Neuroprotective Effect of Plasminogen Activator Inhibitor-1 Antagonist in the Rat Model of Mild Traumatic Brain Injury

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Abstract——Plasminogen activator inhibitor-1 (PAI-1) antagonists are known for their neuroprotective effects. In this study, it was aimed to investigate the possible protective effects of PAI-1 antagonists in a rat mild traumatic brain injury (TBI) model. Sprague–Dawley male rats were grouped as sham (n = 7), TBI (n = 9), and TBI + PAI-1 antagonist (5 and 10 mg/kg TM5441 and TM5484; n = 6–7). Under anesthesia, TBI was induced by dropping a metal 300-g weight from a height of 1 m on the skull. Before and 24-h after trauma neurological examination, tail suspension, Y-maze, and novel object recognition tests were performed. Twenty-four hours after TBI, the rats were decapitated and activities of myeloperoxidase, nitric oxide release, luminol-, and lucigenin-enhanced chemiluminescence were measured. Also, interleukin-1β, interleukin-6, tumor necrosis factor, interleukin-10, tumor growth factor-β, caspase-3, cleaved caspase-3, and PAI levels were measured with the ELISA method in the brain tissue. Brain injury was graded histopathologically following hematoxylin–eosin staining. Western blot and immunohistochemical investigation for low-density lipoprotein receptor, matrix metalloproteinase-3, and nuclear

Highlights
• After mild TBI, PAI-1 antagonists TM5441 and TM5484 were used for the first time in the literature.
• PAI-1 inhibition reduced oxidative stress, inflammation, and neuronal damage in a TBI model.
• PAI antagonist treatment also improved corticospinal pathway functions and behavioral results.

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Abbreviations CL, Chemiluminescence; DG, Dentate gyrus; DI, Discrimination index; DS, Difference score; IL, Interleukin; LDLR, Low-density lipoprotein receptor; MMP3, Matrix metalloproteinase-3; MPO, Myeloperoxidase; NF-κB, Nuclear factor-κB; NO, Nitric oxide; PAI-1, Plasminogen activator inhibitor-1; PBS, Phosphate-buffered saline; PMNs, Polymorphonuclear leukocytes; RI, Recognition index; ROM, Reactive oxygen metabolites; ROS, Reactive oxygen species; TBI, Traumatic brain injury; TGF, Tumor growth factor; TNF, Tumor necrosis factor; tPA, Tissue plasminogen activator
factor-κB were also performed. Data were analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) and expressed as means ± SEM. Values of $p < 0.05$ were considered to be statistically significant. Higher levels of myeloperoxidase activity in the TBI group ($p < 0.05$) were found to be suppressed in 5 and 10 mg/kg TM5441 treatment groups ($p < 0.05$–$p < 0.01$). The tail suspension test score was increased in the TBI group ($p < 0.001$) and decreased in all treatment groups ($p < 0.05$–0.001). The histologic damage score was increased statistically significantly in the cortex, dentate gyrus, and CA3 regions in the TBI group ($p < 0.01$–0.001), decreased in the treatment groups in the cortex and dentate gyrus ($p < 0.05$–0.001). PAI antagonists, especially TM5441, have antioxidant and anti-inflammatory properties against mild TBI in the acute period. Behavioral test results were also improved after PAI antagonist treatment after mild TBI.

**KEY WORDS:** antioxidant; anti-inflammatory; neuroprotection; Plasminogen activator inhibitor-1 antagonist; traumatic brain injury

**INTRODUCTION**

Traumatic brain injury (TBI) is defined as a change in brain function, or the presence of another brain pathology as a result of an external force [1]. TBI is one of the main causes of disability and death worldwide, especially in the young population [2–5]. At least 2–5 million new cases of TBI occur each year in European countries [5]. In the USA, it is estimated that 52,000 people die annually, and 530,000 people experience disability due to TBI [6]. TBI is a process, not a static injury, and long-term symptoms in TBI survivors are known to cause structural and functional damage. It is estimated that 10 million people in the USA and Europe experience permanent disability and cognitive impairment due to TBI [7, 8]. TBI also contributes the formation of the neurological diseases such as epilepsy, Alzheimer’s disease, Parkinson’s disease, and chronic neuritis occurring years after initial injury [9]. Patients and their caregivers can experience serious economic and social consequences [5]. TBI can be classified as mild, moderate, or severe according to the patient’s neurological signs and symptoms [5]. As an estimate, 75–90% of all TBI patients are categorized as mild TBI [10, 11].

The effects of TBI are divided into primary and secondary injuries. Primary injury is the mechanical damage that develops at the time of trauma and occurs directly in neurovascular structures and glial cells. Secondary injury begins minutes after the trauma, and all available treatment modalities are directed at the secondary injury. Secondary injury is associated with pathophysiological mechanisms such as excitotoxicity, ionic imbalance due to lack of energy, inflammation, oxidative stress, and apoptosis [12, 13]. Studies aimed at treating TBI have often focused on neuroprotective agents, and a drug that has reached clinical efficacy has not yet been found [14, 15].

PAI-1 is a key endogenous inhibitor of plasmin-mediated fibrinolysis and increases clot formation after injury [16]. It is a member of the serine protease inhibitor superfamily [17]. PAI-1 is found in plasma, vascular endothelium, liver, adipose tissue, neutrophils, astrocytes, and platelets [18–21]. It provides a tight balance between clot formation and clot breakdown. Normally, clot formation begins when a tissue factor is released following the damage of the vessels and the parenchyma [22]. At the same time, tissue plasminogen activator (tPA) is also released from the damaged endothelium to prevent clot formation and vascular occlusion [23]. PAI-1, which binds to active centers of tPA and uPA, causes irreversible inhibition of these fibrinolytic molecules [24, 25]. Because tPA is used for thrombolytic purposes in various medical conditions such as cerebral infarction and embolism, it is logical to assume that inhibition of known tPA inhibitors such as PAI-1 would be beneficial in preventing clot formation. However, PAI-1 is not just a tPA or a uPA inhibitor and has diverse biological actions, and its production increases under some pathological conditions. Various inflammatory stimuli and mediators induce PAI-1 release [26].

PAI-1 has clinical significance in pathologies accompanied by ischemia, inflammation, and vascular dysfunction [16, 27]. High PAI-1 levels after ischemic stroke are a risk factor and adversely affect survival [28,
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29]. PAI-1 deficiency has been reported to reduce brain damage in an experimental ischemic stroke model [16]. It was reported that PAI-1 is associated with increased coagulation response after TBI, and the absence or inhibition of PAI-1 reduces the lesion area after TBI [17]. In a human study, an increase in PAI-1 levels was observed after head trauma and has been associated with poor prognosis [30]. In 12 h after TBI the PAI mRNA level reaches its peak value. Demonstration of a role for PAI-1 in the pathophysiology of inflammation as well as cerebrovascular injury drove considerable attention to the development of PAI-1 antagonists, and no studies are testing the possible anti-inflammatory actions of PAI-1 antagonists during the secondary brain injury after TBI.

In this study, the possible neuroprotective effects of PAI-1 antagonism against mild TBI were investigated with various parameters. It is thought that the administration of PAI-1 antagonists after mild TBI will show neuroprotective activity by reducing inflammation, oxidative stress, and apoptosis and will have a positive impact on corticospinal tract functions and learning. In order to understand the role of inflammation and oxidative stress, activities of myeloperoxidase (MPO), nitric oxide (NO) release, luminol- and lucigenin-enhanced chemiluminescence, interleukin-1β (IL-1β), IL-6, TNF, IL-10, and tumor growth factor (TGF)-β levels were measured. To evaluate the apoptosis, caspase-3 and cleaved caspase-3 levels were measured. To understand the impact of PAI-1 antagonists on matrix metalloproteinase (MMP) activity, we assayed levels of signaling molecules relevant to the MMP pathway including MMP-3, low-density lipoprotein receptor (LDLR), and nuclear factor-kB (NF-kB). Since there are no comprehensive studies evaluating treatment responses in behavioral tests that deteriorate after TBI, another objective of this study is to investigate the PAI-1 antagonism on behavioral tests after mild TBI.

MATERIALS AND METHODS

All experimental procedures used in this investigation were reviewed and approved by the Marmara University Animal Care and Use Committee (March 12, 2019). Animal care and all experiments were conducted in concordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) on the protection of animals for experimental use. The ARRIVE guidelines were applied for this study. Forty-one adult male (10–12-week-old) Sprague–Dawley rats weighing 250–400 g were used. Animals were housed in an air-conditioned room with 12 h light and dark cycles maintained at constant temperature (22 ± 2 °C) and relative humidity (65–70%). Rats were fed standard laboratory chow and had free access to water.

The rats were randomly assigned to four groups as follows:

1. Sham group (n = 7): rats underwent only a skin incision under anesthesia and received a single intragastric dose of saline (0.9% NaCl, 0.1 ml/100 g) 15 min after surgery.
2. TBI group (n = 9): rats underwent TBI as described below and received a single intragastric dose of saline (0.9% NaCl, 0.1 ml/100 g) 15 min after TBI.
3. TM5441-5 mg group (n = 6): rats underwent TBI as described below and received a single intragastric dose of TM5441 (5 mg/kg in 0.5% carboxymethylcellulose, Tohoku University Miyagi, Japan) 15 min after TBI [31].
4. TM5441-10 mg group (n = 6): rats underwent TBI as described below and received a single intragastric dose of TM5441 (10 mg/kg in 0.5% carboxymethylcellulose, Tohoku University Miyagi, Japan) 15 min after TBI.
5. TM5484-5 mg group (n = 6): rats underwent TBI as described below and received a single intragastric dose of TM5484 (5 mg/kg in 0.5% carboxymethylcellulose, Tohoku University Miyagi, Japan) 15 min after TBI [32].
6. TM5484-10 mg group (n = 7): rats underwent TBI as described below and received a single intragastric dose of TM5484 (10 mg/kg in 0.5% carboxymethylcellulose, Tohoku University Miyagi, Japan) 15 min after TBI.

Anesthesia and Induction of TBI

The animals were anesthetized by an intraperitoneal injection of 0.5 mg/kg chlorpromazine (Largactil, Eczacibaşi, Turkey) and 50 mg/kg ketamine (Ketalar, Parke Davis, Turkey) combination and were allowed to breathe spontaneously. A mild TBI model, described by Marmarou et al. [33] and modified by Ucar et al. [34], was applied for head trauma. A lead object weighing 300 g was allowed to fall freely from a height of 1 m through a copper tube onto the metal disc over the skull of the rat. In order to reduce mortality, thicker metal disc was used during experiments.
In rats, weight-drop TBI models have high mortality rate [33]. Ucar et al. [34] modified the original weight-drop model in order to decrease the mortality and suggested to drop 300-g weight from 1-m height as a mild TBI model. Since height and weight are the major determinant for the severity of the TBI, the age, weight, and species of the rats also affect the clinical outcome. The severe models have over %30 mortality, and it is difficult to follow-up those rats for hours. In this study, we aimed to replicate the clinical situations where patients suffer from mild TBI, and their cognitive deteriorations were overlooked because there are no major symptoms such as unconsciousness and seizure. It is thought that, because the hippocampus is a delicate structure and has thin caliber vascular feeders, they are more prone to hypoxia which is observed after TBI. One of the underlying mechanisms is that, after TBI, microclots effect the circulation negatively. With PAI-1 antagonism, we tried the overcome this microclot formation and try to sustain the patency of hippocampal vasculature in the early stages of the TBI where most of the damage occur due to hypoxic state.

Collection and Storage of Brain Tissue Samples

All the animals were decapitated 24-h after trauma, and the brains were carefully removed. Same parts of the brain for the analysis of each parameter obtained from each animal. Brain parts that were used for biochemical analysis were stored at −80 °C, and brain parts that were used for histological investigations were stored at paraformaldehyde solution.

Biochemical Analyses

Measurement of Myeloperoxidase Activity in Brain Tissue

Myeloperoxidase, an enzyme that is mainly located in the azurophilic granules of polymorphonuclear leukocytes, is commonly used to demonstrate the accumulation of neutrophils in tissues [35]. Tissue MPO activity was evaluated as previously described [36].

Chemiluminescence Measurements in Brain Tissue

Chemiluminescence (CL) is a direct, noninvasive method for the measurement of reactive oxygen radicals that utilizes luminol and lucigenin as enhancer probes. When added to in vitro biological systems, luminol and lucigenin produce high levels of excited products. Excited electrons from these compounds generate radiating light energy or CL that can be detected by a luminometer. Luminol detects radicals such as hydroxyl ions, hydrogen peroxide, and hydrochloric acid, whereas lucigenin is selective to superoxide anions [37]. Reactive oxygen species (ROS) were numerically measured after the addition of 0.2 mM of enhancers luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (bis-N-methylacridinium nitrate) (Sigma-Aldrich, USA). Purified luminol-hydrogen peroxide system was used for nitric oxide measurement. For this purpose, potassium carbonate (K₂CO₃) (0.4 mM), desferal (60 µM), H2O2 (4 mM), and purified luminol (3.6 M) were added to the tissues in 2 mL of phosphate-buffered saline (PBS) + HEPES buffer [38, 39]. Counts were measured at room temperature using a luminometer (Junior LB 9509 luminometer; EG&G Berthold, Germany) and obtained at 1-min intervals for 5 min, the area under the curve was determined, and data expressed as relative light units after counts were normalized to the weight of the brain tissue sample. Results were expressed as relative light units/mg tissue (rlu/mg).

Enzyme-Linked Immunosorbent Assay Measurements

To determine the levels of IL-1β, IL-6, TNF, IL-10, TGF-β, PAI-1, caspase 3, and cleaved caspase 3 in the brain tissue, commercial kits (Sunlong Biotech Co. Ltd., China) was used according to the manufacturer’s instructions. Supernatants of tissue homogenates were used for measuring the levels of cytokines.

Western Blot

The frozen tissues were weighed and homogenized in ice-cold 10 mM Tris–HCl (pH = 7.2) buffer containing 1 mM EDTA and protease inhibitors (0.2 mM PMSF, 1 µg/ml leupeptin, 1 µM pepstatin, 10 µg/ml soybean trypsin inhibitors) with Ultra-Turrax homogenizer. Whole homogenates were used in Western blots. The protein content of the whole homogenate was determined with the Lowry method [40]. Mixed with loading buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromphenol blue) samples containing 50 mcg protein have been denatured at 100 °C for 3–5 min and electrophoretically transferred onto nitrocellulose
membranes (Schleicher & Schuell, 0.45 μm, Germany) for 120 min at 80 V. The membranes were blocked with Tris-buffered saline containing 1% bovine serum albumin (Sigma-Aldrich, USA) and 0.05% Tween-20 (Sigma-Aldrich, USA) at room temperature for 60 min and incubated overnight at 4 °C with antibodies against MMP-3 (1:500; Bioss, USA), LDLR/CREB (1:1000; Bioss, USA), NF-κB (1:500; Novus, Co, USA) and β-actin (1:1000). β-Actin (Sigma-Aldrich, USA) was used as an internal control.

The secondary antibodies were purchased from Sigma (St Louis, MO, USA). All chemicals were obtained from Sigma unless stated otherwise. The blots were washed three times with TBS containing 0.05% Tween-20 (TBS-T) and incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h at room temperature (20 °C). The antibody-antigen complex was detected with NBT-BCIP (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium method). The apparent molecular weights of MMP3, CREB, NF-κB, and β-actin 50 kDa are 65 kDa and 47 kDa, respectively. The densitometric analyses were carried out with Image Studio Lite ver5.2 software (Lincoln, Nebraska, USA).

**Histopathological Examinations**

**Light Microscopy**

The brain samples were fixed in the 4% paraformaldehyde in phosphate buffer (pH 7.4) for 24 h at 4 °C. Tissues were then embedded in paraffin, and 5-μm-thick coronal sections were created using a rotary microtome. The sections were stained with hematoxylin and eosin stains. Finally, sections were examined under a photomicroscope (Olympus BX51, Japan). The severity of neuronal damage in the cortex was scored semiquantitatively as follows: 0 = no damage, 1 = mild damage, 2 = moderate damage, and 3 = severe damage. Pyknotic nuclei and intense staining of the shrunken neuronal perikarya were considered in scoring the degree of neuronal degeneration. The histopathological scorings were performed by investigators blinded to treatment groups to reduce the potential for bias.

**Immunohistochemical Investigations**

For immunohistochemical studies, sections were deparaffinized with xylene and rehydrated with 96% ethanol. Then, the sections were incubated with 3% hydrogen peroxide solution to inhibit endogenous peroxidase, incubated in the microwave with citrate buffer solution (pH 6.0, 60 °C) for antigen retrieval (20 min), and were cooled at room temperature. Then, the slides were washed in PBS and treated with protein blocking solution (EXPOSE Rabbit specific HRP/DAB Detection IHC Kit, Abcam, Cambridge, UK). Then, the slides were incubated with anti-MMP-3 (1:300, BS-0413R, Bioss Inc, MA, USA), anti-NF-KB (1:1000, 8242 T Cell-Signaling Danvers, MA, USA), and anti-CREB-1 (1:200, BS-0035 M, Bioss Inc, MA, USA) primary antibodies overnight at 4 °C. After washing with PBS, the slides were incubated with biotinylated secondary antibody (UltraTek Hrp Anti-Polyvalent, Scytek, USA) for 20 min at room temperature and then incubated with streptavidin peroxidase for 10 min. Sections were then washed in PBS, exposed with liquid diaminobenzidine (DAB Chromogen/Substrate Kit, Scytek, USA). Slides were counterstained with hematoxylin and were examined with an Olympus BX51 photomicroscope and photographed with a digital camera (Olympus C-5060). Sections were semiquantitatively scored in terms of staining intensity from 0 to 3 (0 = none; 1 = mild; 2 = moderate; 3 = severe). The immunohistochemical scorings were performed by investigators blinded to treatment groups.

**Behavioral Tests**

The behavioral tests performed two times for each rat. One is before TBI, and the second one is 24 h after TBI. The scorings were performed by investigators blinded to treatment groups.

**Novel Object Recognition Test**

The novel object recognition test, which is efficient in evaluating short-term memory, has become a widely used model for the investigation of memory alterations. Alterations in the test results are accepted to be indicative of both hippocampal and cortical lesions [41].

The test is applied as previously described [42]. Difference score (DS; in centisecond), discrimination index (DI; in seconds (s)), and recognition index (RI; in s) are noted accordingly.

**Y-Maze Test**

The Y maze is separated by three 120° branches from each other and is similar to the T maze. The rat
starts at the end of one arm then chooses between the other two. In this setup, the number of entries into the arms and spontaneous alternation is measured. Spontaneous alternation is measured to assess spatial working memory [43]. The number of entries is counted, and spontaneous alternation % is then calculated with the following formula [43].

\[
\text{Spontaneous alternation } \% = \frac{\# \text{ spontaneous alternations}}{\text{total number of arm entries}} - 2 \times 100
\]

RESULTS

Analysis of Biochemical Data

Measurements of Myeloperoxidase activity, Luminol, Lucigenin Enhanced CL, and Nitric Oxide Release in Brain Tissues

A statistically significant increase in MPO values \((p < 0.05)\) was observed in the trauma group when compared to the sham group. A statistically significant decrease was observed in TM5441-5 mg \((p < 0.01)\) and TM5441-10 mg \((p < 0.05)\) group values when compared to the trauma group (Fig. 1A). A statistically significant difference was also found between the treatment groups TM5441-5 mg and TM5484-5 mg \((p < 0.05)\).

When NO release was examined, an increase was observed in the trauma group when compared to the sham group (not statistically significant). At the treatment doses of TM5441-5 mg and TM5484-5 mg, NO values were close to the sham group, but there was no statistical significance (Fig. 1B). When the treatment groups were compared, a statistically significant difference was found between TM5441-5 mg and TM5484-5 mg \((p < 0.05)\).

A statistically significant increase was observed in the trauma group when compared to the sham group \((p < 0.01)\). Statistically significant decrease was observed in the treatment groups of TM5441-5 mg \((p < 0.001)\), TM5441-10 mg \((p < 0.05)\), and TM5484-10 mg \((p < 0.05)\) when compared to the sham group. A statistically significant increase was observed in the TM5484-5 mg group when compared to the sham group \((p < 0.05)\) (Fig. 1C). When the treatment groups were compared, there was a statistically significant difference among TM5441-5 mg and TM5484-5 mg \((p < 0.001)\), TM5441-10 mg and TM5484-5 mg \((p < 0.05)\), TM5484-5 mg and TM5484-10 mg \((p < 0.01)\).

Lucigenin values, which increased statistically significantly in the trauma group when compared to the sham group \((p < 0.01)\), were decreased significantly with TM5441-5 mg treatment \((p < 0.05)\) (Fig. 1D). An increase was observed in the TM5441-10 mg treatment group when compared to the sham group \((p < 0.05)\). When the treatment groups were compared, a significant difference was found between TM5441-5 mg and TM5441-10 mg \((p < 0.05)\), TM5441-5 mg, and TM5484-5 mg \((p < 0.05)\).
Evaluation of PAI-1 Level in the Brain Tissue at the 24th Hour After TBI

A statistically significant increase in the PAI-1 level was observed in the trauma group when compared to the sham group ($p < 0.01$) (Fig. 2). PAI-1 antagonist treatments did not cause any significant change.

Effects of PAI-1 Inhibition on Inflammatory Cytokines After TBI

Levels of IL-1β and IL-6 were significantly increased between trauma and sham-treated animals ($p < 0.05; p < 0.01$, respectively); however, neither inhibitor significantly mitigated these increases at either dose.
Kuru Bektaşoğlu, Koyuncuoğlu, Akbulut, Akakın, Eyüboğlu, Erzik, Yüksel and Kurtel tested (Fig. 3A, B). There was a statistically significant difference in IL-1β level between TM5484-5 mg and TM5484-10 mg ($p < 0.05$).

Levels of TNF, IL-10, and TGF-β were not statistically elevated in trauma compared to sham-treated animals (Fig. 3C–E), although treatment with 10 mg/kg of TM5484 was associated with increased levels of IL-10 compared to sham ($p < 0.05$). Also, for IL-10, there was a statistically significant difference between TM5484-5 mg and TM5484-10 mg groups ($p < 0.05$).

**Effects of PAI-1 Inhibition on the Apoptotic System After TBI**

A statistically significant increase was observed in the caspase-3 activity in the trauma group and the TM5484-10 mg group when compared to the sham group ($p < 0.05$) (Fig. 4A).

Although there was an increase in the cleaved caspase-3 activity in the trauma group when compared to the sham group and a decrease in the treatment groups compared to the trauma group, no statistically significant difference was found between the groups (Fig. 4B).

**Measurements of LDLR, MMP3, and NF-κB Protein Levels in Brain Tissue by Western Blot Method**

A statistically significant increase in LDLR /β actin level was seen in the trauma group when compared to the sham group ($p < 0.001$; Fig. 5A). A statistically significant decrease was observed in the TM5441-5 mg and 10 mg and TM5484-10 mg groups when compared to the sham group ($p < 0.01–0.001$). A statistically significant decrease was observed in all treatment groups when compared to the trauma group ($p < 0.05–0.001$). A statistical significance was also found between TM5441-10 mg group and TM5441-5 mg, TM5484-5 mg, and 10 mg groups ($p < 0.05–0.001$). A statistically significant difference was also found between TM5484-5 mg and TM5484-10 mg groups ($p < 0.01$).

There was a statistically significant increase in MMP3/β actin level in the trauma group when compared...
to the sham group \((p < 0.01; \text{Fig. 5B})\). A statistically significant decrease was observed in all treatment groups when compared to the sham and the trauma groups \((p < 0.05–0.001)\). A statistically significant difference was also observed between TM5441-10 mg and TM5441-5 mg and TM5484-5 and 10 mg groups \((p < 0.001)\).

There was a significant increase in the level of NF-κB/β actin in the trauma group when compared to the sham group \((p < 0.001; \text{Fig. 5C})\). A statistically significant increase was observed in the TM5441-5 mg group when compared to the sham and the trauma group \((p < 0.001)\). A statistically significant increase was observed in the TM5441-5 mg group when compared to the sham group \((p < 0.01)\). A significant decrease was observed in the TM5441-10 mg group when compared to the trauma group \((p < 0.01)\). There was a significant difference between TM5441-5 mg and TM5441-10 mg and TM5484-5 mg groups \((p < 0.01–0.001)\). There was a statistically significant difference between TM5484-5 mg and TM5441-10 mg and TM5484-10 mg groups \((p < 0.001)\).

When the scores of NF-κB immunohistochemistry were evaluated, no statistical significance was found between the groups.

**HISTOPATHOLOGICAL EVALUATION RESULTS**

**Light Microscopic Evaluation and Histological Damage Score Results**

Cortex, dentate gyrus (DG), and CA3 regions of the hippocampus were evaluated with a semi-quantitative
method in terms of neuronal cell damage with H&E staining. In the cortex of the rats in the sham group, regular neuropil structure, large nucleus, and prominent nucleolus structures and neurons in uniform morphology were observed (Fig. 6). In the cortex of the rats in the trauma group, neuronal damage, pycnotic cell nuclei, irregularity of the cell structures, and cytoplasmic deterioration were observed when compared with the sham group.

It was observed that this damage was less in the treatment groups. It was observed that the histological
damage score \( (p < 0.001) \), which increased in the trauma group when compared to the sham group, decreased in the PAI treatment groups \( (p < 0.01–0.001) \) (Fig. 7A). Histological damage score for hippocampal DG was increased in the trauma group when compared to the sham group \( (p < 0.001) \) and decreased in all treatment groups \( (p < 0.05–0.001) \) (Fig. 7B). The histological damage score for the hippocampal CA3 region was increased in the trauma group when compared to the sham group \( (p < 0.01) \) and decreased in the TM5441-5 mg group \( (p < 0.05) \) (Fig. 7C). A statistically significant increase was observed in the histological damage score in the TM5441-10 mg group when compared to the sham group \( (p < 0.05) \).

**BEHAVIORAL TESTS**

**Novel Object Recognition Test**

There was no statistically significant difference in the difference score between the groups in the novel object recognition test. A statistically significant increase was found in the discrimination index in the sham group when compared to the pre-experiment evaluations \( (p < 0.05) \). Although there was a decrease in the recognition index in the trauma group, there was no statistically significant difference between the groups.

**Y Maze Test**

In the Y maze test, a statistically significant decrease was observed in the number of entries in TM5441-5-mg and TM5484-10-mg treatment groups when compared to pre-procedure values \( (p < 0.05, p < 0.01, \text{respectively}; \text{Sidak’s multiple comparison test}; \text{Fig. 8A}) \).

A significant decrease was observed in the percentage of spontaneous alternation in the trauma group compared to the sham group \( (p < 0.05) \), and no statistically significant difference was found in the other groups (Fig. 8B).

**Tail Suspension Test**

A statistically significant increase was observed in the tail suspension test score in the trauma group when compared to the sham group \( (p < 0.001) \), and a statistically significant decrease was observed in all PAI-1 treatment doses \( (p < 0.05–p < 0.001) \) (Fig. 8C).

**Modified Bederson Neurological Examination Score**

The neurological examination score of all experimental animals was 0.
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DISCUSSION

Traumatic brain injury causes microthrombosis and rapid spread of the lesion by disrupting the fibrinolysis mechanisms [16, 46]. In vivo studies demonstrated the formation of microscopic clot areas around the contusion as early as the 1st hour after the injury [47, 48]. Appropriate fibrinolysis with plasminogen activators is required to balance clot formation [16]. Anti-inflammatory, anti-apoptotic, and neuroprotective activities of the PAI-1 antagonism in neuroinflammatory animal models were previously reported [16, 31, 32]. In this study, TM5441 and TM5484 compounds were used as PAI-1 antagonists in two different doses (5 and 10 mg) for the first time in a mild TBI animal model. TM5484 has a lower molecular weight than TM5441, and it has been shown to pass through BBB with a higher penetration rate [32]. Acute PAI-1 inhibition has been shown to reduce MPO activity, luminol, and lucigenin-enhanced CL, and these results indicated antioxidant and anti-inflammatory properties of these molecules. Histological damage was ameliorated, and corticospinal pathway damage was reversed with PAI-1 antagonism.

In the present study, brain MPO levels, which is an indicator of polymorphonuclear leukocytes (PMNs), significantly increased in the trauma group when compared to the sham group. Similar results were demonstrated in our previous study, where MPO levels were found to be elevated at the 24th hour after TBI [49]. There was a significant decrease in tissue MPO activity with TM5441 treatment indicating a role of PAI-1 in PMN recruitment.
after brain injury. When the treatment groups were compared among themselves, significantly lower MPO values were found in the TM5441-5 mg group compared to the TM5484-5 mg group.

PAI-1 plays a chemoattractant role for macrophages and PMNs [50]. In previous studies, it has been reported that TM5484 reduces macrophage migration and suppresses microglia activation in the experimental multiple sclerosis model [32]. Activated macrophages, PMNs, and microglia may release NO that its accumulation has been proposed to be associated with some toxic effects [51]. The brain location of this accumulation as well as the machinery of NO production (inducible vs constitutive enzymatic activation) may play an important role for the course of these harmful effects. Villalba et al. [52] reported that, after TBI profound increase in endogenous, NO production was observed due to increased iNOS expression. In the present study, there was an increase in NO values in the trauma group when compared to the sham group. This increase was leveled off with TMS441-5 and TM5484-5 treatments becoming comparable to the sham group. However, this tendency was not observed with the higher dose of TM5441 (10 mg). Although the increase in NO levels in the trauma group was not significant enough, this short-lived free radical may be quickly reacting with other molecules/ radicals and turning into more stable compounds (e.g., nitrate or nitrite) [53].

It has been previously shown that the levels of luminol and lucigenin-amplified chemiluminescence, which reflect tissue oxidative stress, increase at the 24th hour after mild TBI [49]. In our study, a significant increase in luminol and lucigenin-amplified chemiluminescence was observed in the trauma group when compared to the sham group. It was noted that both parameters significantly decreased after treatments with TM5441 and TM5484 at doses of 5 mg. On the other hand, TM5441 but not TM5484 were also effective in reducing luminol-amplified chemiluminescence at the dose of 10 mg. These results indicate that, after TBI, there was an increase in oxidative stress that somehow involves PAI-1 formation since its antagonism was associated with a reduction in the luminol and lucigenin-amplified chemiluminescence. Although there may be various sources of oxidants following the TBI, one plausible candidate is the accumulated PMNs at the site of injury. Our MPO data supported this hypothesis and suggested that reduced oxidative stress following PAI-1 antagonism might be due to a significant reduction in brain MPO activity after the administration of PAI-1 antagonists especially TM5441. Thus, the significant decrease in inflammatory parameters such as oxidative stress and inflammatory cell accumulation with PAI-1 antagonists was remarkable in terms of the possible therapeutic feature of PAI-1 inhibition.

It has been reported that patients with high PAI-1 levels after TBI have higher neurological damage scores [54]. This situation is thought to be associated with endothelial damage, thrombocyte activation, increased coagulopathy, and venous thrombosis [54]. Consistent with previous studies in the current study, brain PAI-1 level was found to be evaluated at the 24th hour in the trauma group when compared to the sham group. Although the source of increased PAI-1 production after TBI is unclear, it can be speculated that PMNs are the unlikely candidates since their tissue level was significantly reduced after TM5441 treatment despite an unaltered PAI-1 level.

In the study of Griemert et al. [16], it was demonstrated that elevated IL-1β and TNF mRNA levels in the pericontusional area were not altered at the 24th hour after TBI with pharmacological inhibition of PAI-1 with PAI-039. On the other hand, Pelisch et al. [32] reported in a multiple sclerosis model that TM5484 was effective in the outcome of neurological/functional recovery as well as in reducing previously increased IL-6, TNF mRNA levels, and decreased IL-10 activity. In a mild TBI model, IL-10 levels after TBI were found to be increased compared to the control group [55]. In the current study, a significant increase was observed in IL-1β and IL-6 values with trauma, and a decreasing trend was observed in the TM5484-5 mg group. There was no significant difference in the TNF levels between the groups. When we evaluated the anti-inflammatory cytokines, an increasing trend was observed in the trauma group. On the other hand, a significant increase in IL-10 level was observed in the TM5484-10 mg group compared to the sham group. It has been shown that IL-1β, which is a known proinflammatory cytokine after TBI, binds to the IL-1 receptor, causing an increase in NF-κB, cyclooxygenase-2, inducible NO synthase, and reactive oxygen metabolites levels [55]. Although TBI-associated production of cytokines is documented by the current as well as by the previous studies, the role of these alterations in TBI pathophysiology is a difficult issue to resolve because of diverse interactions of cytokines with multiple receptors, transcription factors, with each other, the time course of production and the source, etc.
The caspase family is cysteine proteases that regulate many steps of programmed cell death [56, 57]. Caspase-3 breaks down proteins involved in DNA repair [58, 59]. It is known that cytoskeletal proteins such as actin and spectrin are degraded by caspase-3 activation [60, 61]. Cleaved caspase-3 is the active form of caspase-3, and it was reported that its increased values were associated with the unfavorable outcome [62]. It has been shown that there was an increase in caspase-3 enzyme activity at the 24th hour after TBI [49, 56]. Griemert et al. [17] showed that apoptotic cells decreased at 24 h after TBI following PAI-1 antagonist treatment. In our study, we showed that there was a significant increase in caspase-3 values and that the cleaved caspase-3 level tended to increase after TBI. Treatment with PAI-1 antagonists, on the other hand, resulted in a decrease in cleaved caspase-3 levels indicating decreased apoptotic activity.

Matrix metalloproteinases are the second important proteolytic system in the mammalian brain, and although they may play a role in the protection of neurological functions, they can also cause damage to the BBB. Under ischemic stress, proteolytically active tPA causes MMP-3 induction from endothelial cells through the LDLR and NF-κB pathways [63]. On the other hand, when PAI-1 complexes with tPA or uPA these complexes are then taken into the cell and cleaned by binding to the LDLR from the circulation [64–66]. Pharmacological inhibition of previously increased MMP-3 significantly attenuated neurovascular permeability and improved neurological functions [67]. Similarly, it was found that tPA-PAI complex and MMP-3 levels increased in the CSF of patients with TBI and were associated with a negative neurological outcome. In another study, it was found that the levels of albumin and MMP-3, as indicators of cerebrovascular damage, were increased in the brain tissue of patients with head trauma [68]. It was reported that tPA activity increased by 30% within 1–3 h after trauma and returned to its baseline value at the 24th hour [68]. These studies indicate the importance of biological steps after PAI-1-tPA complex formation and suggest a role of LDLR and MMP in TBI pathophysiology.

In our study, western blotting demonstrated an increase in the LDLR, NF-κB, and MMP3 protein levels in the trauma group compared to the sham group. LDLR and MMP3 levels decreased and NF-κB levels increased in the treatment groups. PAI-1 inhibition may have reduced the possible effects of MMP and LDLR activations on inflammatory pathways. Mettang et al. [69] evaluated the inhibition of NF-κB in the TBI model and reported that the inflammatory response was enhanced, neuronal apoptosis and brain damage were increased, and functional outcomes were negatively affected. The protective role of NF-κB in neurons includes the antiapoptotic effects mediated by inducing endogenous caspase inhibitors or by triggering the expression of antioxidant genes [70, 71], as NF-κB transcription factors regulate a wide range of survival-promoting target genes [72]. In our study, treatment with PAI-1 antagonists increased NF-κB levels that are compatible with this scenario. As seen in western blot results, immunohistochemical expression of NF-κB was also increased in the trauma group, and this increase was higher in the PAI antagonist group. A further increase in NF-κB levels in the treatment groups may indicate the activation of some protective mechanisms following brain damage.

The histological damage score significantly increased in the cortex, hippocampal DG, and CA3 regions and decreased in all treatment groups except CA3. In the CA3 region, a significant reduction was only detected in the TM5441-5 mg group. In a TBI model, Griemert et al. 16 evaluated neuronal loss in the DG with TUNEL staining and reported that PAI-1 inhibition had a positive impact. This was thought to be due to improved cerebral perfusion. Taken together, our biochemical and histological data support each other and suggest that reduced neutrophil infiltration, oxidative stress observed with PAI-1 inhibition, and possible modulation of the peptidase/protease pathway positively affect cell morphology and histological damage scores.

In the current study, changes in cognitive functions and neurological status before and 24-h after TBI were evaluated with Y-maze, novel object recognition, tail suspension, and neurological tests for the first time in the literature. In the novel object recognition test, a decreased tendency in the discrimination and recognition indexes was observed in the trauma group when compared to the sham group. In most of the treatment groups, values were close to the sham. In the Y maze test, the number of entries was decreased in the trauma group when compared to pre-procedure values in all groups, and this response was found to be compatible with the freezing responses observed during the experiment. Similarly, the spontaneous alternation percentages were lower in the trauma group when compared to the pre-procedure values. Additionally, a significant decrease was observed in the trauma group when compared to the sham group, and an increasing trend was noted in all treatment groups.
Griemert et al. [16] evaluated the long-term neurological effect of another PAI-1 antagonist in mice TBI model, and they showed a significant decrease in neurological severity score in the treatment group 5 days after TBI. The long-term effects of PAI-1 antagonist treatment in TBI model on the recovery of cognitive performance could be studied in further studies.

Previously, a loss in locomotor function was shown on the 11th day after mild TBI [55]. In the current study, the application of the tail suspension test to evaluate corticospinal function demonstrated a significant increase in damage score after TBI, and elevated damage score significantly decreased in all treatment groups. Overall, our behavioral tests represented that mild TBI might alter not all but some of the patterns of actions performed by the whole animal indicating that inflammation at the 24th hour of trauma was associated with some cognitive and behavioral changes. Furthermore, it was conceivable to suggest that, while PAI-1 inhibition was effective in reducing PMN infiltration, oxidative stress, and histological damage score, it was also associated with better functional outcomes.

As seen in any laboratory study, there are also limitations in this study. The neuroprotective efficacy of PAI antagonist treatment at different doses and different treatment durations with different TBI models can be studied more comprehensively. Especially in learning-memory tests, the number of subjects is insufficient to show possible differences among groups because the data does not show a normal distribution. The changes in other periods could also be evaluated. In this study, the aim of applying PAI antagonist treatment immediately after TBI is to reach the effective blood concentration immediately and try to reduce the effects of the injury mechanisms. However, since it is not possible to provide treatment immediately after TBI in the clinical settings, the effectiveness of the treatment can be examined in a more realistic model by applying PAI antagonist treatment after a certain time after TBI.

Another limitation of this study is there are only young-male rats included in this study. Epidemiological studies show that TBI is much more common in young adult males [73]. However, in aged-female group, they need more neurosurgical intervention then male counterparts who suffer from TBI-related consequences. PAI-1 is increased in senescence and aging-related pathologies [74]. PAI-1 antagonists were also thought of as a treatment alternative for those aging-related pathologies such as cardiovascular disease and diabetes. So, for aged-female TBI suffering patients, PAI-1 antagonists would show a greater beneficial effect. Further studies are needed in order to make comment on this topic.

In some studies, PAI-1 has also been reported to have a neuroprotective effect through the mitogen-activated protein kinase/extracellular signal-mediated kinase signaling pathway [75]. It has been reported that intracerebral PAI-1 administration is also neuroprotective in the neonatal cerebral hypoxia model [76]. For this reason, it should be kept in mind in future studies that PAI-1 may show a dual effect in different conditions.

**Conclusion**

In this study, PAI-1 inhibition was achieved after TBI with TM5441 and TM5484 PAI-1 antagonists for the first time, and their effects on inflammation, oxidative stress, apoptosis, neuronal damage, and functional outcomes after TBI were investigated. PAI-1 inhibition has been shown to reduce oxidative stress and neuronal damage and improve damage to the corticospinal pathway. Examining the effects of PAI-1 inhibition on both pathophysiological and functional results in TBI and obtaining promising results would shed light on future clinical studies.

**AVAILABILITY OF DATA AND MATERIALS**

The data and materials are available on request.

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**AUTHOR CONTRIBUTION**

Pınar Kuru Bektaşoğlu: conceptualization, methodology, data curation, writing–original draft preparation, writing–reviewing and editing.
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DECLARATIONS

Ethics Approval and Consent to Participate All experimental procedures used in this investigation were reviewed and approved by the Marmara University Animal Care and Use Committee (March 12, 2019).

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Conflict of Interest The authors declare no competing interests.

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