 2h post-TBI followed by 1 mg/kg s.c. at 2 h 15 min, 6, 24, and 30 h after TBI. Vehicle was administered to injured rats in accordance with the same protocol. Normal rats were used as controls. Kruskal-Wallis's test for non-parametric ANOVA followed by Dunn's test for comparison between groups was used (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* control at the same time point. (*)$P < 0.05$, (**)$P < 0.01$, (***)$P < 0.001$: *vs.* TBI vehicle at the same time point).

Cognitive function

Control rats were able to reach the goalbox with less than 2 errors during all the post-training period (Fig. 13). In contrast, 2 days post-TBI injured rats had a significant increase in the number of errors ($P < 0.001$ *vs.* control rats). In the following weeks, this impairment progressively recovered but remained significantly different from the control rats at 6 ($P < 0.001$), 13 ($P < 0.001$), and 20 days post-injury ($P < 0.05$), whereas at day 27

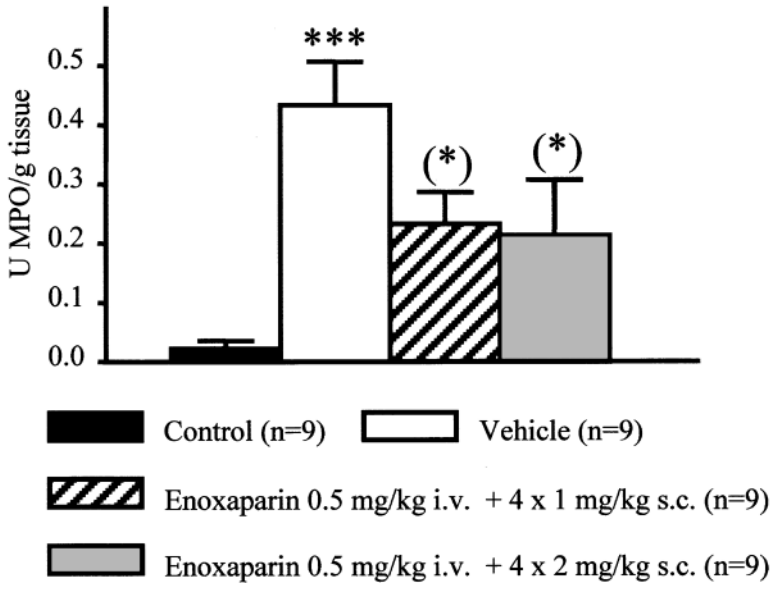

Fig. 14. Effect of enoxaparin on the increase of MPO activity at 48 h after TBI. Enoxaparin was administered at 0.5 mg/kg i.v. at 2 h post-TBI, then at 1 or 2 mg/kg s.c. at 2 h 15 min, 6, 24, and 30 h after TBI. Vehicle was administered to injured, control rats in accordance with the same protocol. ANOVA, PLSD Fischer test were used to determine the statistical differences between groups (***$P < 0.001$ *vs.* control, *$P < 0.05$: *vs.* vehicle).

no significant difference could be demonstrated between control and injured rats. In rats treated with enoxaparin at 0.5 mg/kg + 4 × 1 mg/kg the number of errors was significantly less pronounced important at days 2 ($P < 0.05$ *vs.* TBI-vehicle rats), 6 ($P < 0.001$) up to day 13 ($P < 0.01$) after TBI. At days 20 and 27 as injured rats had slightly recovered, there was no longer a difference between groups. The latency time to reach the goalbox remained constant and normal (less than 20 sec) during all the post-training period in control rats. In contrast, 2 days post-TBI this latency time was significantly increased in TBI-vehicle rats ($P < 0.001$ *vs.* control rats). At later times, this progressively decreased but still remained significant compared to control rats from days 6 ($P < 0.001$) to 13 ($P < 0.001$) and 20 ($P < 0.01$) post-TBI, and was no more significantly different from control rats at the end of the test, on day 27. In injured rats treated with enoxaparin at 0.5 + 4 × 1 mg/kg, increase in this latency was significantly less from day 2 ($P < 0.05$ *vs.* TBI-vehicle rats), 6 ($P < 0.001$) and up to day 13 ($P < 0.01$). Finally untreated rats reached the score of treated rats.

Myeloperoxidase (MPO) activity

In control rats, MPO activity was very low, whereas, in injured rats MPO activity was significantly increased at 48 h after TBI ($P < 0.001$). In contrast, enoxaparin at 0.5 + 4 × 1 mg/kg significantly reduced (45%, $P < 0.05$) the TBI-induced increase in MPO activity. At the higher dose, 0.5 + 4 × 2 mg/kg, enoxaparin significantly reduced (50%, $P < 0.05$) MPO activity (Fig. 14).

DISCUSSION

Cerebral insults of various origins seem to be made up of a small number of interconnected deleterious elements that will interact and in so doing magnify each others effects and increase the brain damage that results from the initial injury and progresses during the days following the insult. Currently, treatment of patients with a high risk of stroke is largely preventative, as illustrated by chronic use of inhibitors of platelet aggregation, such as aspirin. In acute cases of cerebral injury fibrinolytic drugs are used, or clots are removed surgically. Once the first hours of an accident have passed there is a lack of any medium term intervention policy that could break the ring of deleterious effects and reduce cerebral damage. This paper describes the activity of enoxaparin, a low molecular weight heparin, which might have potential as an agent for the treatment of cerebral damage.

Enoxaparin has shown activity against the cerebral edema in models of photothrombosis and cerebral trauma in rats. Even if administered at 18 h after insult enoxaparin significantly reduced cerebral edema in a dose-dependent manner. Edema may also be induced by a mechanical trauma, as seen in the fluid percussion model. In this model enoxaparin reduced lesion size and edema in hippocampus and in parietal cortex in a dose-dependent manner. To our knowledge this is the first time that a LMWH, enoxaparin, has been found to be active in traumatic brain injury in rats with a therapeutic time window of at least 2 h.

Clearly the reduction of brain edema can only be of benefit if it results in a reduction of the neuronal loss following an accident. In the rat TBI model enoxaparin significantly reduced brain contusion volume as observed at one week following injury. In the rat model of transient focal cerebral ischemia (tMCAO) enoxaparin, administered at 2, 5, and 24 h after ischemic insult, significantly reduced the lesion size and improved neuroscore. If administered at 5h after insult enoxaparin also reduced cortical lesion size in a dose-dependent manner. This significant reduction of the lesion size by enoxaparin was confirmed in the permanent MCAO model.

Stroke is a highly variable clinical state. The location, cause, severity and residual blood flow can all contribute to the variability in outcome. A particularly important variant is the delay in spontaneous reperfusion. The effectiveness of enoxaparin in the permanent MCAO model indicates that its effect is not dependent on reperfusion time. This finding and the fact that the effectiveness of enoxaparin is not limited to a single preclinical model, reinforce our interest in the use of enoxaparin in stroke. Since clinical cases are highly variable, the effectiveness of potential drugs should be demonstrated in different models. Demonstration of the neuroprotective effect of enoxaparin on various parameters using various protocols should be considered relevant to the clinical situation.

Finally, any relevant treatment for brain damage must result in an improvement in the quality of life for the patients. In a large proportion of surviving patients, stroke and brain trauma induce, due to functional impairment, important disability and dependence. In clinical studies functional outcome after an ischemic episode is one of the most useful parameters. Therefore, to provide a more sensitive measure of neuroprotection, we associated the evaluation of functional outcome (neuroscore) to the measure of lesion size. Enoxaparin provided both infarct reduction and functional neurological improvement for up to three weeks after insult. These complementary effects provide another strong argument for a potential activity of enoxaparin in the clinic. Moreover, in the TBI model

enoxaparin, at a dose effective against edema and lesion size, produced significant improvement in memory retention for several weeks after injury.

One of the most important factors to bear in mind when considering acute treatment of stroke is that patients are given medical care only hours after a stroke. Potential neuroprotective drugs such as enoxaparin must be efficacious with a wide therapeutic window. We looked, therefore, at the dose-effect relationship of enoxaparin administered at various time intervals after onset of ischemia or trauma. In the photothrombosis model, when treatment was started at 18 h after lesion, a significant effect of enoxaparin on edema was still observed. In tMCAO as well as in pMCAO, the therapeutic window of opportunity was, at least, 5 h. Finally in TBI, even when treatment was initiated at 2 h after insult, enoxaparin significantly reduced edema and cortical lesions and improved functional recovery (i.e. neuroscore and memory retention). We chose a degree of severity corresponding to a clinical mild TBI in order to be able to maintain a control group of animals alive, as severe lesions produced a high mortality. This may explain why recovery was similar for both groups at the end of the observation period. What is important is that animals treated with enoxaparin recovered more rapidly. All these results are consistent with a suitable therapeutic window of opportunity for enoxaparin in stroke and/or in brain trauma patients.

At a dose active against edema in the photothrombotic model of cerebral ischemia, enoxaparin produced only minor changes in bleeding time of rats. It did not induce major cerebral hemorrhage, as seen with heparin in the tMCAO study. To confirm that this dose was safe, the APTT was determined after treatment with enoxaparin in both non-ischemic and ischemic rats. In either group of rats, enoxaparin induced a transient elevation of APTT (around 2 fold increase compared to controls) within 10 min after its injection. The APTT values return to normal within 1 h post-injection. This slight and reversible elevation of APTT, in addition to the fact that no macroscopic hemorrhage was observed, suggests that enoxaparin is neuroprotective at a non-hemorrhagic dose. At the same dose heparin induced such a pronounced prolongation of APTT that it could not be measured. These results suggest that the increased risk of bleeding following enoxaparin administration is low.

One can hypothesize that improvement of functional recovery is mainly a result of the reduction of edema and of lesion size. But how does enoxaparin work to reduce edema and lesion volume in stroke and trauma models in rats? The first mechanism of action that could explain, at least in part, the neuroprotective effect of heparin and LMWH, especially enoxaparin, in stroke could certainly be the anticoagulant/antithrombotic activity of this class of drugs. Coagulation of blood is one of the key players in the pathology of acute neurodegenerative diseases, either as the primary causative mechanism, as in thromboembolic stroke, or as a secondary detrimental effect as seen in traumatic injury. In both cases blood clots may block the vessels and cause ischemia. Enoxaparin could be producing its anti-edematous action by a number of mechanisms. First, as suggested above, the rose bengal lesion causes endothelial cell injury by producing reactive oxygen species that stimulate thrombin, which in turn induces permeability changes and thrombus formation. Administration of enoxaparin at 18 h after lesion might indicate that the thrombus might still be evolving even 18 h after its creation, and enoxaparin could be preventing thrombus extension into surrounding tissues with new areas of edema being formed. This phenomenon of clot extension, and spreading of the area of blood stasis has been observed in the clinic, although it has not been documented in the photothrombotic lesion. Second-

ly, thrombin and other thrombogenic factors have been shown to disrupt the blood brain barrier and induce toxic effects on vascular endothelial cells (52), and thrombin infusions have been shown to provoke cerebral edema in the rat (46). Inhibition of thrombin activation would thus tend to protect the blood/brain barrier. Enoxaparin, by s.c. administration, has been shown to inhibit thrombin generation in human volunteers, and be resistant to inactivation by platelet factor 4 released from activated platelets (6). Thus, enoxaparin could be anti-ischemic in rat tMCAO due to its antithrombotic activity.

In experimental TBI, blood accumulation in the subarachnoid space (70) and microthrombus formation (48), caused by petechial hemorrhages, occur within hours after experimental trauma. During the first hour after injury the damage is limited to the immediate area of contusion, and afterwards, within 6 h it extends to the peripheral areas (48). Microthrombus formation has also been observed in brains from head trauma patients (44). In addition platelet aggregation has been described in hemorrhagic regions following TBI in rats (23). Blood coagulation is known to be one of the key players in the pathology of acute neurodegenerative diseases, either as the primary event (embolic stroke), or as a secondary injury (traumatic injury). In both cases clotting causes blockade of vessels leading to ischemia. Taken together, these data strongly suggest that anticoagulant properties of enoxaparin could be involved in the efficacy of enoxaparin in our models. However, the risk with an anticoagulant drug is the hemorrhagic transformation. With the emphasis on anti factor Xa rather than factor IIa activities, enoxaparin shows much lower propensity for bleeding than heparin (75% less; 12) for the same anti-Xa activity. Pratt et al. (57) measured hemoglobin content in brain samples from rats subjected to photothrombosis and treated with enoxaparin at doses similar to ours. They observed no increase in hemoglobin content by enoxaparin, indicating absence of hemorrhage. These data, in addition to our findings of no worsening in the outcome and absence of intradural or extradural hematomas, indicated that at the doses used enoxaparin is safe.

In addition to their anticoagulant activity, unfractionated heparin and LMWHs have other pharmacological properties that may contribute to neuroprotection. The pathophysiological cascade of events that have been described after acute injury to the brain has provided various pharmacological targets (for review, see 50). Current knowledge of unfractionated heparin or LMWHs suggests that these targets include: (i) excitotoxicity, (ii) free radical production (iii), cerebral inflammation and (iv) trophic factors.

(i) Excitotoxicity via excessive release of the excitatory amino acid glutamate has been described in ischemic and traumatic brain injury and is a relatively transient phenomenon (for review, see 54). Subsequent activation of the glutamate receptors associated with excessive neuronal depolarization and ionic homeostasis failure, leads to an increase in intracellular Ca^{2+}. This accumulation of Ca^{2+} occurs within hours and remains elevated for 2 to 4 days after injury (28,59). *In vitro* heparins and enoxaparin have been shown to reduce the release of intracellular Ca^{2+} after stimulation of the IP_3 second messenger system (39). In addition, unfractionated heparin was shown to block the glutamate-induced intracellular Ca^{2+} release in Purkinje cells (39,67). Therefore in our model a possible interaction of enoxaparin with the internal stores of Ca^{2+} cannot be excluded.

(ii) Free radical production has been widely reported in models of brain trauma (35) or cerebral ischemia (15), and various antioxidant strategies have been found to be active in experimental models of neurotrauma (for reviews see 50,62). *In vitro*, superoxide anion generated by stimulated PMNs is reduced by heparin (4), and *in vivo* heparin increased the plasma superoxide dismutase activity and reduced the edema provoked by paw ischemia

(56). Another reactive oxygen species, nitric oxide (NO) is strongly implicated as a key actor in the pathophysiology of CNS injuries such as cerebral ischemia or TBI. The production of NO results from activation of 3 isoforms of the nitric oxide synthase (NOS): constitutive (neuronal nNOS and endothelial eNOS), and inducible (iNOS). In experimental TBI, iNOS activity has been reported to occur after a TBI (58,74,78), and we recently demonstrated that selective iNOS inhibition strongly reduced the brain edema in our model (78). Nitrate and nitrites, indicators of NO production, have also been found in the CSF of head trauma patients and have been associated with the severity of the injury (16). Recently, heparin was found to inhibit induction of iNOS provoked by cytokines in endothelial cells by modulation of iNOS mRNA (11). Upchurch et al. (73) also demonstrated that heparin reduced NO production in endothelial cells by modulation of eNOS mRNA. Although no work has as yet been reported with enoxaparin, these data nevertheless suggest that possible interaction of enoxaparin with reactive oxygen species production should be considered.

(iii) Cerebral inflammation. The fact that enoxaparin has been shown to inhibit P-selectin mediated platelet/neutrophil interaction in a plasma medium (20), and to reduce neutrophil accumulation in the myocardial infarct in a canine model of cardiac ischemia with reperfusion (47) could also contribute to its neuroprotective activity. Unfractionated heparin was ineffective under the same experimental conditions, although binding of heparin to L and P selectins and inhibition of acute inflammation have already been described (53). Enoxaparin also inhibits complement activation and neutrophil elastase release in a model of extracorporal circulation imitating cardiopulmonary bypass (31), where it appears to accelerate C1 inhibitor activity to a greater degree than heparin. Lastly enoxaparin has been shown to reduce human vascular endothelial cell permeability in culture (1) by mechanisms still poorly understood. Thus enoxaparin may be able to reduce the damage inflicted on the vascular endothelium in a number of ways following an ischemic insult, thus reducing the uncontrolled passage of water out of vessels. One or a number of these properties, acting in synergy, provide enoxaparin with a potentially very interesting antiedematous action.

The use of heparins for the treatment of stroke remains controversial, due to their potential for increasing secondary hemorrhage. However, the antiedematous properties of enoxaparin, combined with its reduced anti-factor IIa activity and, therefore, lower potential for hemorrhage, as well as its effectiveness by s.c. administration and its longer half-life, make enoxaparin an attractive candidate for its further evaluation in the treatment of severe cerebral edema.

Endothelial leukocyte adhesion has been described in human disease (8). In experimental trauma leukocyte infiltration into the cerebral parenchyma due to hemorrhage and/or endothelial disruption has been described (60,64). PMN infiltration has been observed in lesions with concomitant BBB damage (64), although maximal BBB permeability occurs prior to the peak of neutrophil ingression suggesting that neutrophils may not mediate BBB permeability (79). Leukocytes may aggravate cerebral injury by prolonging or intensifying an existing ischemic condition. This effect may be primarily due to physical microvascular occlusion (17) or release of reactive oxygen species (80). Indeed, neutrophil depletion was shown to reduce TBI-induced hyperemia (72). However, whenever a positive correlation between early PMN infiltration and edema development has been shown, it depended on the severity of the injury (18,60). Also neutrophil depletion failed to reduce TBI-induced brain edema (72). Leukocyte rolling and adhesion to endo-

thelial cells depends on the expression of specific cell adhesion molecules like selectins. Endothelial E-selectins are present in endothelial cells, and platelet P-selectins are expressed by both endothelial cells and platelets (for review, see 14). Endothelial P-selectins can be rapidly redistributed to the cell surface after an acute brain injury and are present early within and around the injured tissue (27). Recently, P-selectin blockade has been shown to reduce myeloperoxidase (MPO) activity (reflecting neutrophil infiltration) post-TBI together with a trend toward behavioral improvement (32). This study indicates that enoxaparin is capable of inhibiting TBI-induced MPO activity by 50% in rats and demonstrates that PMN leukocyte infiltration resulting from an injury to the brain can be significantly reduced by enoxaparin. Futhermore, these data strongly suggest that enoxaparin has antiinflammatory properties. Reduction of MPO activity as well as neurological improvement was also seen with a LMWH and heparin in experimental cerebral ischemia (82,83). Interestingly, enoxaparin was shown to reduce both neutrophil accumulation and platelet aggregation in the myocardial infarct in a canine model of ischemia-reperfusion (47), and to modulate P-selectin mediated platelet-neutrophil adhesion in a plasma medium (20). Another component in inflammation is the complement system that has also been involved in TBI (for review, see 66). Activation of the complement cascade has been described around the lesion following experimental TBI (5), and inhibition of the classic pathways of complement activation has been shown to reduce the TBI-induced MPO activity in the lesioned hemisphere (40). Complement inhibition with a C1-esterase inhibitor also reduced the brain infarction in a model of photothrombosis (36). Interestingly, Gikakis et al. (31) reported that enoxaparin inhibits complement activation in a model of cardiopulmonary bypass. Therefore, these data support the hypothesis that in our model enoxaparin could be neuroprotective by acting on cerebral inflammation.

(iv) Trophic factors: The use of various neurotrophic factors has provided neuroprotection in different models of brain injury including cerebral ischemia (for review, see 50). Lafont et al. (43) reported that heparin enhanced axonal outgrowth in cell culture. Likewise fibroblast growth factor requires the presence of heparin to stimulate dopaminergic neurons growth *in vitro* (51). The activity of aFGF, whether endogenous or exogenous, is potentiated by heparins, including enoxaparin (65). Moreover, Barbeito, et al. (personal communication) have shown that enoxaparin possesses a trophic effect mediated by spinal astrocytes on motoneurons in culture. These data suggest, therefore, a beneficial role of enoxaparin in pathological conditions involving growth factors, such as following ischemic or traumatic brain injury.

Therefore, in addition to its anticoagulant activity, enoxaparin possesses different mechanisms of action, which alone or in synergy may be involved in its neuroprotective profile.

In conclusion, enoxaparin, a LMWH, protects rats from neurobehavioral sequelae of an ischemic insult or a TBI. The measured outcomes, i.e. cerebral edema, brain contusion, as well as motor and memory impairments, were significantly improved by enoxaparin at doses that do not induce bleeding. These data also indicate that enoxaparin is likely to have a large window of therapeutic opportunity and a wide neuroprotective profile. It could, therefore, be a useful candidate for the treatment of acute neurodegenerative disease.

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