Influence of the adaptive immunity in the experimental traumatic brain injury in the rat and the effects of enoxaparin

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Diplomski rad (Master's thesis)

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Prologue

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Contents

List of abbreviations and acronyms

TBI *Traumatic brain injury*

CNS *Central nervous system*

GCS *Glasgow Coma Scale*

DAI *Diffuse axonal injury*

NMDA *N-methyl-D-aspartate*

AMPA *α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid*

ROS *Reactive oxygen species*

RNS *Reactive nitrogen species*

BBB *Blood-brain barrier*

DAMP *Damage-associated molecular pattern*

HMGB1 *High mobility group box 1*

PRR *Pattern recognition receptor*

TLR *Toll-like receptors*

TGF-ß1 *Transforming growth factor-beta 1*

IGF-1 *Insulin- like growth factor -1*

IL-1ß *Interleukin – 1 beta*

TNFα *Tumor necrosis factor-alpha*

IL *Interleukin*

NK *Natural killer*

Th *T helper*

MHC *Major histocompatibility complex*

Iba-1 *Ionized calcium-binding adapter molecule 1*

APC *Antigen presenting cells*

MBP *Myelin basic protein*

ENX *Enoxaparin*

LMWH *Low molecular weight heparin*

LFPI *Lateral fluid percussion injury*

COX-2 *Cyclooxygenase-2*

FPI *Fluid percussion injury*

CCI *Controlled cortical impact injury*

WDI *Weight drop injury*

PBBI *Penetrating ballistic-like brain injury*

rmTBI *Repetitive mild TBI*

PFA *Paraformaldehyde*

IHC *Immunohistochemistry*

HIER *Heat induced antigen retrieval*

EDTA *Ethylenediaminetetraacetic acid*

DAB *Diaminobenzidine*

IF *Immunofluorescence*

BSA *Bovine serum albumin*

DAPI 4*′,6-diamidino-2-phenylindole*

mNSS *Modified neurological severity score*

CA *Cornu ammonis*

1. Introduction

1.1. What is a traumatic brain injury?

Traumatic brain injury (TBI) is a trauma to the central nervous system (CNS) caused by a penetrating or a blunt impact, resulting in an open or closed injury. The severity of the injury depends on the impact object, mechanism of injury, force, and motion (1). According to the Working Group of the International and Interagency Initiative toward Common Data Elements for Research on Traumatic Brain Injury and Psychological Health, TBI is defined as "an alteration in brain function, or other evidence of brain pathology, caused by an external force"; alterations in the function being any changes in the state of consciousness, retrograde or anterograde amnesia, impairments in the neurological status, and deterioration in the mental state (2,3). TBI can be clinically classified as mild, moderate, or severe, in accordance with the Glasgow Coma Scale (GCS) score (3).

TBI is one of the leading causes of disability and death worldwide. In the European Union, TBI results in 57 000 deaths and 1.5 million hospital admissions every year. TBIs most commonly occur in traffic accidents, falls, and (attempted) suicides; more than half affected being male (4). The age-adjusted incidence rate for Europe amounts to 287.2/100 000 person-years, respectively (5,6). Survivors suffer from long term consequences which inevitably reduce the quality of life, increasing years of life lost and as such represent a socioeconomic and a healthcare burden (4). TBI should not be considered as a single event, but as a trigger to a neuroinflammatory and neurodegenerative cascade that continues over time (7). Such long-term repercussions include limitations in daily functioning, cognitive deficits, neurodegenerative disorders and neuropsychiatric illnesses, emotional disturbances, ischemic insults, and mortality (7).

1.2. Pathophysiology of TBI

1.2.1. Primary brain injury

Trauma to the head may result in the damage of the skull, blood vessels, or the brain parenchyma. Primary brain injury refers to the mechanical damage as a result of focal injury, diffuse, or a combination of both (1,8).

Depending on the type and the degree of injury, different pathophysiological entities such as contusion, diffuse axonal injury, and concussion, can be distinguished. Contusions take a form of *coup* or *contrecoup* injury, depending on the energy and the size of the object at the collision site (8). *Coup* injury occurs on the side of the impact, as a result of direct energy transmission from the object. Brain, being confined within the intracranial cavity bordered with rigid cranial bones, hits the opposite side of the initial contact, resulting in a *contrecoup* injury. Contusions develop in the gyral crests, being situated anatomically closest to the injury site (1). Mechanical damage causes shearing of the blood vessels and the brain parenchyma. Depending on the location of the blood vessels and the subsequent hemorrhage; epidural or subdural hematoma may develop, along with the intracerebral or the subarachnoid hemorrhage (9). Interrupted blood supply induces ischemia and necrosis of the brain tissue. As the aftermath of cardiac compensation mechanisms and increased intracranial pressure, cerebral autoregulation leads to the vasodilatation of cerebral blood vessels, and, consequently, intracranial hypertension (8). Rotatory motion of one part of the brain relative to the other can produce diffuse axonal injury (DAI). This type of injury is characterized by the breakage of the neurofilaments in the cytoskeleton, impairing cellular function. As axons diverge through the encephalon, this leads to a widespread axonal disconnection (8,9). Their *loci minoris resistantiae* are the brain stem and the angles of the lateral ventricles (1); explaining the prognosis which is rather poor,

regularly with a comatose state as an end result. Lastly, a concussion is a reversible deterioration in the neuronal function accompanied by amnesia without a patho-anatomical substrate as a result of mild trauma.

1.2.2. Secondary brain injury

Once initiated, a plethora of secondary molecular events following the primary trauma may persist from days to weeks, even years in case of chronic neurodegenerative processes such as chronic traumatic encephalopathy (10,11). Ischemia alters depolarization properties of injured neurons, which release excess glutamate in the synaptic cleft, upregulating glutamatergic receptors (12). Glutamate excitotoxicity dysregulates ion trafficking, inducing Ca^{2+} influx acting as a positive feedback loop of further glutamatergic drainage in the extracellular space (9). On the other side, downregulated and dysfunctional glutamate transporters on astrocytes are incapable of removing excess glutamate (12,13). Glutamate excitotoxicity is tightly bound with mitochondrial dysfunction. A surplus of intracellular Ca^{2+} disrupts mitochondrial membrane potential and induces permeability, inevitably ensuing leakage of enzymes. These changes cease ATP synthesis while increasing the oxygen demand and contribute to the formation of reactive oxygen species (ROS). Oxidative stress undeniably contributes to secondary damage. ROS and reactive nitrogen species (RNS) are extensively produced in the mitochondria, phagocytic processes, and hemoglobin oxidation. They interact with proteins and DNA, but also with phospholipids in membranes. Lipid peroxidation generates disturbances in transport across the cell membrane (12,14). Moreover, trauma-induced tearing of blood vessels deranges continuity of blood-brain barrier (BBB), allowing the formation of vasogenic edema (15). The hypertensive response of the vasculature, in addition to the endothelial permeability brought upon arachidonic acid metabolites, also

promotes this type of edema. On the other side, cytotoxic edema directly affects cells. A hypoxic injury that occurs during the primary trauma corrupts normal functioning of the ATP dependent Na^+/K^+ pump, leading to the osmotic buildup of Na^+ and water in the intracellular compartment. Excessive glutamate levels also cause an imbalance in the ion transport, consecutively resulting in the cellular swelling (9). Neuroinflammatory processes have a complex role that is both detrimental and beneficial in the evolution of secondary brain injury and will be extensively discussed in the text below.

1.3. Neuroinflammation

1.3.1. Innate immunity

Inflammation appears to have a pivotal role in the evolution of tissue injury following brain trauma (16). The onset of every immune response begins with the non-specific, innate arm, followed by the specific arm of the adaptive (or acquired) immunity, greatly responsible for the shaping of secondary injury (16,17). Innate immunity acts as a primary response to the noxious stimulus, infiltrating the site of the injury. The purpose of the initial immune response is to limit the injury, remove the cellular debris and mobilize components of cellular adaptive immunity from the periphery, which is achieved through chemotactic signaling and cytokine release (16). Once a mechanical insult occurs, damage-associated molecular patterns (DAMPs) are released through the disrupted cellular membrane. DAMPs include DNA, RNA, S-100 protein, high mobility group box 1 (HMGB1), and other chaperone proteins (13,18). Resident cells such as microglia and astrocytes recognize DAMPs through pattern recognition receptors (PRRs), more precisely Toll-like receptors (TLRs). Astrocytes face morphological changes, such as hypertrophy and processes extension, as well as functional alterations, in terms of the expression of various pro-

and anti-inflammatory factors. Sum of these cellular responses is termed reactive astrogliosis. However, reactive astrocytes tend to express heterogeneous phenotypes, dependent on their location to the injury site and signals they receive (13). Astrocytes secreting transforming growth factor-beta 1 (TGF-ß1) and insulin-like growth factor -1 (IGF-1) are thought to have a role in attenuating neurotoxicity, while astrocyte phenotypes secreting interleukin – 1 beta - (IL-1ß) and tumor necrosis factor-alpha (TNFα) - promote it. In addition to cytokine signaling, astrocytes play a role in shaping a physical barrier in a form of glial scar around the injured tissue, as well as in the uptake of excess glutamate (10,13).

Activated astrocytes, microglia, and likely neurons release various chemokines and cytokines in an immediate response that recruits other resident and peripheral immune cells (19). Chemokines and cytokines also help in the activation of PRRs. Chemokines are small (10 kDa or smaller) proteins that activate peripheral immune cells, such as neutrophils, macrophages, and monocytes through G-protein coupled receptors and chemotactically guide them to the location of the injury (16). Cytokines such as IL-1ß with the co-occurrence of TNFα, and the interleukins (IL) IL-6, and IL-18 are the main drivers of the neuroinflammatory cascade (16,20). On the other side, IL-10 has been associated with the suppression of the pro-inflammatory Th1 pathway and beneficial effects on neurological recovery. Nonetheless, the division of signaling molecules on strictly pro- versus anti-inflammatory serves mostly logistical purposes; as it should be kept in mind that this categorization is far more complex. For instance, IL-10 can mediate harmful effects via B-cell signaling, and IL-1ß induces hallmarks of neurotoxicity when influenced by TNFα (20).

Cellular components of the innate arm include granulocytes, macrophages and microglia, mastocytes, dendritic cells, and natural killer (NK) cells (16). Neutrophils

are the first line of the cellular arm defense. They migrate to the tissue injury site within hours, attracted by the chemotaxis of CXC chemokines. Their peak is between 24 to 48 hours after injury; during that time they induce endothelial permeability through integrins – adhesion molecules interaction, also release chemokines, cytokines, and ROS which act directly by damaging the tissue or indirectly, recruiting other immune cells (16,18,19).

Microglia are the brain's resident immune cells. They originate from the same myeloid precursor as macrophages, sharing functions such as antigen-presentation and phagocytosis (13). Through the expression of TLRs, microglia respond to DAMPs, the signals of necrosis, and are activated early following the neutrophil invasion. DAMPs, but also ROS and pro-inflammatory cytokines, commence the polarization of microglia across the continuum of the pro-inflammatory M1 phenotype, to the anti-inflammatory M2 phenotype expression (18). They represent a link between the innate and adaptive immunity, reacting early after the injury and persisting along with the infiltration of the cells of the adaptive immunity. Both human and rodent studies have demonstrated upregulation in major histocompatibility complex (MHC) II Class molecule, CD68, and ionized calcium-binding adapter molecule 1 (Iba-1) in chronic injury, substantiating as the markers of activation (13).

1.3.2. Adaptive immunity

T lymphocytes penetrate the CNS 24 hours after the injury, with a maximum in their number within the end of the first week (19,21). Under physiological conditions, T cells reside in the meningeal blood vessels and the choroid plexus (21,22), from where brain parenchyma is invaded in case of the BBB disruption or chemotactic signaling. Proliferation and differentiation of lymphocytes is mediated by cytokines, which promote maturing of naive CD4+ T cells to the T helper (Th) 2 effector cells, the

survival of CD8+ cells, and the production of antibodies by stimulated B cells. However, to achieve the proper adaptive immunity activation, co-stimulation by the cytokines should be followed with appropriate antigen presentation and recognition (so-called “two-signal hypothesis“) (23). Once the BBB has been damaged, antigens from formerly immunologically privileged brain enter the bloodstream and become exposed to the antigen-presenting cells (APC), recognized by T cells. Viewpoints on the adverse versus protective influence of adaptive immunity on the recovery after injury are opposed. On the one hand, T lymphocytes autoreactive to brain antigens, such as myelin basic protein (MBP), have shown to have a beneficial effect by limiting the injury through the release of neurotrophic factors (16,20,21). This phenomenon was described as protective autoimmunity and appears to be specific for CNS antigens only (22). The function of the effector T cells goes beyond the injury, even so, extends to the homeostatic maintenance of the neural functioning such as plasticity, learning, and neurogenesis (22). Moreover, effector T cells must be in equilibrium with the regulatory T cells, as the latter seems to carefully influence the inflammatory sequence (21,22,24).

On the contrary, infiltration of T cells may aggravate harmful neuroinflammation, alongside with concurrent microglia/macrophage activation. According to the study conducted by Daglas et al. (25), mice depleted of the CD8+ T cells have exhibited better performance in neurological scores and reduced myelin vacuolization, without compensation in immunological over reactivity, comparing to the CD8+ control. Furthermore, Ndode-Ekane et al. (26) found a positive correlation between the number of CD3+ T cells in the brain parenchyma and impaired neurological recovery. As a result of these contrary evidences, further investigations are to be done to disentangle the baffling relationship between cellular immunity and the progression of TBI.

1.4. Current therapeutic options and challenges: a (promising) role of enoxaparin

Despite better understandings of the pathophysiology of TBI, therapeutic and management options are still limited. The aim of the treatment is to reduce the damage caused by processes involved in secondary brain injury. Up to now, therapeutic options are comprised of securing the airway and adequate ventilation (including prophylactic hyperventilation), maintaining the cerebral perfusion pressure, and reducing cerebral metabolic requirements, either by medically induced coma or therapeutic hypothermia (27). Furthermore, TBI has been associated with a higher risk of thrombotic events (28). Anticoagulant therapy is given with caution in order to prevent hemorrhage and further damage to the brain tissue.

A vast number of therapeutic options have been tested in both animal and human studies, from pharmacological drugs to biological and non-invasive therapy, all of which targeting secondary events of TBI. Mostly investigated ones include erythropoietin, cell cycle inhibitors, and anti-inflammatory drugs such as minocycline and peroxisome proliferator-activated receptors agonists. Biologic treatments include gene therapy, stem cell therapy, growth factors, and peptide therapy. Stem cells appear to have multiple beneficial traits proven in preclinical trials by supporting neuroregeneration and modulating immunoregulation (29).

Enoxaparin (ENX), an anticoagulant drug, has shown to exhibit beneficial effects in both preclinical studies and clinical trials (28,30,31). Its main mechanism of action, as low molecular weight heparin (LMWH), is the inhibition of the coagulation factor Xa and, to a minor extent, factor IIa (thrombin). In comparison with the unfractionated heparin, enoxaparin has longer bioavailability, a lesser affinity for thrombin, and a safer profile when it comes to adverse bleeding events (32). The effect

on the reduction of brain edema, lesion volume, and improved recovery of cognitive functions following the trauma has been demonstrated in different animal TBI models (32–35). Pro-coagulant state leads to occlusion of the blood vessels and, consecutively, ischemia; blood stasis forming in occluded blood vessels contributes to edema. Effects on the decrease of edema correlate with improved motor and cognitive functions in neurological tests (32). In the context of clinical studies, enoxaparin was primarily evaluated as prophylaxis of venous thromboembolism, which has not led to the further progression of the cerebral hemorrhage comparing to the initial injury (31,36). Adjacent to these anticoagulation features, enoxaparin has also been evaluated for its anti-inflammatory and neuroprotective properties, making it an interesting drug of choice that would target secondary TBI at various pathophysiological steps. An *in vitro* study by Jonas et al. (1997) proposed that enoxaparin can act in both extracellular and intracellular compartments, conducive to lowering cytoplasmic calcium contents and opposing glutamate-induced depolarization (37). Previous research done in lateral fluid percussion injury (LFPI) model has shown that enoxaparin reduced astrocytosis, levels of cyclooxygenase-2 (COX-2), and certain parameters of oxidative stress (38). Also, activated vascular endothelium, through signaling cascade, leads to increased leukocyte adhesion, platelet aggregation, and blood vessel permeability. Enoxaparin reduced endothelial damage evaluated by a decrease in leukocyte rolling and adhesion (28,39), albumin leakage (28), and microthrombi formation (32,34).

1.5. Animal models of TBI

TBI in experimental conditions can be studied using animal models, *in vitro* models or computational modelling (40). *In vitro* models simulate injury on whole tissue or isolated cell types. *In vivo* injury is mostly performed in rodent or porcine animal

models. Firmly established and widely recognized animal models include fluid percussion injury (FPI), controlled cortical impact injury (CCI), blast, and weight drop injury. Each model is specific in regards to the type of injury produced (focal, diffuse, or mixed), adaptation to the species of the experimental animal (rodents versus non-rodents), and biomechanics (40,41).

1.5.1. Fluid percussion injury model

Midline or lateral, FPI is a well-established model of TBI. First described in cats, nowadays widely used with rodents, it serves as a model of mixed (both focal and diffuse) injury (42,43). Animals are placed in a stereotaxic frame and a craniotomy is performed (41). Intact dura is exposed, and a Luer-Lock fitting is implanted in the trephinated side. The injury is generated with the pendulum, released from a certain angle, which hits a piston on one side of the cylinder filled with saline, connected to a Luer-Lock counterpart fitting. The hub implanted on the dura is also filled with saline and jointed with its counterpart on the cylinder. Once a pendulum is struck, it generates a rapid fluid pressure pulse resulting in a cerebral injury, which is being monitored with an oscilloscope (41,42). The severity of the injury is dependent upon the force of pendulum striking.

Depending on the craniotomy location, different types of FPIs can be inflicted. Midline FPI is carried out over sagittal sinus, between bregma (junction of sagittal and coronal suture) and lambda (junction of sagittal and lambdoid suture) points, while craniotomy for the lateral FPI (LFPI) is done over the parietal cortex, minimally 3.5 mm lateral to the midline (41). Parasagittal craniotomy takes place between up to 3.5 mm to the midline. Careful choice of trephining position is crucial, as even small stereotaxic differences can yield different histopathological results. Tissue compression by a fluid pressure leads to focal, unilateral cortical damage, diffuse subcortical destruction,

accompanied by intracerebral bleeding, edema, and subarachnoid hemorrhage (42). Midline FPI is associated with higher mortality rates, causing bilateral damage to the cortex and diffuse injury in the subcortical structures, sometimes with brainstem hemorrhage or even displacement, resulting in apnea and death (43). However, this model subtype has been regaining interest, as it reproduces trauma seen in sports and blasts (40).

1.5.2. Controlled cortical impact injury model

Controlled cortical impact (CCI) injury is a type of focal TBI model (44). Injury is inflicted as a pneumatic or electromagnetic CCI device with accelerating rod, impacting the exposed dura, and leading to brain contusion (45). A unilateral craniotomy is performed between bregma and lambda (40). CCI model has several advantages that make it widely used in preclinical settings. The first advantage is the susceptibility of modulations in the mechanistic compartment, such as the size and the shape of the impactor (making pediatric TBI easily reproducible), velocity, angles, and other parameters (45). Biomechanical characteristics can be therefore more precisely defined, in comparison with the other experimental models (40). Another main advantage is the ability to correlate pathophysiological findings to those seen in clinical TBI, especially due to tissue contusion, next to the BBB disruption and DAI; as well as chronic changes such as volume loss, ventricular expansion and chronic inflammation (40,45).

1.5.3. Weight drop injury model

This model of TBI is used to reproduce both focal or diffuse injury in animal models with a free-falling weight to the exposed skull (40). Feeney's model was the first to introduce this type of injury and requires a craniotomy. This leads to cortical contusion and, in case of severe forces, subcortical hemorrhaging and necrotic

cavities. Neurological deficits may persist up to 90 days post injury (46). On the other side, free-falling weight drop injury (WDI) can create a model of diffuse injury. Such an example is the Marmarou's acceleration model, constructed so it recreates TBI caused by falls or traffic accidents (40,46). Marmarou et al. were the first to introduce a closed head WDI. Skull exposure is necessary to mount a steel helmet or a disk, which distribute weight across the cranium. This way aids in the prevention of the fractures to the cranium and spread weight impact across the skull, causing DAI (40,44,46). Marmarou's closed head WDI model has been modified (Maryland's model) to simulate frontal impact with sagittal rotation and acceleration as seen in sports or automobile accidents (40,44). Although WDI may be more convenient due to its price and simplicity of use, the main disadvantage remains inconsistency in reproducibility of injury as a result of weight rebound effect and possible skull fractures (40,44,47).

1.5.4. Blast injury model

Blast TBI is a result of the indirect impact leading to a diffuse injury. Pneumatic shock tube blast, live blast explosives, or laser-induced shock wave (41) can be used to demonstrate a blast injury, commonly seen on the battlefield. Parameters of the shock tube blast model are more reproducible comparing to the use of live explosives (44). In this model, compressed air (or other gas) disperses through a cylindrical tube, releasing blast overpressure. Different parameters can be modified; such as the angle of the head, animal positioning against the tube, duration, and intensity of pressure. However, this scope of variations makes results from different research groups and settings less comparable (40,41). On the contrary, explosive-induced blast injury induces a mixed lesion. The detonation of explosives creates shock waves, transmitted to the animal on the other side of a blast tube (47). Blast injuries cause motoric, behavioral, and functional deficits, even at mild TBI with lower blast energy

(40). Although blast TBI models are intriguing as they mirror TBI seen in war casualties, main setbacks in wider usage are expensiveness and complexity of equipment, aside from potential safety concerns (41).

1.5.5. Penetrating ballistic-like brain injury model

In the previous decade, a rat model of penetrating ballistic-like brain injury (PBBI) was developed as a substitute for previously used large animals, such as a cat. Adaptations had been made in the gear to correspond with the experimental animal's cranium size, as well as to induce penetrating trauma without firing projectile (41). A rodent is placed in a stereotaxic frame and a craniotomy is performed over the site of interest. A trephine is formed to stereotactically insert a surgical probe in the cortex. Ballistic-like, moderate to severe injury is induced as a balloon inflate in the cavity under pressure at a certain rate (40,48). The pathohistological hallmark of this model is the extensive intracranial hemorrhage, followed by an increase in intracranial pressure (48).

1.5.6. Mild injury models

Concussions appear to be the most commonly encountered type of TBI (49). It also causes hefty disagreements regarding the definition and classification, which can result in inadequate epidemiological data. However, in order to distinguish terms concussion and mild TBI (mTBI), it can be said that mTBI is an objective diagnosis at the time of injury, while concussion relates to symptoms and signs that may persist, even for a longer period of time (49). Mild injury is of specific interest when designed as a repetitive mTBI (rmTBI), as it can mimic contact sports or military accidents (40). This is commonly done with the FPI model, WDI, or blast TBI model. Kane et al. (50) developed a modified Marmarou's weight drop impact acceleration model of rmTBI using minimal sedation, omitting craniotomy, or body restraint, resulting in a better

imitation of the concussion in humans. Closed head injury models are generally preferred as mTBI implies minimal damage with a low fatality rate (51). Histopathological findings usually found in moderate to severe TBI, such as cell death, BBB damage, brain edema, or extensive glial reaction, are not seen in mTBI (40,46). Nevertheless, tau pathology and amyloid deposition, as hallmarks of chronic traumatic encephalopathy, can be detected and evaluated as a bridge between TBI and chronic neurodegenerative changes (11). Cognitive deficits do not occur after a single hit, but are rather noticeable after repetitive injuries, and can be accompanied by psychiatric abnormalities such as anxiety and depression (46).

1.6. Aims and objectives

The purpose of this study was to elucidate mechanisms of an adaptive immune response using the LFPI model in the rat; in particular, infiltration of T cells in the cerebral cortex within the first week following TBI. Furthermore, this research wanted to highlight the potential of low molecular heparin, administered through the acute phase after the injury, on the migration and distribution of CD4+ helper T-cells and CD8+ cytotoxic T-cells throughout the cortex, in conjunction with posttraumatic neurological deficits. Argumentation for these objectives is based on the previous research done in TBI on the subject of adaptive immunity; however, currently there is no evidence regarding immune response in the LFPI model. Moreover, due to the previously reported properties of enoxaparin that have been targeting on the components of the innate response, we wanted to explore its further capacity in cellular immunity. The outcomes measured include number of CD3+ and CD4+ cells per mm^2, presence of CD8+ cells in the parenchyma and the evaluation of motor deficits with a neurological severity score.

2. Materials and methods

2.1. Experimental animals and study design

Experiments were conducted on adult male Wistar-Hannover rats (body weight 350 g to 450 g at the time of injury). Rats were maintained on a 12-hour light-dark cycle in a controlled environment (room temperature of 20 to 24 °C, 45% to 65% humidity), with access to food and water *ad libitum*. All experiments were carried out between 10:00 AM and 2:00 PM. All animal procedures were approved by the Faculty Ethical Committee and conducted in agreements with the national laws and regulations (Animal Welfare Act, NN 19/99; and Regulation on maintenance of laboratory animals, special conditions for animal facilities and types of experiments NN 176/2004) alongside with the guidelines set by the European Community Council Directive of November 24, 1986 (86/609/EEC).

Experiments were divided into two parts. First, we wanted to determine the time course of the T cell infiltration into the rat cerebrum post-LFPI. For this experiment the animals were randomly divided to two major groups: sham-operated rats were assigned as the control group, and the animals exposed to moderate LFPI as the TBI group. Control animals underwent the exact craniotomy procedure as the TBI groups, only were not injured. Both animals from the control and the TBI group were sacrificed at different time points, i.e. at 1, 3 or 7 days after LFPI or sham procedure. Since we found no differences between the sham injured groups sacrificed at different time points, for the analyses were used animals sacrificed 1 day following sham treatment. In the second part of the experiments, animals were assigned into three experimental groups: Control group, TBI/vehicle group, and TBI/enoxaparin group. The group treated with enoxaparin received a total of 8 doses, 1 mg/kg administered subcutaneously (s.c.). The first dose was given 1 hour post-injury, and the following 7

doses over the course of 48 hours, in periods of 6 hours (Figure 1). Control group and TBI/vehicle group received vehicle, which contained sterile water for injections, at the same time points as the TBI/vehicle group. In this part of the experiment all the animals were sacrificed after 48 hours.

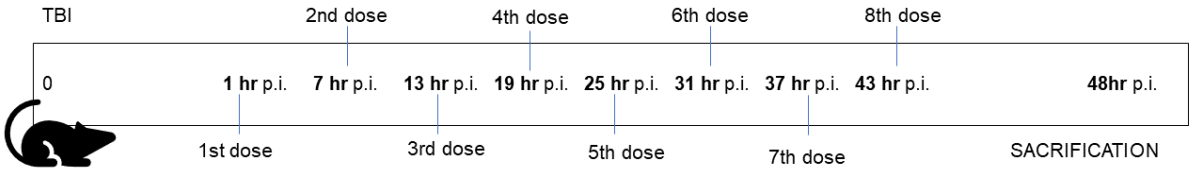

Figure 1. Graphic representation of the enoxaparin administration in 8 doses throughout a course of 48 hours following TBI. First dose was given one-hour post injury (p.i.), and every following 6 hours after the previous one. Animals were sacrificed 48 hours after TBI.

2.2. Induction of TBI

TBI was inflicted using the aforementioned LFPI model (42). Animals (N=10 to 11) were anesthetized with isoflurane, using a mixture of oxygen and nitrous oxide in ratio 1:2, and in concentration of 4% for the induction of anesthesia and 2% for the maintenance. After placement in the stereotaxic device, an incision over the sagittal suture was made to expose the cranium, following subcutaneous injection of 0.5% bupivacaine solution. A craniotomy was performed over the left parietal cortex, 3.4 mm lateral to the sagittal suture and midway between coronal and lambdoid suture, leaving the dura intact. The skull was trephined in order to place an empty Luer-Lock fitting and fixed using dental acrylic. The Luer – Lock fitting was filled with saline (0.9 % NaCl) and connected to the FPI system. Prior to the injury induction, animals were deprived of anesthesia; in other words, the injury was inflicted after first signs of consciousness had appeared. Moderate lateral fluid percussion injury was achieved using fluid pressure of 1.8-2.2 atm. After induction, duration of apnea was monitored.

This study included only those animals with apnea duration shorter than 60 seconds. After the procedure, Luer-Lock fitting was removed, and the wound was sutured. Antibiotic ointment was applied to the wound and 5 mL of saline was given intraperitoneally (i.p.). As aforementioned, sham-injured animals received the same treatment, except for the induction of TBI, and represented the control.

2.3. Tissue preparation

Animals were killed by transcardial perfusion after being anesthetized with sodium thiopental. Perfusion was carried out using cold phosphate buffered saline (PBS) solution, followed with the prefixation step with 4% paraformaldehyde (PFA). Brains were extracted from the cranial cavity and further fixated in PFA over the course of 20-24 hours. Furthermore, tissue was dehydrated through the ethanol gradient (50%- 100%), xylene, and embedded in paraffin. Coronal slices were cut at 4 μm thickness for the purposes of the immunohistochemical (IHC) and histochemical analyses.

2.4. Immunohistochemistry

Expression and distribution of CD3 positive cells was analyzed using IHC. Paraffin embedded brain tissue sections were deparaffinized through immersion in xylene (2 times, for 10 minutes) and rehydrated through alcohol serial of decreasing concentrations from 100 % to 70%, 5 minutes in each. After the final step of rehydration in water, heat induced antigen retrieval (HIER) was performed. Water bath with ethylenediaminetetraacetic acid (EDTA) buffer (pH 9.0) was heated to 98°C. After reaching the desired temperature, rack with slides containing brain sections was immersed in the buffer and boiled for 20 minutes. Next, slides were cooled on a room temperature for 20 minutes and transferred to a humidified chamber. Non-specific binding sites were blocked with 2.5% Normal Horse Serum (ImmPRESS HRP

Universal Polymer Kit, Vector Laboratories, USA) for 20 minutes. Sections were incubated with Purified Mouse Anti-Rat CD3 primary antibody (BD Pharmingen, BD Biosciences, USA), overnight at 4°C. The following day, secondary ImmPRESS Universal Antibody (anti-mouse IgG/anti-rabbit IgG, Peroxidase) Polymer Reagent was applied. Prior to that, endogenous peroxidase was quenched with 3% hydrogen peroxide, diluted in methanol, for 10 minutes. Diaminobenzidine (DAB) chromogen (Dako Liquid DAB+ Substrate Chromogen System, Agilent, Denmark) was used to label CD3+ cells. All sections were dyed with haematoxylin for 60 seconds in order to visualize the nuclei. Slides were dehydrated through increasing alcohol concentrations and xylene, and finally mounted in Entellan (Merck Millipore, USA).

2.5. Immunoflourescence

Immunofluorescent (IF) technique was applied to identify CD4+ and CD8+ cells in fluorescence histochemistry. The same protocol for deparaffinization, rehydration and HIER as the one described previously was followed. Blocking of non-specific binding was performed with 1% bovine serum albumin (BSA) in TBS-Triton-X-100 (0.025%) for 2 hours. Sudan Black B (Sigma Aldrich, USA) staining was used to inhibit autofluorescence. Slides were further incubated with Mouse CD4 antibody or Mouse Anti-CD8 antibody (Abcam, UK), both diluted 1:200, overnight at 4°C. For visualization, fluorochrome conjugated secondary antibody (AlexaFluor 488; Cell Signaling Technology, USA) was utilized. Detection of nuclei was done with 4′,6-diamidino-2-phenylindole (DAPI) counterstaining. Slides were dried and mounted using Mowiol mounting media (Merck Millipore, USA).

2.6. Histochemistry

Cresyl violet staining (Nissl staining) was used to demonstrate patterns of tissue injury and neuronal degeneration. Deparaffinization and rehydration was performed

as previously described in Immunohistochemistry section. Slides were stained using 0.1% Cresyl Violet acetate (Sigma Aldrich, USA), incubating for 10 minutes at room temperature. Slides were further immersed in 95% alcohol with glacial acetic acid, added to the alcohol solution to make it acidic and enhance differentiation reaction. This step is carried out in order to whiten the background, while the nuclei remain stained. Finally, brain sections were dehydrated in alcohol, cleared in xylene and mounted with Entellan (Merck Millipore, USA).

2.7. Neurological evaluation

In the animals from the experiments in which we tested the effects of the application of enoxaparin, motor functions were evaluated 1 day and 2 days post-injury using the modified neurological severity score (mNSS). Baseline evaluation was performed 1 day before TBI. Seven tests were performed to assess neurological deficits, each scoring between 0 (complete motor impairment) to 4 (complete capability), with a total number of points in the scoring system ranging between 0 and 28. Tests included left and right forelimb flexion, left and right hindlimb flexion (both during suspension by the tail), resistance to lateral pulsion and power to withstand movements in different directions (left, right, vertical) on an inclined plane (52,53).

2.8. Image quantification, data collection and analysis

Quantitative analyses of the immunohistochemical and the immunofluorescent photomicrographs were done by random selection of 4 to 6 visual fields on 40x magnification. Microsoft Excel and Microsoft PowerPoint were used to collect data and prepare tables and figures (Microsoft Corporation, USA). Analysis of photomicrographs was performed using the ImageJ software (NIH, USA). Statistical analyses were obtained using Statistica software, version 13.0, (Dell, USA). The

results are expressed as means ± standard errors of the mean (SEM) and considered statistically significant if *P* value was less than 0.05, unless stated otherwise.

3. Results

3.1. Pathohistological changes following traumatic brain injury

Neuron-specific, cresyl violet staining was used to visualize neuronal somata in the brain injured animals. Neurodegeneration was observed in the cortex, hippocampus, and thalamus of the injured animals of the animals from both experimental groups with TBI. As Figure 2. shows, pyknotic nuclei of apoptotic neurons are darker and dense. Patterns of neurodegeneration can be seen in the ipsilateral cortex as a perturbation of the cytoarchitecture. Furthermore, diffuse damage in the ipsilateral hippocampi and thalami is noticed. A considerable dispersion in the cellular layer appears in the cornu ammonis 2 and 3 (CA2 and CA3) regions of the hippocampus. Regarding the thalamus, Figure 2. demonstrates diffuse distribution of cell death in the neurons of the ventroposteromedial and ventroposterolateral nuclei.

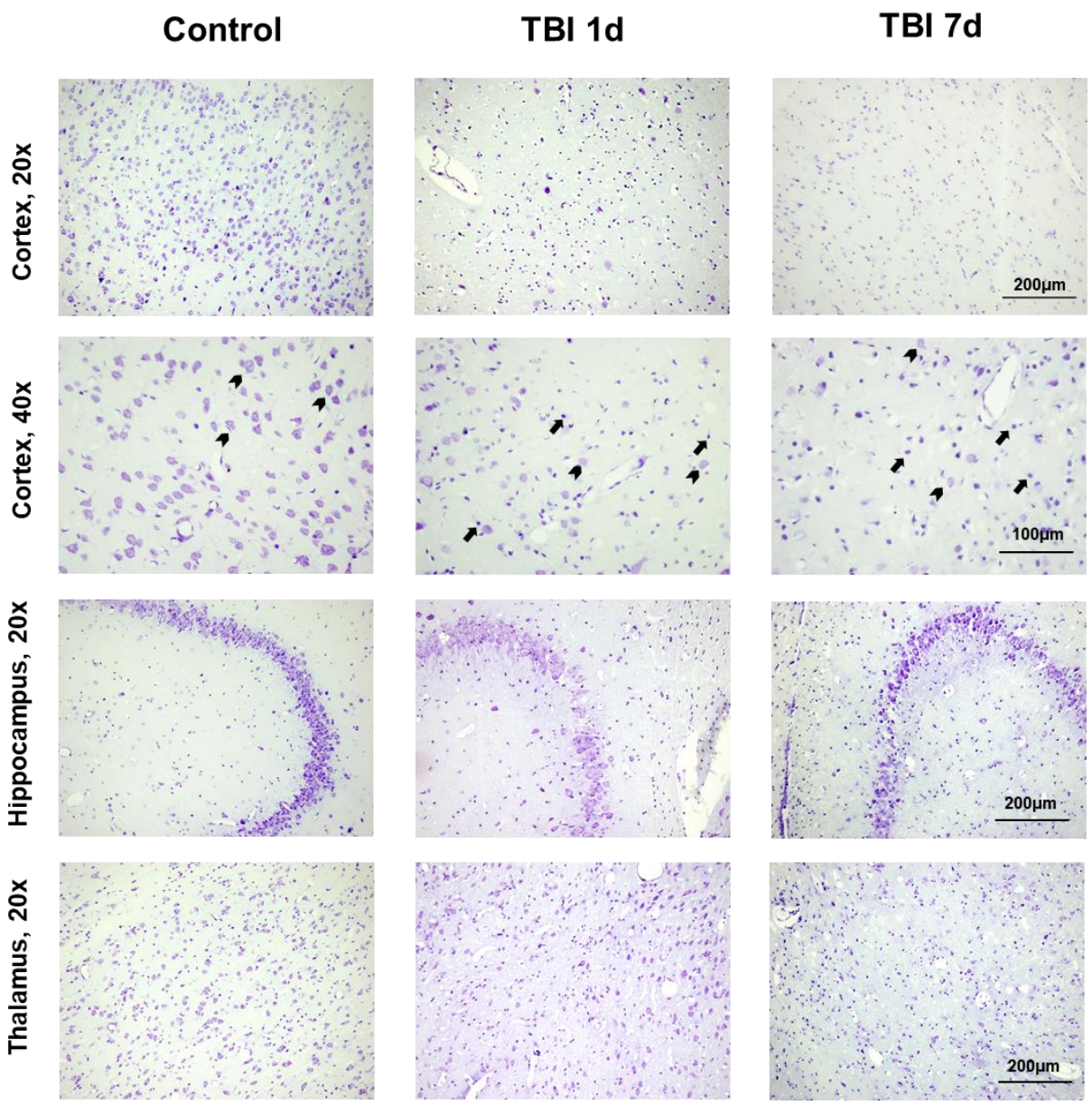

Figure 2. Pathohistological evaluation of neural degeneration after traumatic brain injury (TBI) in the rat. Cresyl violet (Nissl) stained ipsilateral cortices, hippocampi, and thalami of the sham-operated animal (Control) and rats sacrificed 1 day (TBI 1 d) and 7 days (TBI 7d) after the traumatic injury are shown. Arrows indicate apoptotic neurons, some of which have corkscrew-like dendrites. Arrowheads indicate healthy neurons with Nissl bodies. Note neuronal loss in all three examined regions; especially disarrangement in the cortical cytoarchitecture as well as the demarcation of the CA2/CA3 border. Scale bars: 200 μm at 20x magnification; 100 μm at 40x magnification.

3.2. Immunohistochemical analysis of cortical CD3+ cells distribution following LFPI in the rat

We next analyzed immunohistochemically distribution of the cells expressing CD3 protein, T cell co-receptor, in the rat parietal cortex within the first week following LFPI (Figure 3). As Figure 4. demonstrates, an upsurge in the number of CD3+ cells was detectable at 24 hours after TBI compared to the results from the cortices of the sham-treated animals [one-way ANOVA: F (3, 18)=4,3214, *P*=0.018], which decreased over the next days following injury. Differences in the distribution across the cortex to the lesion respectively were not observed.

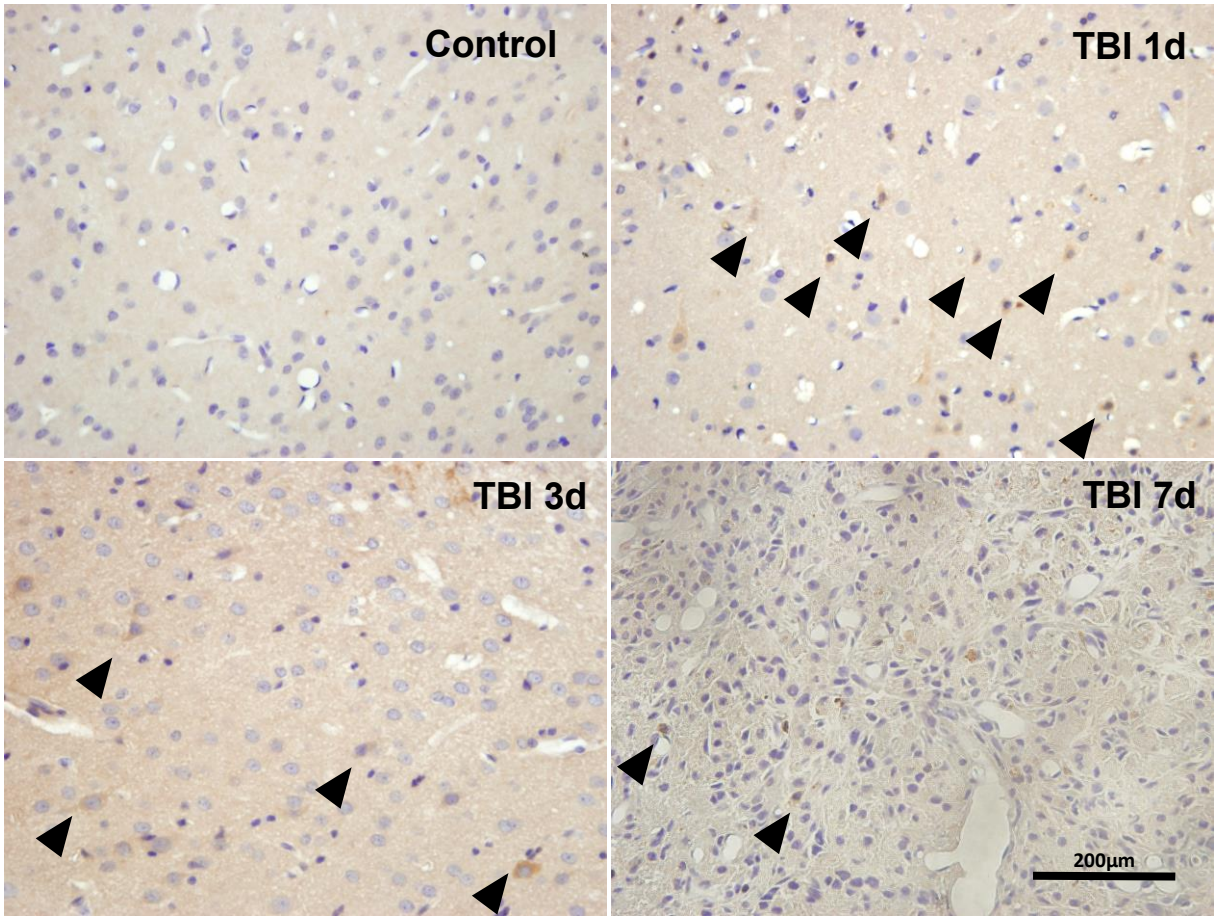

Figure 3. Immunohistochemical staining of CD3 in the ipsilateral parietal cortex after traumatic brain injury (TBI) in the rat. Representative photomicrographs of CD3-stained cells in the control (Control) animal and rats sacrificed at 1 day (TBI 1d), 3 days (TBI 3d) or 7 days (TBI 7d) after brain trauma. Arrowheads indicate CD3+ cells. Note the abundance of infiltrating cells in the cortex of the animal from the TBI 1d group. Scale bar: 200 µm.

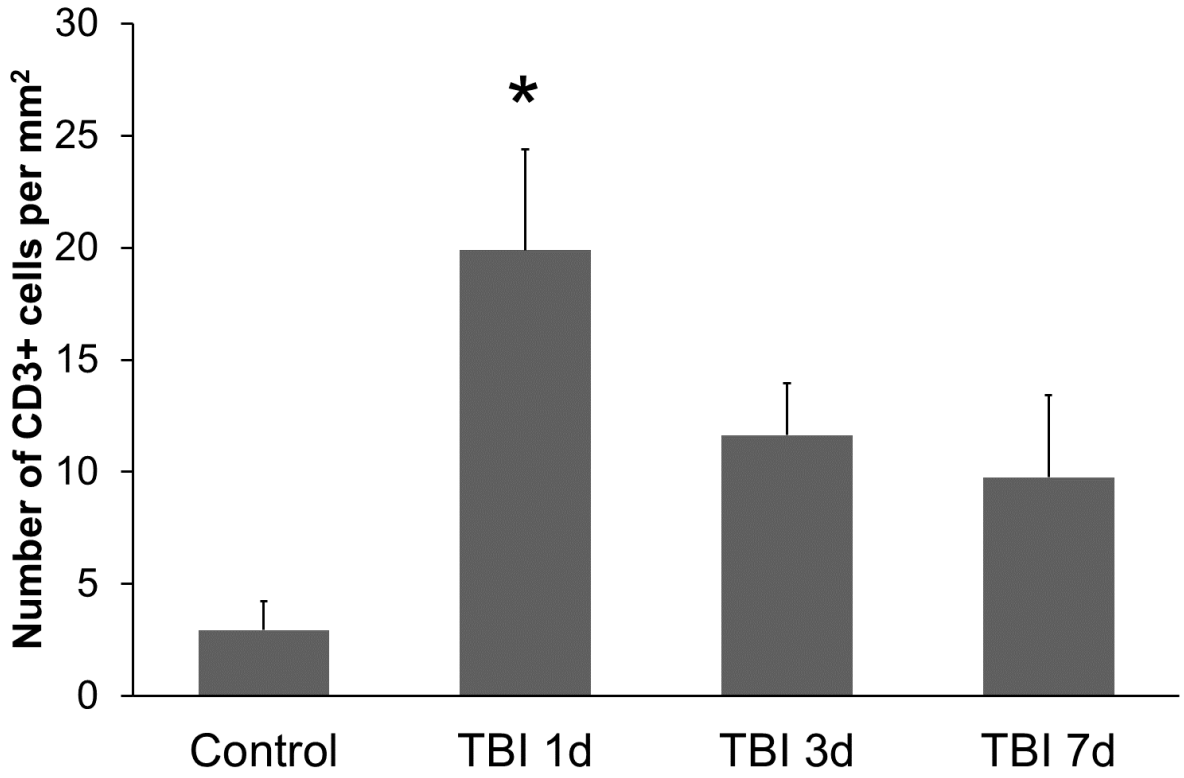

Figure 4. **Quantitative analysis of the CD3+ cells' cortical infiltration at different time points after traumatic brain injury (TBI) in the rat.** Histogram shows the quantitative analysis of CD3+ cells with data expressed as number of cells per mm^2. Sham-treated animals (Control) were sacrificed 1 day, and the injured rats were sacrificed at 1 day (TBI 1d), 3 days (TBI 3d) or 7 days (TBI 7d) following brain trauma. Data is represented as means ± SEM (N = 4-7 animals/group). *P<0.05, significantly different from the Control group.

3.3. Immunohistochemical analysis of cortical CD4+ and CD8+ cells distribution following LFPI in the rat

Presence of the cells expressing CD4+ and CD8+ markers was evaluated by immunofluorescence. Cells that are most likely to be CD8+ are cytotoxic T lymphocytes and M1 microglia/macrophages, both part of the pro-inflammatory cascade. Invasion of cytotoxic lymphocytes signals damage to the blood-brain barrier. Infiltration of CD8+ cells was noticed in the subpial space (Figure 5.) and the ipsilateral

cortices of the injured animals at all investigated time points. (Figure 6.). Cells were not quantified due to their sporadic presence in the investigated brain region.

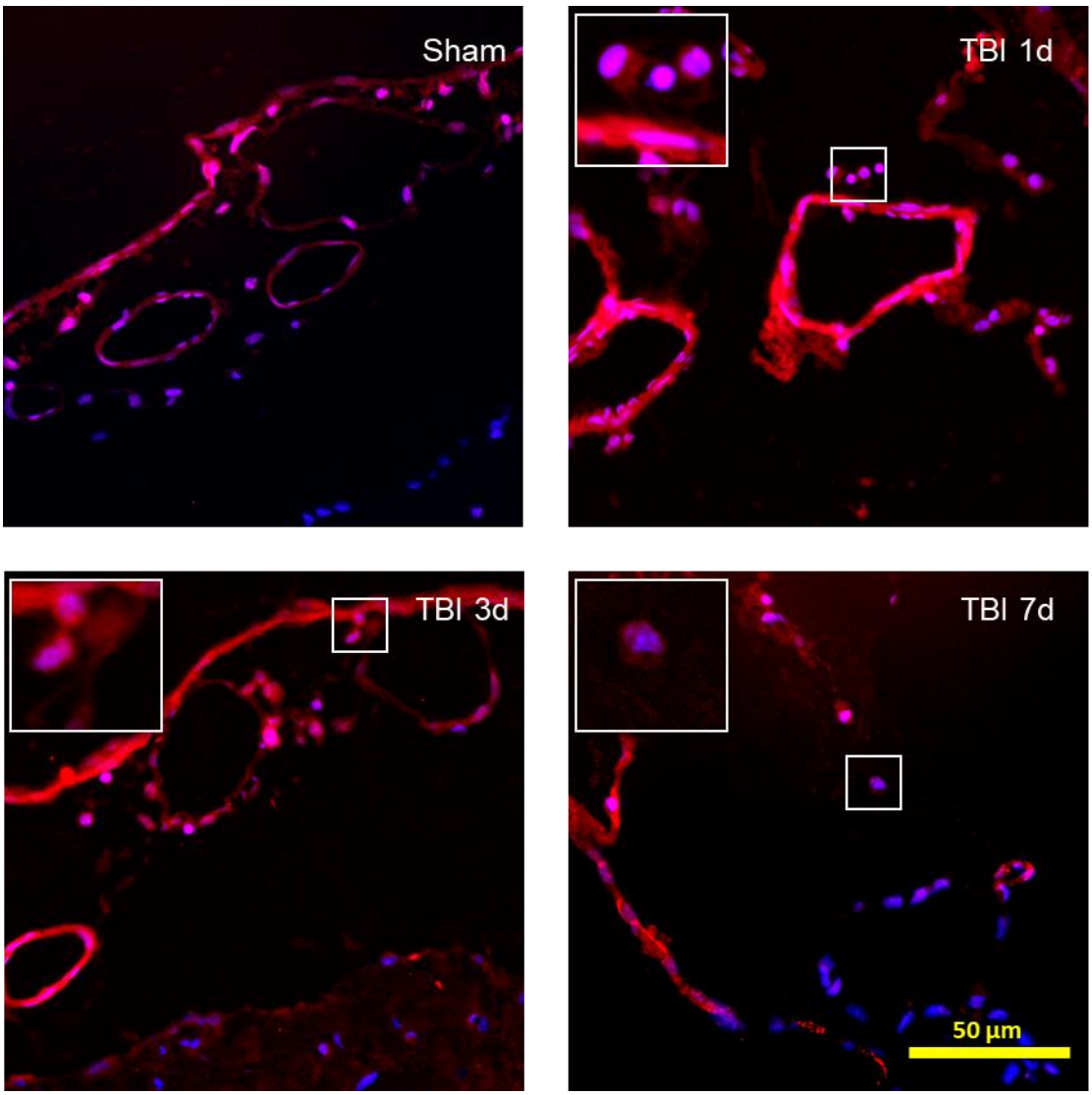

Figure 5. Immunofluorescent detection of CD8+ cells in the ipsilateral subpial space after traumatic brain injury (TBI) in the rat. Representative photomicrographs show CD8-immunostained sections from the sham-treated animal (Control) group and the rats sacrificed 1 day (TBI 1d), 3 days (TBI 3d), or 7 days (TBI 7d) following brain trauma. Inserts show detail at a higher magnification. Scale bar: 50 µm.

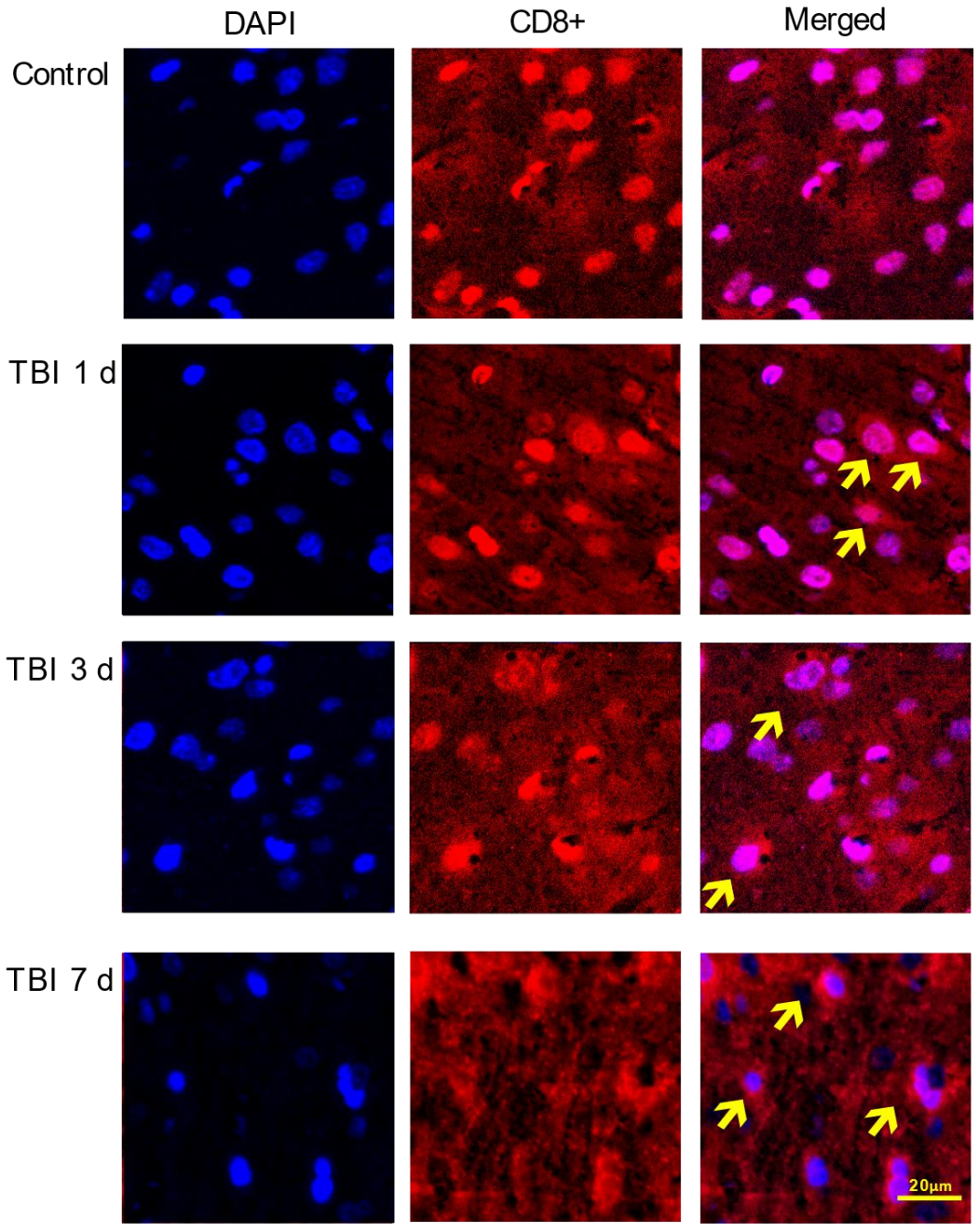

Figure 6. Immunofluorescent detection of CD8+ cells in the ipsilateral perilesional part of the parietal cortex after traumatic brain injury (TBI) in the rat. Representative photomicrographs show cortical sections from the rat of the control group (Control), and the animals sacrificed at 1 day (TBI 1d), 3 days (TBI 3d), or 7 days (TBI 3d) following brain trauma. Arrows point to CD8-immunostained cells. DAPI staining shown in blue in all images. Scale bar: 20 µm.

Congruously, the number of cells exhibiting CD4+ phenotype (Figure 7.), a marker of helper T cells, also rose upon injury [one-way ANOVA: $F(3,9) = 5.667$; $P = 0.018$] (Figure 8.). We observed a slight, but non-significant rise in the number of CD4+ cells at 1 day post-trauma compared to control group, which was followed by a statistically significant peak on the third post-injury day (post-hoc Duncan's multiple range test: $P = 0.004$). At the last investigated time point, i.e. on the seventh day following TBI, the number of CD4+ cells was not significantly different from the result detected in the control animals.

Furthermore, regarding the cellular morphology of CD4+ stained cells, we observed different types of cells presenting this molecular marker. CD4+ cells were mostly observed as cells with small round nuclei and spherical cell shape, which is characteristic for the lymphocytes. However, some of the CD4+ cells resembled microglia/macrophages in their morphology, as they were of amoeboid shape with scant branching, a morphological hallmark of activated microglia (Figure 9.)

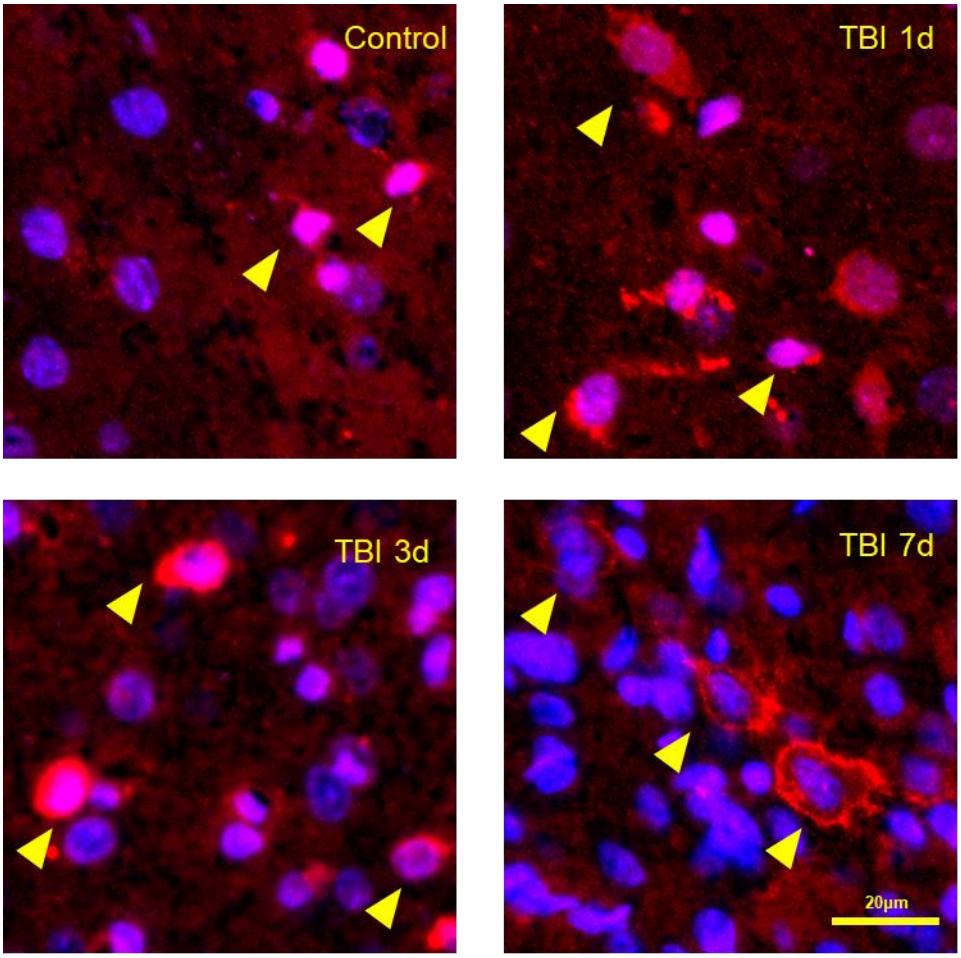

Figure 7. CD4+ cells in the ipsilateral cortex after traumatic brain injury (TBI) in the rat. Representative photomicrographs show cortical sections from the rat of the control group (Control), and the animals sacrificed at 1 day (TBI 1d), 3 days (TBI 3d), or 7 days (TBI 3d) following brain trauma. Arrowheads point to CD4-immunostained cells. Scale bar: 20 µm.

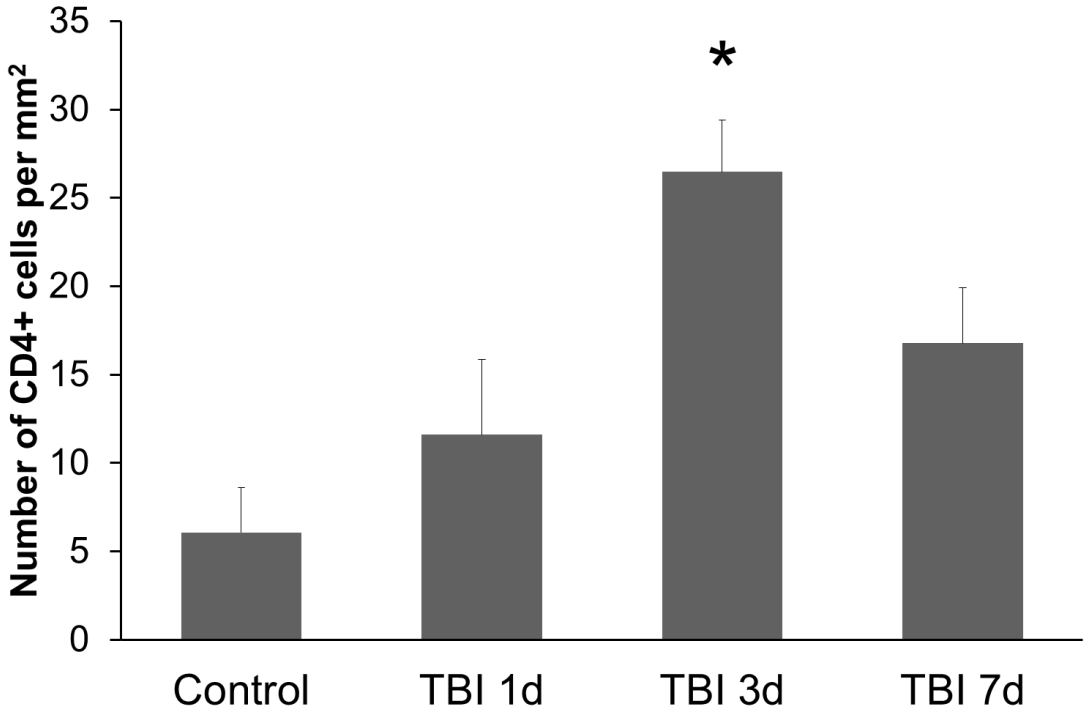

Figure 8. Quantitative analysis of the CD4+ cells' cortical infiltration at different time points after traumatic brain injury (TBI) in the rat. Sham-treated animals (Control) were sacrificed 1 day, and the injured rats were sacrificed at 1 day (TBI 1d), 3 days (TBI 3d) or 7 days (TBI 7d) following brain trauma. Data is represented as means ± SEM (N = 3-4 animals/group). **P*<0.05, significantly different from the Control group.

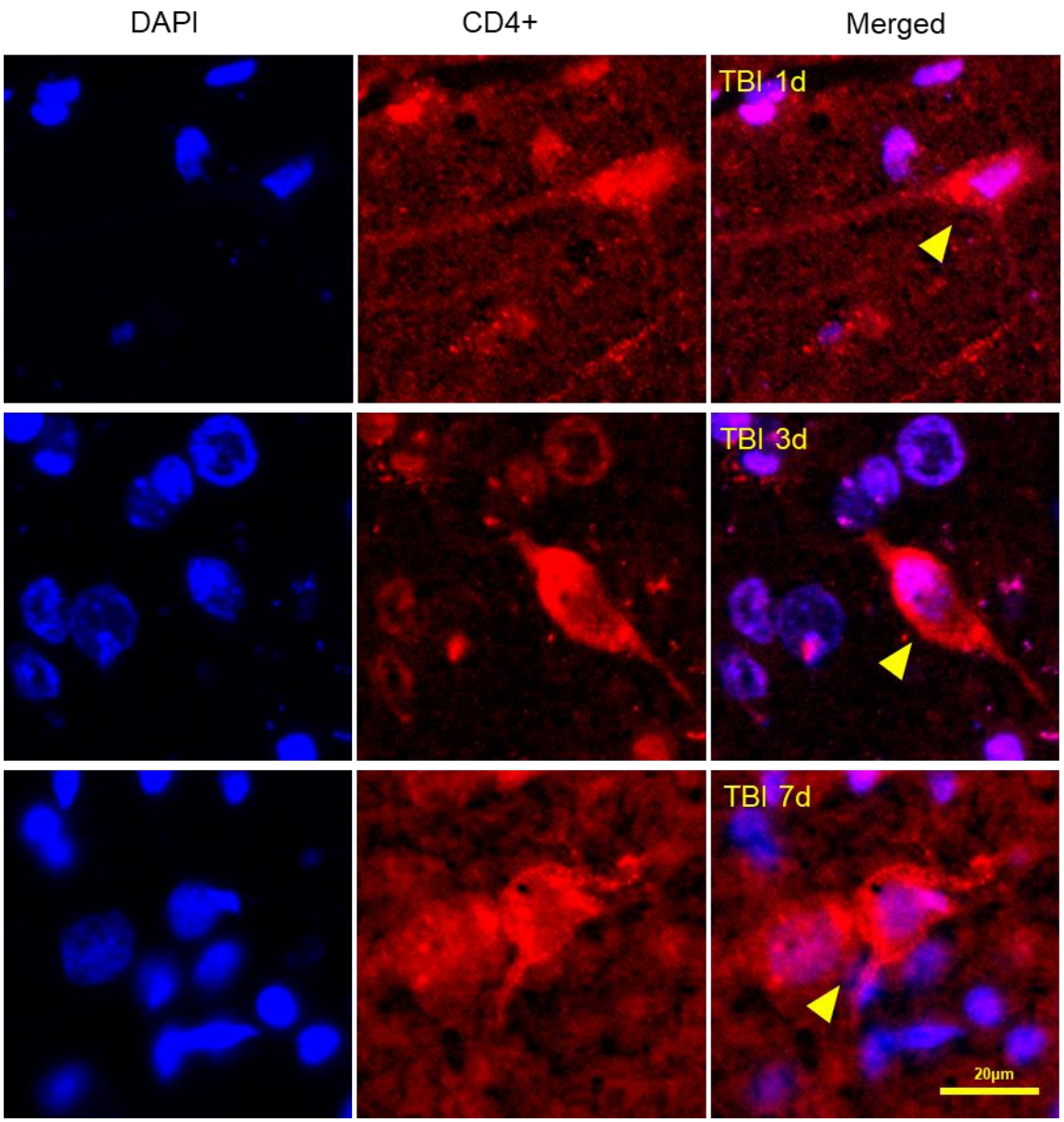

Figure 9. CD4+ cells exhibiting glial morphology in the parietal cortex after traumatic brain injury (TBI) in the rat. Representative photomicrographs of anti-CD4+ immunostained cortices in the rats killed at 1 day (TBI 1d), 3 days (TBI 3d), 7 days (TBI 7d) post-injury. Some of the CD4+ cells resemble activated microglia, with increased cell body size and thick, retracted rami (highlighted with arrowheads). Scale bar: 20 µm.

3.4. The effects of enoxaparin following traumatic brain injury in the rat

3.4.1. Neuromotor impairment after LFPI and the effects of enoxaparin

To determine how the TBI affects motor skills and the effectiveness of enoxaparin on this aspect of the brain injury, scoring of the severity of neurological outcomes was performed in the rats exposed to moderate LFPI. Neuromotor functions were evaluated on the first and the second day after the injury using a 28-point neuroscore scale. Non-parametric, Kruskal-Wallis test was used to analyze the differences between the experimental groups, followed by a Mann-Whitney U test for post-hoc comparisons. Brain-injured animals from both groups (vehicle only and enoxaparin group) exhibited a statistically significant decrease in the overall motor functioning on both 1 [$H(2, 32) = 22.070$; $P < 0.001$] and 2 days [$H(2, 32) = 21.385$; $P < 0.001$] after TBI in comparison with the results observed in the animals of the control group (Figure 10.) No difference was found between the NSS test results obtained in the enoxaparin- and vehicle-treated animals (day 1: $P = 0.170$; day 2: $P = 0.622$).

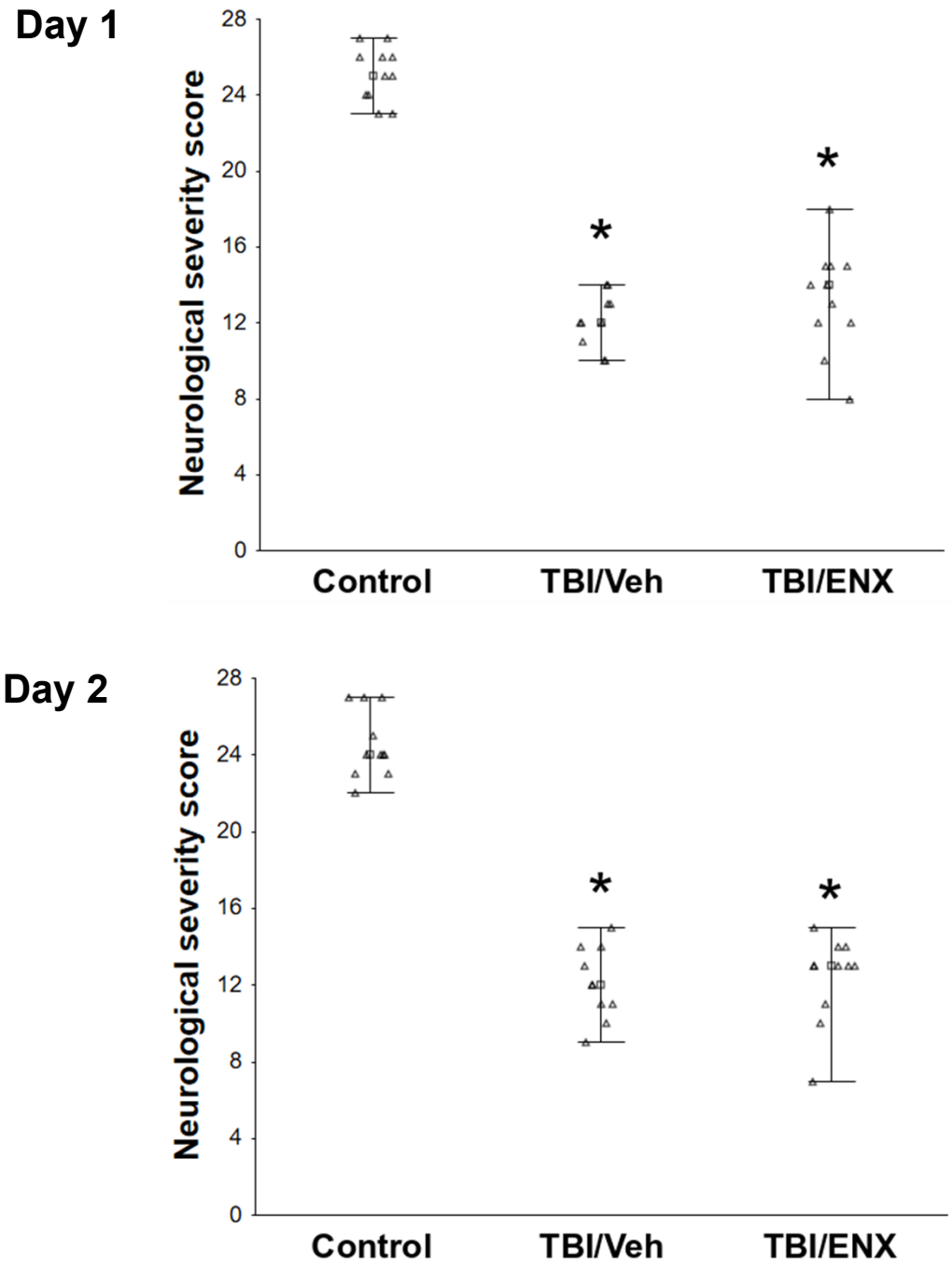

Figure 10. Neurological severity score evaluated 1 and 2 days after traumatic brain injury (TBI) in the rat. Control group (Control), TBI group treated with vehicle (TBI/Veh), TBI group treated with 1 mg/kg of enoxaparin (TBI/ENX). Data is represented as means ± SEM (N = 10-11 animals/group). *P<0.01, significantly different from the Sham/Veh group.

3.4.2. The effects of enoxaparin on the number of CD3+ and CD4+ cells in the rat parietal cortex following traumatic brain injury

Immunohistochemical analysis of the presence of CD3+ cells in the parietal cortices of rats treated with enoxaparin or vehicle following TBI was conducted (Figure 11.). No significant difference in the number of CD3+ cells was detected in our experimental conditions (Student's t-test: $P = 0.845$).

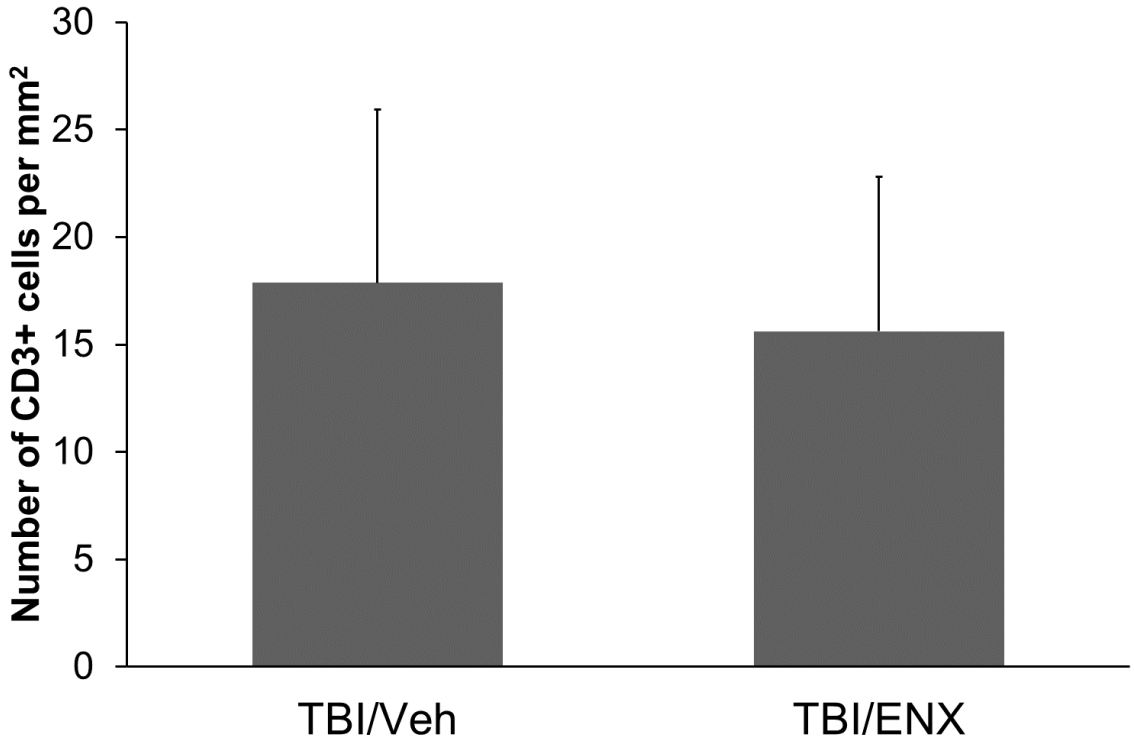

Figure 11. The effect of enoxaparin application on the CD3+ cells' cortical infiltration after traumatic brain injury (TBI) in the rat. Histogram shows the quantitative analysis of CD3+ cells expressed as number of cells per mm^2. Injured rats were administered vehicle (TBI/Veh) or 1 mg/kg of enoxaparin (TBI/ENX) and sacrificed 48 h following brain trauma. Data is represented as means ± SEM (N = 3 animals/group).

Analysis of the number of cortical CD4+ cells in the brain-injured rats treated with the investigated LMWH or vehicle did not reveal a difference between the two experimental groups (Student's t-test: *P* = 0.774) (Figure 12.).

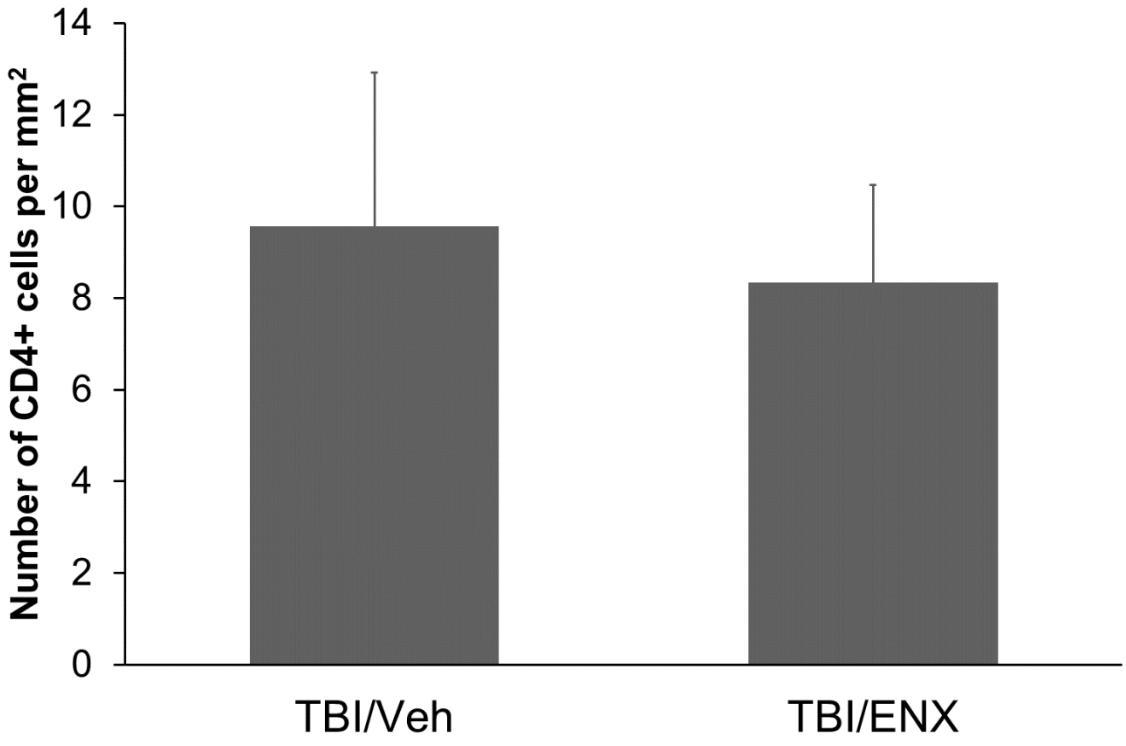

Figure 12. The effect of enoxaparin application on the cortical expression of CD4+ cells after traumatic brain injury (TBI) in the rat. Histogram shows the quantitative analysis of CD4+ cells expressed as number of cells per mm^2. Injured rats were administered vehicle (TBI/Veh) or 1 mg/kg enoxaparin (TBI/ENX) and sacrificed 48 h following brain trauma. Data is represented as means ± SEM (N = 3 animals/group).

4. Discussion

The results of this study show T-cell mediated immune response in the first 7 days following TBI, particularly early infiltration of CD3+ and CD4+ in the brain parenchyma with the maximum number after the first 72 hours. The effects of enoxaparin were also evaluated for alleviating T cell invasion and motor deficits after TBI; however, results were insignificant.

4.1. Posttraumatic cortical infiltration of T cells

TBI affects the continuity of BBB, leading to the exposure of CNS antigens to the peripheral immune system. In normal conditions, immune surveillance of the CNS takes place in the meningeal lymph vessels and through the glymphatic system, involved in the clearance in the CNS connecting it to the peripheral lymphatics (16,21,23,54). Once the BBB becomes dysfunctional both by mechanical and molecular disruption, peripheral immune cells can migrate to the CNS. While microglia as antigen-presenting cells native to the brain do not exhibit self-reactivity towards CNS antigens (as it would be hazardous), peripheral T-cells do recognize MHC II molecules expressed on microglial cells (17). The role, as well as specific mechanisms, of activation of adaptive immunity following CNS trauma, is not defined. Nevertheless, the infiltration of T cells in the brain parenchyma due to antigen retrieval must be more than an incidental finding, as several studies in the past two decades have shown a relationship between TBI outcome and levels of activity of T cells (21,25,26,54–57).

Here we discuss the invasion of T cells in the cortex over 7 days after TBI. Cortex was chosen as the region of interest as it is subjected to the highest force of injury after LFPI along with its anatomical closeness to the lymph vessels in the dural venous sinuses.

4.1.1. CD3 positive cells in the ipsilateral cortex

The CD3 molecule is a surface marker of T lymphocytes expressed on all T cells, which makes it a pan-T cell marker (23). There is contrasting evidence on the fluctuation of lymphocyte numbers in the peripheral blood, discussing both in the favor of lymphocyte expansion (58) and lymphopenia (54) occurring after TBI, proposing that lymphocytes migrate to the injured tissue. Results of this study demonstrate cortical invasion in the ipsilateral cortex at the site of the lesion. CD3 positive cells can be detected in the cortex 24 hours after the injury, with a decrease on the 3rd day and further decline on the 7th day. One interpretation of the given data is that the resolution of inflammatory processes begins between 1st and 3rd day post-TBI and settles toward the 7th day.

4.1.2. Cytotoxic and T helper cells

Naïve CD4+ T helper (Th) cells differentiate to Th1 or Th2 CD4+ cells (23). Nevertheless, the purpose and impact of Th response in TBI remain vague. It is traditionally deemed as beneficial (17), but Fee et al. (57) demonstrated that treatment with CD4+ T cells after TBI worsened outcomes in immunodeficient, T and B cell depleted mice.

Our results show the presence of CD4+ cells in the cortex after TBI. Although CD4 is a classic marker of T cells, we suggest that it could also be expressed by activated microglia, based on the morphology of the cells. All CD4+ T helper cells also express CD3+ marker, but our results do not show correspondence in peaks in these T cell subtypes, suggesting that another cell line exhibits CD4 positivity and promotes inflammatory cascade. Further studies, possibly double immunohistological staining of Iba1 or MHC II with CD4, is needed to confirm this hypothesis. Another possible

explanation of our findings is the cross-reactivity of the chosen antibody with other CD molecules of similar structure.

Another observation has been made on the entrance of cytotoxic, CD8+ cells. Daglas et al. (25) have found a negative correlation between CD8+ count and recovery after TBI. We wanted to show the presence of CD8+ cells, most likely T cells, beneath pia mater and in the cortex, to confirm infiltration of this T cell type in our TBI model.

Sham craniotomy disrupts the continuum of the BBB by causing damage in the small blood vessels. These alterations can be a product of a direct mechanical hit, or it could be molecule-mediated (59). The above mentioned could explain the occasional presence of T cells in the examined cortices of the animals of the control group, as CNS is considered an immunologically privileged organ protected from the peripheral immune cell infiltration (23).

The precise role of T cells in LFPI has not been studied in this research. It cannot be confirmed whether these findings are detrimental or not, or their effect on the outcome of TBI. Further research is needed to show correlations between T cells and glia, signaling between peripheral immune system and CNS-invading T cells, dependence on antigen-mediated mechanisms and finally, the ability to modify pathophysiological course of TBI and recovery.

4.2. Effects of enoxaparin in the rats following traumatic brain injury

TBI-induced cortical damage manifests with cognitive and motor dysfunctions, a consequence of neuronal damage, as our results of Nissl staining show. Behavior tests are often employed to evaluate animal performance, which directly implies a CNS lesion. We have evaluated neurological severity scores 1 and 2 days after injury, with baseline evaluation beforehand. A statistically significant difference was observed

between the control and the injured groups. However, there was no difference between injured animals treated with vehicle comparing to the group given enoxaparin. The explanation for such results could be that the neurological evaluation was performed too early after the injury, and that difference could be observed after the acute phase following the injury. Wahl et al. (34) noted improvement in the neurological recovery after enoxaparin administration 1 week after injury, which remained consistent throughout the following weeks.

From the data presented, it is evident that enoxaparin treatment did not yield any compelling advancement in the outcomes in comparison with TBI-only groups. The reasoning could be justified by the study design; perhaps higher doses (currently administered 1 mg/kg) or higher frequency (currently administered 8 times in total) of administration could be more potent and result in different outcomes. However, one should be wary of adverse bleeding risks. Another answer on the effectiveness could lie in the ability of enoxaparin to cross the blood-brain barrier. Its penetrance to the CNS is enhanced under hypoxic conditions (32). Although mimicking hypoxia to alter penetrance of the drug in the CNS would be counterproductive and unethical at least, perhaps a novel formulation of the drug could lead to better bioavailability.

5. Conclusion

To our knowledge, this is the first study to investigate the infiltration of T cells in the cortex using the LFPI model of TBI in the rat. Our findings point out that the invasion of peripheral immune cells occurs early after brain injury, with both cytotoxic and helper T cells being activated, which indicates a prompt response of the cellular arm of adaptive immunity. Glia-like cells expressing CD4+ markers were found at all time points after injury. Moreover, enoxaparin was tested, and its effects were studied at 48 hours following TBI. Despite trends in the decline of investigated molecules in the enoxaparin treated group, observed effects were insignificant. Lastly, neuromotor impairment after TBI was observed, with a tendency of improvement in the acute phase of injury. Further research should be done to elucidate the relationship between T cell activation and TBI outcomes, patterns of migration of different T cell subtypes, as well as long term consequences of lymphocyte infiltration on molecular homeostasis in an apparently healthy brain.

Summary

Traumatic brain injury (TBI) is considered a major epidemiological and socioeconomic issue of the modern age, due to traffic accidents, sports, or warfare injuries. Although there are several established animal models of TBI that portray different patterns of injury caused by brain trauma and that shed light upon pathophysiological sequelae, much is still unknown about the link between immune response and TBI. The aim of this research was to investigate the infiltration of T cells, indicating activation of the adaptive immunity in the brain tissue, within the first week after experimental TBI in the rat. Lateral fluid percussion injury (LFPI), a model of TBI causing both focal cortical lesion and diffuse damage in the ipsilateral hemisphere, was induced in male Wistar rats. Experiments were divided into two parts. One experiment was conducted to evaluate the time course of neuroimmune mechanisms, with animals being sacrificed at 1, 3, or 7 days post-TBI. The other arm consisted of the application of the enoxaparin (1 mg/kg), low molecular weight heparin to the rats exposed to LFPI. After the injury, animals were administered with vehicle or enoxaparin over the course of 48 hours. Sham craniotomy and vehicle treatment were performed on the control group. The results of this study show the invasion of CD3+, CD4+, and CD8+ cells in the cortices of the brain-injured animals. Enoxaparin treatment did not affect CD3+ or CD4+ cell numbers in the cortices of injured animals as it also had no significant effect on the TBI-induced neuromotor impairment. In conclusion, the results of this study indicate that the LFPI model of TBI can elicit a cellular immune response, although significant effects of enoxaparin were not observed.

Key words: adaptive immunity; brain injuries, traumatic; enoxaparin; T-lymphocytes; rats

Sažetak

Traumatska ozljeda mozga (engl. traumatic brain injury, TBI) smatra se znatnim epidemiološkim i socioekonomskim problemom modernoga doba, uslijed prometnih nesreća, sportskih ili ratom uzrokovanih ozljeda. Iako je afirmirano nekoliko životinjskih modela traume mozga koji pobliže razjašnjavaju različite patofiziološke mehanizme, i dalje se ne zna mnogo o poveznici između adaptivne imunosti i traume mozga. Cilj ovog istraživanja bio je istražiti infiltraciju T stanica u prvome tjednu nakon eksperimentalne trauma mozga u štakora, što upućuje na aktivaciju mehanizama stanične imunosti u moždanome tkivu. Lateralna ozljeda tlakom tekućine (engl *lateral fluid percussion injury*, LFPI) vrsta je TBI koja uzrokuje žarišnu kortikalnu leziju i difuzno oštećenje u ipsilateralnoj hemisferi. LFPI uzrokovana je na Wistar štakorima muškog spola. Pokusi su podijeljeni u 2 dijela. Prvi je pokus planiran za evaluaciju vremenskog slijeda neuroimunih mehanizama gdje su životinje žrtvovane 1, 3, odnosno 7 dana nakon traume. U drugome su pokusu dani enoksaparin (1 mg/kg), niskomolekularni heparin, ili vehikul tijekom 48 sati životinjama kojima je inducirana LFPI. Lažno žrtvovane, kraniotomiji podvrgnute životinje kojima je apliciran vehikul, sačinjavale su kontrolnu skupinu. Rezultati ovog istraživanja pokazuju invaziju CD3, CD4, i CD8 pozitivnih stanica u korteksima ozlijeđenih životinja.. Tretman enoksaparinom nije utjecao na broj CD3+ i CD4+ stanica u korteksima ozlijeđenih životinja , niti je pokazao značajan utjecaj na traumom uzrokovan deficit u motornim funkcijama. Navedeni rezultati govore u prilog aktivacije mehanizama stanične imunosti u LFPI modelu, iako nisu uočeni značajni učinci enoksaparina.

Ključne riječi: adaptivna imunost; enoksaparin; traumatska ozljeda mozga, štakor , T limfociti

Curriculum Vitae

Ljerka Delač was born on the 6th of February 1996, in Rijeka, Croatia. There she attended elementary school and high school. After graduating from Gymnasium „Andrija Mohorovičić Rijeka“in 2014, she enrolled in medical studies at the Faculty of Medicine, University of Rijeka. During her studies, she has been an active member of the Croatian Medical Student Association (CroMSIC) and has engaged in, or organized herself, various activities, ranging from mental health project for adolescents to charity fundraisers. Early on she has acknowledged her interest in science, especially neuroscience, which has been strengthened through participation in IFMSA and Erasmus+ research exchanges in different laboratories as well as volunteering at the Department of Pharmacology, where she carried out her master thesis. Likewise, she has been a member of the Organising Committee of the Student Congress of Neuroscience at her university. For 4 consecutive years, she was working as a student assistant at the Department of Medical Biology and Genetics, Faculty of Medicine, University of Rijeka. She is fluent in English and can speak and write German.

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