Antithrombin III and Enoxaparin Treatment Inhibit Contusion-Triggered Cell Death, Inflammation, Hemorrhage and Apoptosis after Severe Traumatic Brain Injury in Rats

Antitrombin III ve Enoksaparin Tedavisi, Sıçanlarda Oluşturulmuş Travmatik Beyin Yaralanması Modelinde Hücre Ölümü, İnflamasyon, Hemoraji ve Apoptozu Azaltmaktadır

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ABSTRACT

AIM: In this study, we aimed to show the neuroprotective effects of AT III and Enoxaparin after severe traumatic brain injury.

MATERIAL and METHODS: The animals were divided into four groups as Group 1; control group, Group 2; trauma group, Group 3; AT III group and Group 4; Enoxaparin group. Severe trauma was performed by the weight dropping technique. These animals were killed 48 hours after injury. Histopathological and immunohistochemical analysis were performed. Specimens were graded for cell death, inflammation, hemorrhage and apoptosis.

RESULTS: The control group showed normal ultrastructure of brain tissue. Trauma produced obvious damage. 8 rats (80%) in the trauma group demonstrated minimal inflammation and grade 5 cell death. Trauma increased hemorrhage and apoptosis scores to statistically significant levels (p<0.001). Enoxaparin was found to reduce neuronal cell death but not as effectively as AT III. A statistically significant difference was observed between the AT III and Enoxaparin group according to inflammation grades. Significant antiapoptotic properties of AT III were observed while hemorrhage was more common in the Enoxaparin group.

CONCLUSION: Anticoagulants such as AT III and enoxaparin are promising drugs in the treatment of traumatic brain injuries.

KEYWORDS: Traumatic brain injury, Antithrombin III, Enoxaparin, Cell death, Hemorrhage, Apoptosis

ÖZ

AMAÇ: Çalışmamızda, travmatik beyin hasarı sonrasında verilen AT III ve Enoksaparin'in nöroprotektif etkinliğini göstermeyi amaçladık.

YÖNTEM ve GEREÇ: Denekler 4 gruba ayrıldı: Grup 1 kontrol grubu, grup 2 travma grubu, grup 3 AT III grubu ve grup 4 Enoksaparin grubu. Ağırlık düşürme tekniği ile ağır kafa travması oluşturuldu. 48 saat sonra denekler sakrifiye edildi. Histopatolojik ve immünohistokimyasal değerlendirmeler yapıldı. Spesimenler hücre ölümü, inflamasyon, apoptoz ve hemoraji açısından derecelendirildi.

BULGULAR: Kontrol grubundaki denekler normal beyin dokusu ile uyumlu iken, travma grubundaki deneklerde belirgin hasar saptandı. Travma grubundaki 8 denekte (%80) minimal inflamasyon ve grade 5 hücre ölümü görüldü. Nöronal hücre ölümü değerleri göz önüne alındığında AT III tedavisinin, Enoksaparin tedavisinden daha etkili olduğu görüldü. İnflamasyon skorları değerlendirildiğinde AT III ve Enoksaparin grupları arasındaki fark istatistiksel olarak anlamlı bulundu (p<0,001). AT III tedavisinin antiapoptotik etkinliği daha belirgin iken Enoksaparin grubunda hemoraji daha sık görüldü.

SONUÇ: AT III ve Enoksaparin gibi antikoagülan ilaçlar travmatik beyin yaralanmasında umut vaat eden ajanlardır.

ANAHTAR SÖZCÜKLER: Travmatik beyin yaralanması, Antitrombin III, Enoksaparin, Hücre ölümü, Hemoraji, Apoptoz

INTRODUCTION

The loss of function after traumatic brain injury (TBI) results from both the primary mechanical insult and the subsequent, multifaceted secondary response. Unlike the primary mechanical trauma, which is immediate and beyond therapeutic management, the secondary injury occurs over the hours and days after the TBI, further exacerbating tissue loss and functional impairments. The primary injury results from the deformation of gray and white matter leading to the distortion and/or disruption of cell membranes and release of intracellular contents. The evolution of secondary damage represents a cascade of biochemical and molecular events that collectively mediate cell damage. Some of the earliest findings in the injured brain include damage to axons, excessive neuronal activity, widespread changes in neurotransmitter levels, altered cerebral blood flow, hemorrhage and disruption of the blood-brain barrier. Coincident with these events are physiologic disturbances including hypotension, hypoxemia, elevated intracranial pressure, decreased cerebral perfusion pressure, edema, ischemia and silent seizures (1,12-14,19).

Cerebral ischemia is one of the major etiologic factors acting on the formation of brain injury following cerebral trauma. Although vasospasm certainly occurs in some cases, intravascular microthrombosis is suspected of being a potential cause of ischemia in TBI. Local hypercoagulation and release of procoagulants into the systemic circulation cause consumption of clotting factors and stimulation of fibrinolysis. The resulting coagulopathy is a common finding in severe TBI (6,7,12,13).

Antithrombin III (AT III) is the most important physiological inhibitor of blood coagulation. Besides its role in coagulation, AT III has also been shown to have marked anti-inflammatory and anti-platelet effects. Furthermore, enoxaparin, a low molecular weight heparin (LMWH) that is widely used for anticoagulation, has also been reported to have neuroprotective effects in experimental models of traumatic brain and spinal cord injuries (2,5,14-17).

Since intravascular microthrombosis is associated with adverse outcomes in TBI, we hypothesized that inhibition of coagulation in the early course of trauma might reduce neuronal tissue loss after TBI. We therefore aimed to investigate the potential therapeutic effects of AT III and enoxaparin in a contusion model of TBI in rats.

MATERIAL AND METHODS

This experimental study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Principles of Laboratory Animal Care, National Institutes of Health, publication no. 86-23, revised 1985. The protocols were approved by the Baskent University, Ankara, Turkey) Institutional Animal Care and Use Committee for ethics in animal experiments.

Animal Preparation

A total of 40 adult male Sprague Dawley rats weighing 200-290 grams were used in this study. Each rat was given at least 1 day to acclimate to its new surroundings, so as to reduce the effects of stress from transportation. Temperature was kept at

Table I: Histopathological Grading System for Quantitative Evaluation of TBI

Neuronal cell death (%)	Inflammation	Hemorrhage
0 (normal neurons)	0	0
1 (<1% of neurons dead)	Minimal	1
2 (1-5% of neurons dead)	Mild	2
3 (6-15% of neurons dead)	Moderate	3
4 (16-30% of neurons dead)	Severe	4
5 (>30% of neurons dead)	Very severe	5

Table II: Scores of Trauma Parameters

	Control Mean±SD Median	Trauma Mean±SD Median	AT III Mean±SD Median	Enoxaparin Mean±SD Median	P
Cell Death	0.0±0.00 0.0	4.6±0.84 5.0	3.8±0.63 4.0	4.4±0.51 4	<0.001
Inflammation	0.0±0.00 0.0	0.8±0.42 1.0	0.3±0.48 0.0	0.5±0.52 0.5	<0.05
Hemorrhage	0.0±0.00 0.0	3.0±1.33 3	0.4±0.51 0.0	1.3±1.70 0.0	<0.01
Apoptosis	0.0±0.00 0.0	4.0±0.00 4.0	2.4±0.51 2.0	2.8±0.91 2.5	<0.001

20°C±2°C and humidity was 50% ± 10% at the animal facility. Rats were fed ad libitum and remained under 12-hour light-dark conditions.

Surgical Procedure and Head Trauma

General anesthesia was achieved with an intraperitoneal (ip) injection of ketamine 60 mg/kg (Ketalar®, Pfizer, Turkey) and xylazine 2% 10 mg/kg (Rompun®, Bayer, Turkey). The animals were ventilated spontaneously; body temperature was maintained at 37°C using heated gel packs, the femoral artery was cannulated and arterial blood samples (PaCO2 and PaO2) were taken from each animal for blood gas analysis. Preoperative doses of the antibiotic enrofloxacin (10 mg/kg; Baytril K, Bayer, Turkey) was applied subcutaneously to all rats. The frontal region was prepared with povidone-iodine (Betadine®, Pharma, Turkey), and the scalp was shaved. Briefly, the rats were positioned in the prone position. The heads of the animals were fixed in a frame to prevent movement. A skin incision was made to expose the frontal part of the skull

before the force was applied by the weight-drop method (1). A cylindrical Plexiglas® tube (100 cm long and inner diameter of 1 cm) was used to guide the weight during falling onto the cranium. The tube was positioned at a 90 degree angle in front of the bregma, and a cylindrical weight of 150 g was dropped through it. The calculated value of the force applied to the cranium corresponded to 1.47 Newton meter (N m). The surgical site was sutured in layers, and an antibacterial spray (Pyedif®) (Sanofi-Dif, Turkey) was applied onto the skin incision. Immediately after surgery, all rats were given lactated Ringer solution (3 cc) subcutaneously and postoperative care was provided.

Experimental Groups

The animals were randomly allocated into 4 groups of 10 rats each, as follows. Group I (control group): Trauma was not applied to this group. Cerebral tissue and blood samples were used to obtain the basal values. Group II (trauma group): Rats were subjected to severe closed head injury (150 g/m/cm²)
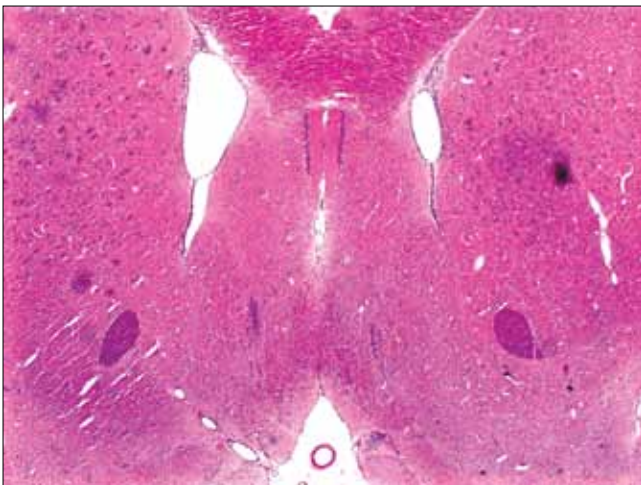

Figure 1: Brain tissues showed no specific morphologic evidence of injury in control group (HEx20).
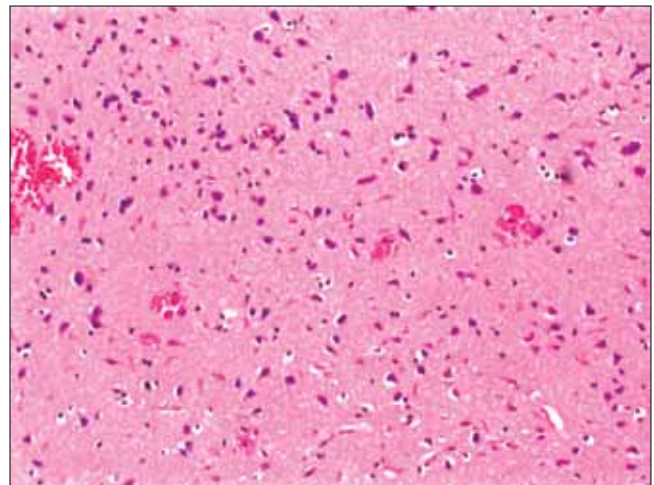

Figure 3: Dead neurons (grade 4) and mild inflammatory infiltrate in Trauma group (HEx20).
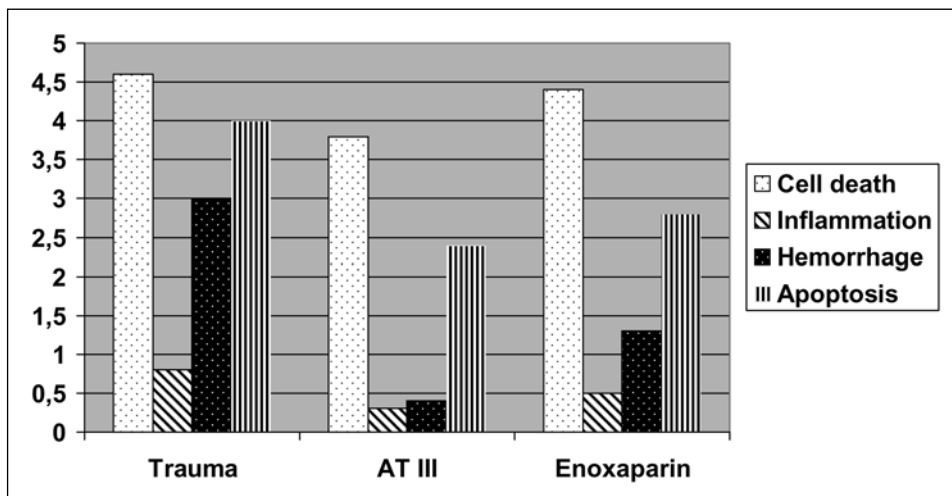

Figure 2: Bar graph showing histopathological and immunohistochemical scores of trauma parameters using a quantitative grading system.
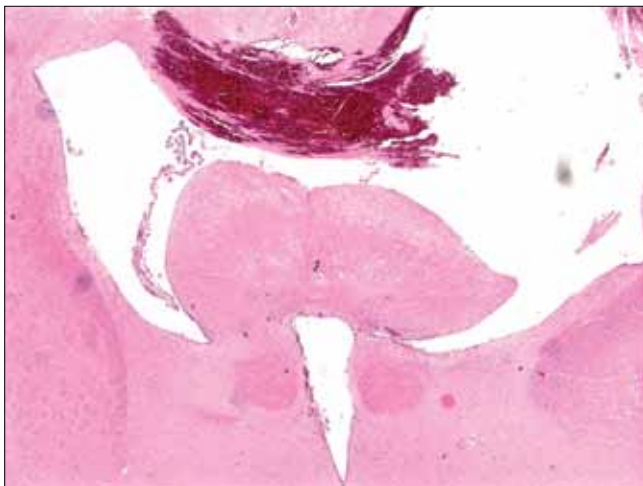
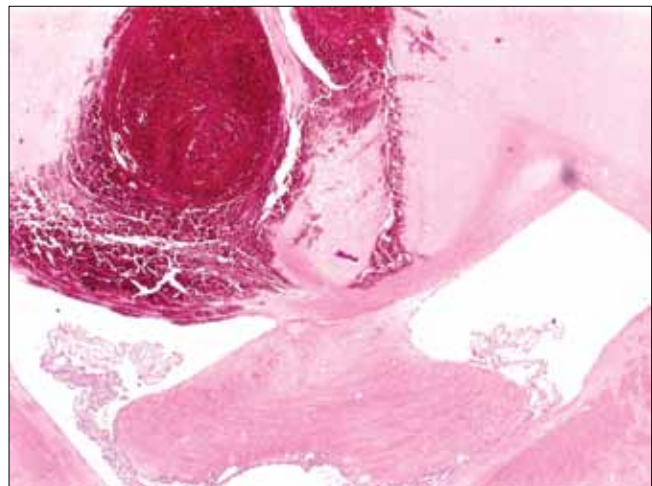**Figure 4:** Intraparenchymal hemorrhage (grade 3) in the corpus callosum region in ATIII group (HEx20).

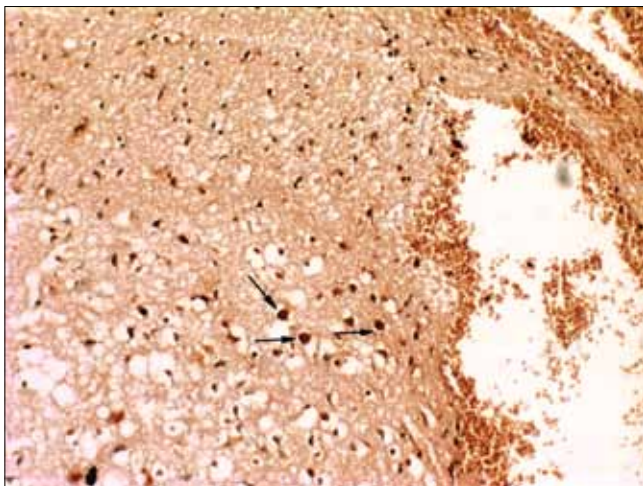**Figure 5:** Massive hemorrhage (grade 4) in Enoxaparin group (HEx20).

Figure 6: Apoptotic cells (arrows) – grade 3 in ATIII group (TUNELx200).
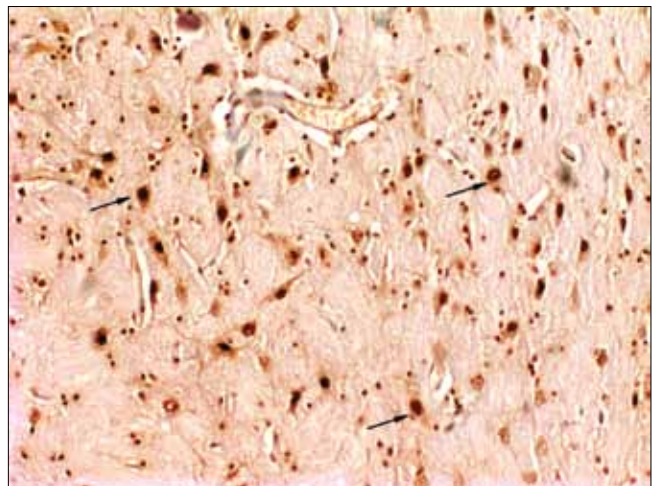

Figure 7: Apoptotic cells (arrows) – grade 4 in Enoxaparin group (TUNELx200).

but no medication was administered to the rats. Group III (antithrombin III group): Rats were subjected to severe closed head injury (150 g/m/cm²) and received an intraperitoneal injection of antithrombin III (250 IU/kg) immediately after the injury and a second dose 24 hours later. Group IV (enoxaparin group): Rats were subjected to severe closed head injury (150 g/m/cm²) and enoxaparin was administered as an intravenous (iv) bolus at 0.5 mg/kg at 15 minutes after TBI and additional subcutaneous (sc) injections of enoxaparin, 1 mg/kg, were administered at 30 minutes and at 6, 24, and 30 hours after TBI.

Animals in all groups were killed by decapitation on the second day postoperatively. After transcardiac perfusion with 10% formalin, the brains were removed en bloc and immersed overnight in 10% formalin. The injured cortex was sectioned for immunohistochemical and histological examination.

Histological Examination

The brains were fixed in 10% formalin and embedded in paraffin. Coronal slices were obtained at the level of dorsal hippocampus. Hematoxylin and eosin-stained slides containing frontoparietal cortex, corpus callosum and hippocampus were evaluated for neuronal cell death, inflammation and hemorrhage. Necrotic cells were identified by apyknotic nucleus or no nucleus, along with a swollen, eosinophilic cytoplasm. The slides were scored semiquantitatively as shown in Table I.

Immunohistochemical Analysis (TUNEL Staining and Evaluation of Apoptosis)

The In Situ Cell Death Detection Kit (Roche) was used to demonstrate apoptosis in coronal sections of the brains. Paraffin-embedded samples were deparaffinized and re-

hydrated in decreasing concentrations of alcohol. Samples were first treated with proteinase K (20 mg/mL) for 30 minutes (min.) at room temperature to increase permeability of the samples. The samples were then treated with terminal deoxynucleotidyl transferase (TUNEL reaction mixture), which catalyzes polymerization of nucleotides to free 39-OH DNA ends in a template independent manner and is used to label DNA strand breaks, in a humidified chamber for 1 hour at RT. Sections were then incubated with Converter-POD for 30 min. and then peroxidase substrate (DAB) was applied for 3-6 min to develop color. Next, they were counterstained with methyl green and mounted. Between steps, the slides were washed in PBS.

The number of total neurons was counted by taking series of non-overlapping pictures and then analyzing them with a software package (Image J). In this study, only TUNEL-positive cells displaying nuclear karyorrhexis and minimal cytoplasmic change were defined as apoptotic cells. The percentage of apoptotic neurons was calculated from apoptotic neurons to all neuron ratios in all brain areas and scored as follows: grade 0, 0%; grade 1, 1 to <5%; grade 2, 5 to <25%; grade 3, 25 to <50%, and grade 4, ≥50%.

Statistical Analysis

Data are presented as mean ± standard deviation (SD) and were analyzed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Statistical comparisons between groups were tested with the Kruskal-Wallis test followed by the Dunn test. A p value of <0.05 was considered statistically significant.

RESULTS

The control group showed normal ultrastructure of brain tissue (Figure 1). Trauma produced obvious damage. Treatment groups exhibited statistically significant neuroprotection (Figure2). Quantitative grading was evaluated based on the changes of trauma parameters such as cell death, inflammation, hemorrhage and apoptosis (Table II).

Cell death

Control group demonstrated normal neuronal structure. In the trauma group, 8 rats (80%) were scored as grade 5. In the AT III treatment group, 3 rats (30%) were scored as grade 3 while 6 rats (60%) were scored as grade 4. Only 1 rat (10%) was scored as grade 5. Enoxaparin-treated 6 rats (60%) were scored as grade 4 while the rest were scored as grade 5. Trauma increased cell death to statistically significant levels (p<0.001). Enoxaparin was found to reduce neuronal cell death but not as effectively as AT III. Statistically significant difference was noted between AT III and Enoxaparin groups (p<0.05).

Inflammation

No inflammatory change was not observed in the control group. 8 rats (80%) in the trauma group demonstrated minimal inflammation (Figure 3). AT III treated 3 rats (30%) showed minimal inflammation while 5 rats (50%) in the Enoxaparin group also showed minimal inflammation. There

was statistically significant difference between AT III and Enoxaparin treated rats (p<0.05).

Hemorrhage

Control group did not demonstrate any hemorrhage. Trauma increased hemorrhage scores to statistically significant levels (p<0.01). The best results were observed in the AT III group (Figure 4). 4 rats (40%) showed only grade 1 hemorrhage. 6 rats (60%) both in the AT III and Enoxaparin group did not show any hemorrhagic areas. In the Enoxaparin treated group, 3 rats (30%) and 1 rat (10%) showed grade 3 and grade 4 hemorrhage, respectively (Figure 5). Statistically significant difference was observed between AT III and Enoxaparin treated rats (p<0.05).

Apoptosis

Apoptosis was not observed in the control group. Trauma increased apoptosis scores to statistically significant levels (p<0.001). All rats (100%) demonstrated grade 4 apoptosis. However, in the AT III treatment group, 6 rats (60%) were scored as grade 2 while the others were scored as grade 3 (Figure 6). In the Enoxaparin treatment group, 5 rats (50%) showed grade 3 and grade 4 apoptosis (Figure 7). Statistically significant difference was observed between AT III and Enoxaparin groups (p<0.05).

DISCUSSION

Trauma is known to release tissue thromboplastin, of which the brain is an especially rich source. Thromboplastin stimulates intravascular coagulation locally in the brain (12, 13). If enough thromboplastin is released, uncontrolled activation of clotting factors or DIC results (7). This syndrome is characterized by systemic coagulopathy, along with widespread intravascular coagulation and hemorrhage after clotting factors are consumed. Stein et al. showed a strong link between intravascular microthrombosis and neuronal death after brain trauma in humans (12,13). Tissue factor and other coagulants released after TBI may enter the systemic circulation, thereby initiating systemic coagulopathy (7,12,13).

Blood coagulation disorders frequently complicate head trauma in more than 60% of patients with severe TBI. Coagulopathy presents an obvious risk of hemorrhage after TBI and has been implicated in delayed posttraumatic bleeding and poor clinical outcome (15). Since coagulopathy plays a major role in the pathogenesis of ischemic-traumatic brain injury, we decided to use AT III and Enoxaparin as the therapeutic agents in our study. We have evaluated the presence of neuronal cell death, apoptosis, inflammation and hemorrhage after severe cortical contusion injury following treatment with AT III and Enoxaparin.

We used the cortical contusion injury model in this model but did not perform craniectomy prior to trauma, in contrast to classical studies, in order to mimic the real human traumatic brain injuries. This model is inexpensive, simple and easy to use. Histopathological changes in the present study were evaluated by light microscopy. Immunohistochemical analysis for apoptosis was evaluated by TUNEL staining.

Antithrombin III is one of the most important natural coagulation inhibitors, also exhibiting beneficial effects in both sepsis and blunt head injury (5,12,13). The literature on antithrombin treatment in severe head injury is not extensive. Hoots et al. found a significant correlation between the severity of head injury and the degree of abnormality in coagulation parameters (4). Ungerstedt et al. indicated that the clotting onset time may be a clinically relevant variable with prognostic value (15). However, Grenander et al. could not detect any obvious favorable influence of antithrombin treatment, although there was a slightly greater reduction in the levels of the hypercoagulation tests (3). In our study, AT III treatment significantly reduced the cell death, inflammation and apoptosis after severe TBI.

In addition to their anticoagulant activity, 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 including excitotoxicity, free radical production, cerebral inflammation and trophic factors (14). Stutzmann et al demonstrated a neuroprotective profile of enoxaparin in an experimental model of TBI in rats (14). Moreover, Wahl et al. reported that enoxaparin reduces brain edema, cerebral lesions, and improves motor and cognitive impairments after an experimental TBI model (16). In this experimental TBI model, we also showed that enoxaparin reduced cell death, inflammation and apoptosis but this decline was not as effective as AT III treatment.

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 (6,7,12,13,19). Taken together, these data strongly suggest that anticoagulant and neuroprotective properties of enoxaparin and AT III could be suspected for the significant benefit in our model.

The risk with an anticoagulant drugs is hemorrhagic transformation. With the emphasis on anti factor Xa rather than factor IIa activities, enoxaparin shows much lower propensity for bleeding than heparin for the same anti Xa activity. Pratt et al. observed absence of hemorrhage by enoxaparin in an experimental stroke model (11). Stutzmann et al. also indicated enoxaparin is safe that at the doses used (14). However, enoxaparin treatment significantly increased the rate of hemorrhage seen after TBI as compared to that of the AT III treatment in our study.

TBI disrupts tissue homeostasis resulting in pathological apoptotic activation (9,10). Larner et al. reported that the caspase-12-mediated apoptotic pathway might play a role in rat TB (8). Wennersten et al. concluded that apoptosis occur after experimental trauma and indicated the activation of different apoptotic pathways at different times after trauma (18). In this study, both AT III and Enoxaparin treatment diminished TUNEL- positive apoptotic neurons after severe TBI. A statistically significant difference was noted between the AT III and Enoxaparin groups.

CONCLUSION

By exhibiting neuroprotective effects on the brain ultrastructure even after severe TBI, anticoagulants such as AT III and enoxaparin are promising drugs in the treatment of these injuries.

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