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RESEARCH ARTICLE

Dose-dependent neuroprotective effect of enoxaparin on cold-induced traumatic brain injury

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Abstract
Recent evidence exists that enoxaparin can reduce brain injury because of its anticoagulant activity. To investigate the potential therapeutic effect of enoxaparin on cold-induced traumatic brain injury, at 20 minutes after modeling, male BALB/c mouse models of cold-induced traumatic brain injury were intraperitoneally administered 3 and 10 mg/kg enoxaparin or isotonic saline solution. Twenty-four hours later, enoxaparin at 10 mg/kg greatly reduced infarct volume, decreased cell apoptosis in the cortex and obviously increased serum level of total antioxidant status. By contrast, administration of enoxaparin at 3 mg/kg did not lead to these changes. These findings suggest that enoxaparin exhibits neuroprotective effect on cold-induced traumatic brain injury in a dose-dependent manner.

Key Words: *nerve regeneration; neuroprotection; traumatic brain injury; cold-induced brain injury; enoxaparin; anti-oxidative; apoptosis; neural regeneration*

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Introduction
Traumatic brain injury (TBI) is the leading cause of disability, and in humans it carries a posttraumatic risk of embolism (Aloizos et al., 2015). Although many researchers continue to investigate neuroprotective agents against damaged nervous tissue, no distinct clinical improvements have been rendered during TBI treatment. Initial trauma causes structural and functional deficits, but the damage continues to evolve through a secondary injury mechanism (Andriessen et al., 2010). Every effort should be made to inhibit the activation of secondary pathways (Župan et al., 2011).

A body of evidence points out the potency of enoxaparin, a low-molecular-weight heparin that is utilized for anticoagulation, as a neuroprotective agent (Stutzmann et al., 2002). When peripherally administered, enoxaparin has the potential to penetrate the blood-brain barrier (BBB) (Sen et al., 2011). The inhibition of coagulation is thought to cut down post-TBI intravascular microthrombosis and neuronal tissue loss (Jonas et al., 1997; Pratt et al., 1998; Mary et al., 2001; Stutzmann et al., 2002; Quartermain et al., 2003; Li et al., 2015, 2016). Adjunct to its anticoagulant effect, enoxaparin possesses powerful anti-inflammatory properties that limit the progression of tissue injury in different organs and may contribute to neuroprotection (Gikakis et al., 1996; Jonas et al., 1997; Mary et al., 2001).

Neuroprotective effects of enoxaparin have not been previously demonstrated in a cold-induced brain injury model. This model type represents several pathophysiological characteristics of human focal cortical contusion, and hence it allows for the assessment of the desired effect of compounds that have a neuroprotective potential (Jonas et al., 1997). The cold-induced brain injury model used in this study is a well-characterized and reproducible model of experimental TBI (Michinaga et al., 2014, 2015).

We designed the present study to further investigate the role of enoxaparin use following cold-induced TBI. To induce TBI, animals were submitted to cold-induced TBI (i.e., a cryogenic injury model), an animal model commonly used to produce brain lesions that, in some respects, resemble TBI in human patients (Murakami et al., 1999; Grasso et al., 2007; Kelestemur et al., 2016).

Materials and Methods
Ethics statement
Our study was approved by the animal ethics committee of Medipol University (approval number: 23-03-2016/29) and all experiments were carried out according to the internationally approved principles for laboratory animal use and care as found in European Community Guidelines. The article was prepared in accordance with the "Animal Research: Reporting of In Vivo Experiments Guidelines" (ARRIVE Guidelines). All efforts were made to minimize animal suffering and minimize the number of animals used.

Animals
Male BALB/c mice, 8–10 weeks old, weighing 20–25 g, were used in this study. Animals were randomly divided into three groups (n = 7 per group) as follows. In the control group, 0.2 mL isotonic saline solution was intraperitoneally adminis-

tered at 20 minutes after TBI. In the low-dose enoxaparin group, enoxaparin (Clexane, Sanofi-Aventis, Istanbul, Turkey) was applied as a single intraperitoneal dose of 3 mg/kg at 20 minutes after TBI. In the high-dose enoxaparin group, enoxaparin was applied as a single intraperitoneal dose of 10 mg/kg at 20 minutes after TBI.

Cold-induced TBI

After general anesthesia by intraperitoneal administration of ketamine hydrochloride (90 mg/kg) combined with xylazine hydrochloride (10 mg/kg), the animals were placed in a stereotactic device (WPI Instruments, Saradota, FL, USA). Throughout the experiments, rectal temperature was kept between 36.5 and 37.0°C through the use of a homoeothermic blanket. Cold induced TBI was executed using the model described by Kelestemur et al. (2016). The skull was exposed *via* midline scalp incision. A 3-mm diameter craniotomy was performed at the parietal bone 2.5 mm posterior to and 2.5 mm lateral to bregma (The Allen Mouse Brain Atlas). The tip (2.5 mm) of liquid nitrogen-cooled (−78°C) copper cylinder rod (Habas Ltd, Istanbul, Turkey) was applied for 60 seconds to produce a cryogenic lesion. Scalp was closed in layers and the animals (except those in the control group) were treated with enoxaparin 20 minutes after TBI induction. Twenty-four hours after trauma, all animals were sacrificed by decapitation.

Assessment of brain infarct volume

The brains from traumatized rats were removed and brain sections were obtained at 1 mm intervals spanning the length of the brain. A total of 12 consecutive coronal sections (5 μm thick) throughout the brain were stained with Cresyl Violet (Sigma, St. Louis, MO, USA). Image J software program (NIH, Bethesda, MD, USA) was used to trace the boundary between the injured and non-injured areas. The area of injury was assessed by subtracting the area of the nonlesioned ipsilateral hemisphere from that on the contralateral side. The volume of injury was calculated by integrating these lesioned areas. All 12 cross sections were individually measured and corresponding volumes were calculated.

TUNEL staining

Brain sections were fixed for 20 minutes at 4°C with 4% paraformaldehyde/0.1 M PBS for DNA fragmentation analysis. TUNEL staining was then performed after labeling with terminal deoxynucleotidyl transferase mix, which contained 12.5 mg/mL terminal deoxynucleotidyl transferase and 25 mg/mL biotinylated dUTP (both Boehringer-Mannheim, Mannheim, Germany); sections were stained with streptavidin-FITC (Sigma-Aldrich). DNA-fragmented cells (apoptotic cells) were microscopically evaluated under 180× magnification using an AxioZoom V16 microscope (Carl Zeiss AG; Oberkochen, Germany) by counting TUNEL-positive cell profiles in predefined arrays consisting of six regions of interest (ROI) in the cortex, 250 μm apart (each ROI measuring 62,500 μm^2). Mean values were calculated for all areas.

Measurement of serum levels of total antioxidant status (TAS) and total oxidant status (TOS)

Before sacrifice of animals, blood samples obtained *via* jugular vein were centrifuged for 5 minutes at 4.500 r/min at 4°C to separate the serum and plasma. Serum TAS and TOS levels were determined using an automated analyzer (Chromate Manager 4300, Palm City, FL, USA). The values are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 equiv/L).

Statistical analysis

All data were analyzed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Differences among groups were analyzed by Kruskal-Wallis tests followed by Mann-Whitney *U* tests. Values for $P \leq 0.05$ were considered statistically significant. All values are given as the mean ± SEM.

Results

Brain infarct volume

At 24 hours after surgery, the reduction in infarct volume was found to be statically significant in the 10 mg/kg enoxaparin-treated group than in the control group ($P < 0.05$; **Figure 1**).

Cell apoptosis

The number of apoptotic cells in the 10 mg/kg enoxaparin group was significantly decreased than that in the control group ($P < 0.05$; **Figure 2**).

Serum TAS and TOS levels

Serum TAS level was significantly increased in the 10 mg/kg enoxaparin than in the control group ($P < 0.05$). However, there was no significant difference in serum TOS level between enoxaparin-treated groups and control group ($P > 0.05$; **Figure 3**).

Discussion

Results from this study confirm the neuroprotective effect of enoxaparin use in a cold-injury TBI model. Enoxaparin reduced cell apoptosis and alleviated brain injury greatly. In addition, the use of enoxaparin significantly increased total antioxidant activity.

It is well known that multiple mechanisms underlie secondary brain damage following TBI. Mechanical trauma can kill neural cells by rupturing their membranes. But ischemia that accompanies brain swelling and elevated intracranial pressure to trauma can indirectly harm neurons (Stoicaand and Faden, 2010). The formation of microthrombi has been reported to occur in TBI and may lead to secondary ischemic injury (Babaee et al., 2015). Fibrin has been reported to be deposited in cerebral microvessels and, along with platelets and leukocytes, contributes to the occlusion of microvessels (Sen et al., 2011).

Stutzmann et al. (2002) reported the neuroprotective character of enoxaparin in an experimental model of TBI. They also pointed out that enoxaparin is free from the risk of harm at the doses used. Later, other animal studies showed the neuroprotective effects of enoxaparin, thus proposing its inherent capacity in the treatment of TBI (Jonas et al., 1997; Pratt et al., 1998; Wahl et al., 2000; Mary et al., 2001; Grasso et al., 2007; Li et al., 2015, 2016). In these studies, enoxaparin decreased

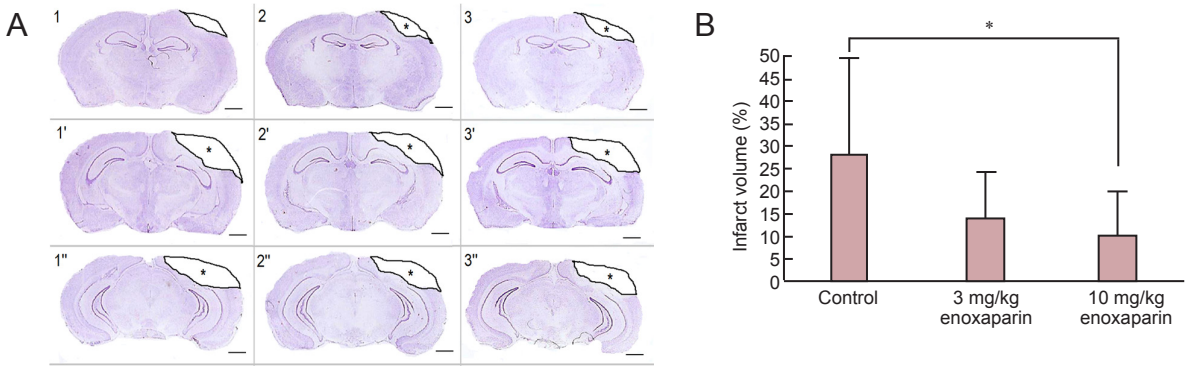


Figure 1 Effect of enoxaparin on infarct volume in the cortex of mice with cold-induced traumatic brain injury (TBI).
(A) Damaged tissue is defined by a decrease in staining intensity and an example of border demarcation is illustrated in these consecutive images (represented as 1, 1', 1" and so on) (Cresyl violet staining) (scale bars: 1mm). 1–1" indicates control group (TBI + isotonic saline solution), 2–2" indicates "3 mg/kg enoxaparin group (TBI + 3 mg/kg enoxaparin)" and 3–3" indicates "10 mg/kg enoxaparin group (TBI + 10 mg/kg enoxaparin)". * indicates the infarct area. (B) Infarct volume at 24 hours after surgery. All values are given as the mean ± SEM (mm^3). Seven rats were used in each group. *$P < 0.05$, *vs.* control group (Kruskal-Wallis tests followed by Mann-Whitney *U* tests).

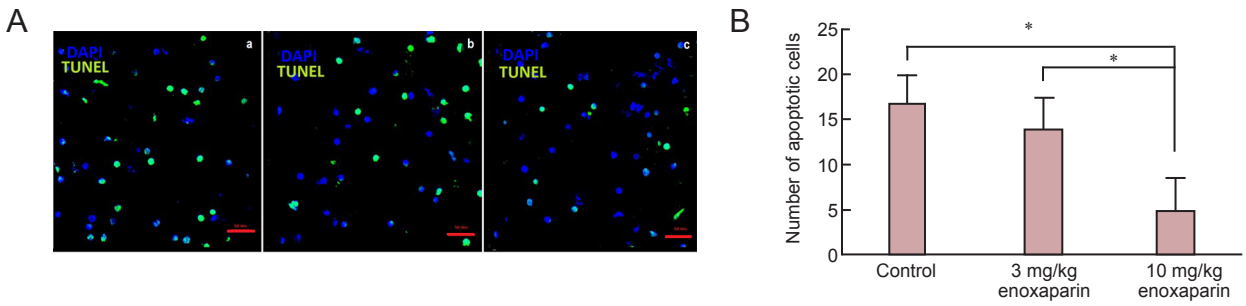
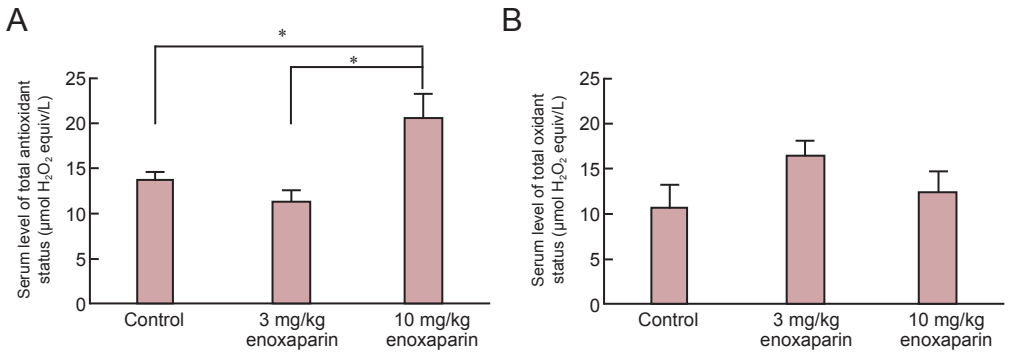


Figure 2 Effect of enoxaparin on cell apoptosis in the cortex of mice with cold-induced traumatic brain injury.
(A) Representative fluorescent microscopic images of brain sections of (a) control rats receiving isotonic saline solution, (b) 3 mg/kg enoxaparin-treated rats, (c) 10 mg/kg enoxaparin-treated rats (TUNEL staining). Green staining represents the apoptotic cells. (B) Quantification of TUNEL-positive cells (/μm^2). All values are given as the mean ± SEM. *$P < 0.05$, *vs.* control group (Kruskal-Wallis tests followed by Mann-Whitney *U* tests). Seven rats were used in each group.



(A) Serum total antioxidant status. (B) Serum total oxidant status. All values are given as the mean ± SEM. *$P < 0.05$ (Kruskal-Wallis tests followed by Mann-Whitney *U* tests). Seven rats were used in each group.

Figure 3 Effect of enoxaparin on serum levels of total antioxidant and total oxidant status in mice with cold-induced traumatic brain injury.

the extent of brain edema, reduced the lesion size, and ameliorated cognitive impairment and neurological deficits following experimental TBI. In subsequent years, the number of studies regarding how enoxaparin demonstrates neuroprotective action in TBI has increased, and these studies demonstrated the molecular mechanism between the two entities. Sen et al. (2011) demonstrated that enoxaparin reduced cell death, inflammation, and apoptosis in the brain tissue after experimentally induced severe focal TBI. Župan et al. (2011) showed that following TBI, enoxaparin use significantly reduced hippocampal thiobarbituric acid-reactive substances and oxidized protein levels, COX-2 overexpression, and reactive gliosis. These evidences suggest that enoxaparin may reduce oxidative damage and inflammation following TBI.

Secondary ischemia is due in part to the edema-induced compression of blood vessels. Even when administered at 18 hours post-insult, the use of enoxaparin was found to significantly reduce cerebral edema in a dose-dependent manner in rat models of cerebral trauma (Wahl et al., 2000). Enoxaparin was shown to attenuate brain edema and improve neurological recovery after TBI, through the blunting of cerebral leukocyte recruitment diminished live leukocytes rolling on the pial endothelium, and endothelial cell activation while accelerating neurological recovery (Li et al., 2015). This was associated

with concurrent reductions in microvascular leakage. Enoxaparin was found to reduce the intensity of tissue inflammation by inhibiting leukocyte activation and adhesion to endothelial cells. In particular, heparinization before hemorrhage and trauma resulted in blunted endothelial activation and restored the ability of endothelial cells to release nitric oxide. In different *in vivo* TBI models, enoxaparin use has been shown to reduce brain edema and lesion size, without increasing intracranial bleeding (Li et al., 2016). Although it has been frequently emphasized, the risk with such an anticoagulant drug is the possibility of a hemorrhagic transformation. However, enoxaparin shows a much lower tendency for bleeding than heparin for the same anti-Xa activity (Pratt et al., 1998).

The current study has many limitations; therefore, the molecular mechanism underlying enoxaparin in cold-induced TBI requires further investigation. Additionally, the dosage regimens for the neuroprotective effect of enoxaparin should be clarified. The currently recommended dose for thromboembolic complications is reportedly to be 3.5 mg/kg/d (Quartermain et al., 2003). Meanwhile, according to Kobbi et al. (2016), a dose of 20 mg/kg/d is considered the upper toxic dose in rats. Doses of 3.5 mg/kg/d and 5 mg/kg/d showed no undesirable effects, and are therefore recommended for further pharmacodynamics studies. However, some adverse effects were clearly observed at the dose of 20 mg/kg/d, whereas induced lethality at doses of 100 and 40 mg/kg/d have been reported. According to the results of our study, the greatest neuroprotective effect was observed when enoxaparin was used at a dose of 10 mg/kg/d—a dose still below the upper toxic dose, but above the standard therapeutic dose.

In conclusion, although the effectiveness of enoxaparin as a neuroprotectant has been investigated previously in brain injury models, none has used a cryogenic injury model or pattern. This study is the first to provide the *in vivo* evidence that following cold-induced TBI, enoxaparin is a beneficial drug in treating injuries, as it exhibits neuroprotective effects on the brain tissue.

Author contributions: *IK designed the study, was responsible for data collection, and wrote the paper. MYG performed surgeries and collected experimental data. NA, UK and MO were responsible for data collection and wrote the paper. EK designed the study and wrote the paper. All authors approved the final version of this paper.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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