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Original Article

Tissue plasminogen activator attenuates intestinal ischemia reperfusion injury in rats

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Abstract: Thrombosis and accompanying inflammation have been reported to affect the severity of ischemia-reperfusion (I/R) injury. Tissue plasminogen activator (tPA) initiates fibrinolysis through the conversion of plasminogen to plasmin. Increased fibrinolysis has been shown to lessen the severity of I/R injury in various organs. This study aimed to investigate the effects of tPA on intestinal I/R injury. Twelve Wistar albino rats were randomly divided into two groups (a sham group, and a tPA group). The superior mesenteric artery was occluded by clamping it for 45 minutes. Then, following the clamp removal, serum physiologic was given to the sham group, and tPA was given to the tPA group, and then all the small intestine specimens were examined by a pathologist. The specimens were scored according to the Park/Chiu classification and immunohistochemical staining of anti-IL-6 and TNF-α. The median of the scores was 4 (3-6) in the sham group and 2.25 (2-3) in the tPA group. The difference between the median scores in the sham and tPA groups was statistically significant (P=0.009). The mean anti-IL-6 and anti-TNF-α-stained cell counts were significantly different when the sham group and the tPA group were compared (P=0.004 and P=0.004). It was determined that tPA has an essential role in lessening the severity of intestinal I/R injury and it is suggested that further studies should be conducted on the effects of fibrinolytic management on intestinal I/R injury.

Keywords: Tissue plasminogen activator, fibrinolysis, ischemia reperfusion injury

Introduction

Ischemia/reperfusion (I/R) injury has a significant role in numerous clinical conditions involving hypoperfusion and impaired oxygenation of the end-organs in their pathophysiology [1]. Intestinal I/R injury causes deterioration of the intestinal barrier function, bacterial translocation, and microvascular injury, together with a severe systemic inflammatory response complicated by multiple organ failure [2]. In addition to the damage created by ischemia, additive and more extensive damage occurs during the macrovascular reperfusion of the tissues once the flow is restored. Reactive oxygen species (ROS) such as superoxides, hydrogen peroxide, and hydroxyl radicals, which are produced during the reperfusion state, are responsible for the escalation of the damage severity [1].

The state of oxygen destitution in the tissues during the ischemic period leads to a shifting of the natural procoagulant/anticoagulant balance within the endovascular wall in favor of a tendency towards coagulation [3], and the eventually occurring thrombosis plays a significant role in the development of intestinal infarction [4]. A “no-reflow phenomenon” describing the absence of flow within the microvasculature occurs due to microvascular thrombosis even in the presence of macrovascular reperfusion [5, 6]. Thrombosis has also been determined to be strongly correlated with the development of inflammation [7] amplifying the severity of tissue damage.

Fibrinolysis avoids the formation of clots within the blood vessels, and it is a process essential for maintaining perfusion of the tissues. The essential active protease for the fibrinolytic process is plasmin. The tissue plasminogen activator (tPA) molecule is an enzyme that belongs to the serine protease family, and it is secreted predominantly by vascular endothelial cells. tPA binds to fibrin in the clot, and this complex converts the plasminogen (the zymo-

Table 1. The histological Park/Chiu scoring system

Score	Histopathological appearance
0	Normal mucosa
1	Subepithelial space at villus tips
2	Extension of the subepithelial space with moderate lifting
3	Massive lifting down the sides of villi, some denuded tips
4	Denuded villi, dilated capillaries
5	The disintegration of lamina propria
6	Crypt layer injury
7	Transmucosal infarctions
8	Transmural infarctions

gen form of plasmin) to plasmin. This cascade initiates a fibrinolytic process, eventually leading to thrombolysis [8]. It was demonstrated that increasing fibrinolysis lessens the severity of I/R injury in the lungs [9]. It was also shown that inhibiting coagulation reduces intestinal I/R injury [10].

In this study, we hypothesized that enhancing fibrinolysis before and during reperfusion of the ischemic tissues would reduce the severity of microvascular thrombosis, and thus the accompanying inflammation and consequent infarction as well. Our aim was to investigate the effects of tPA on intestinal I/R injury in rats.

Materials and methods

All applicable international, national, and institutional guidelines for the care and use of animals were followed. This study was approved by the Experimental Studies Ethics Committee of Çukurova University (26/9/2017 no:8).

Twelve Wistar albino rats weighing 250±50 grams were included. These rats were provided by and kept in proper cages, room temperature, and 12-hour day-night cycles in the Çukurova University Faculty of Medicine, Experimental Medical Research and Practice Center. The rats were randomly divided into two groups: the sham group and the tPA group. The experimental study described below was performed as defined by Gandini et al. [11].

Experimental study

The rats were kept warm. They were transferred to the operating room. An intravenous line was established through the dorsal tail vein. Intravenous anesthesia was initiated using Ketalar (Pfizer New York, USA) and Kepro Xylazine (Biopharm, İstanbul, Turkey). After the incision line was shaved, sterilized with 10% povidone-iodine, and covered with sterile drapes, an abdominal midline incision was made. The superior (cranial) mesenteric artery (SMA) was found and isolated. After the isolation of the SMA, it was clamped with a vascular clamp, and the SMA occlusion was verified by the disappearance of distal vascular pulsation in the intestine. The abdomen was closed temporarily with sutures for 45 minutes to avoid hypothermia while the SMA clamp was kept in position. After 45 minutes, the abdominal closure sutures were removed, the abdomen was reopened, and then the vascular clamp was removed. The abdominal wall was closed.

Following the closure of the abdominal wall, serum physiologic was given intravenously via the dorsal tail vein in the sham group.

In the tPA group, following the closure of the abdominal wall, the standard dose of tPA was used. A total of 9 mg/kg tPA was administered via the dorsal tail vein. 10% of the total dose of tPA was given as a bolus, and the remaining dose was given in a one-hour reperfusion period intravenously. After 45 minutes of ischemia and one-hour of reperfusion time, the abdominal wall was reopened, and the small intestine was totally excised. The samples were fixed with 10% formalin and then embedded in paraffin. The samples were obtained at intervals of a maximum of 5 cm, and the number of samples in one rat was at least 10. After staining the samples with routine hematoxylin and eosin stains, the specimens were examined by a single pathologist who was blinded to the study. The specimens were evaluated according to the Park/Chiu scoring system [12]; the scoring system is shown in **Table 1**.

Immunohistochemical staining of the obtained specimens was performed on 5-mm sections of the formalin-fixed, paraffin-embedded tissue using antibody to TNF-α (ab6671; Abcam, Cambridge, UK) and IL-6 (ab6672; Abcam, Cambridge, UK). The visualization system used was BenchMark XT with heat-induced epitope retrieval (CC1 solution) and an iView DAB detection kit (Ventana, Tucson, AZ). Slides stained with anti-TNF-α and anti-IL-6 were examined in

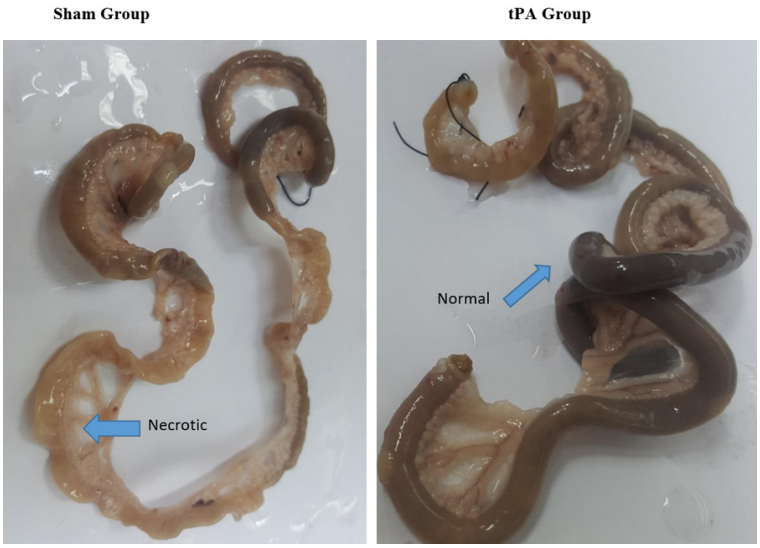

Figure 1. The macroscopic appearances of the specimens belonging to the sham and tPA groups.

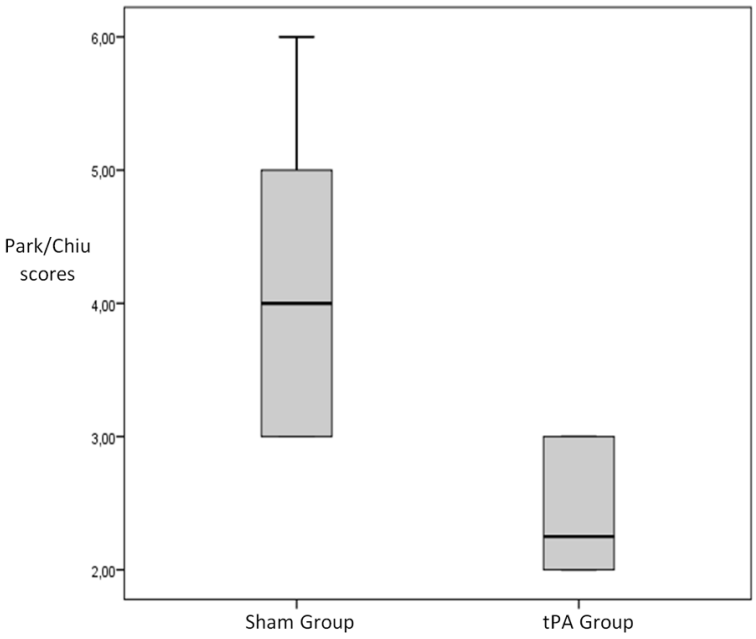

Figure 2. Distributions of the medians of the Park/Chiu scores in the sham and tPA groups.

five high-power fields (Olympus BX40). Cytoplasmic and membranous staining was considered positive, and the positive cells were counted.

Statistical analysis

The results were analyzed using the Statistical Package for the Social Science, version 20 (SPSS 20). The differences between the two groups were evaluated using Mann Whitney U tests. The *p* values lower than 0.05 were considered significant.

Results

The macroscopic appearances of the specimens belonging to the sham group were observed to be more necrotic than those of the specimens belonging to the tPA group (**Figure 1**). This subjective observation was verified using microscopic images.

The median of the Park/Chiu scores was determined to be 4 (range 3-6) in the sham group, but the median of the Park/Chiu score in the tPA group was determined to be 2.25 (range 2-3). The difference between the sham group and the tPA group regarding the medians of the Park/Chiu score was statistically significant (P=0.009). The distributions of the medians of the Park/Chiu scores in the sham and tPA groups are shown in **Figure 2**.

The mean anti-IL-6-stained cell count was determined to be 74.0 cells in the sham group, and 42.1 cells in the tPA group. The difference between the sham group and the tPA group regarding the anti-IL-6-stained cell count was statistically significant (P=0.004).

The mean anti-TNF-stained cell count was determined to be 72.6 in the sham group, and 48.6 in the tPA group. The difference between the Sham and tPA groups regarding the anti-TNF-α-stained cell count was statistically significant (P=0.004) (**Figure 3**).

Discussion

In our study, we determined that tPA, when administered through the dorsal tail vein following the creation of acute intestinal ischemia,

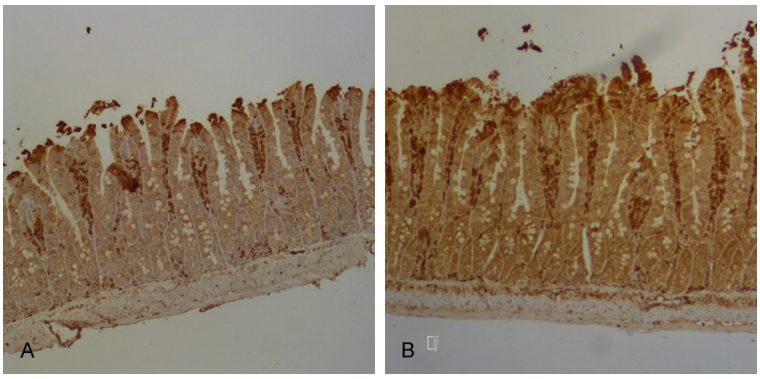

Figure 3. Microscopic appearance of anti-IL-6 and anti-TNF-α. A: Immunohistochemical staining of interleukin-6 positive cells (×40). B: Immunohistochemical staining of tissue necrosis factor-α positive cells (×100).

significantly reduced the overall histopathologic score indicating the severity of the I/R injury. We also saw significant reductions in the anti-IL-6-stained and anti-TNF-α-stained cell counts in our histopathological evaluation of the specimens.

It has been often reported that the formation of macrovascular/microvascular thrombosis and inflammation play active roles in the I/R injury of various organs. Bertuglia and Colantuoni, in their study on I/R injury of the lungs in leukopenic hamsters, determined that thrombus formation might be an independent factor in I/R injury working through leukocyte-mediated pathophysiological mechanisms [3]. It has also been reported that intestinal I/R injury is also strongly associated with the formation of thrombosis. Schoots et al., in their study conducted on rats, showed that 20 and 40 minutes of intestinal ischemia followed by reperfusion for 3 hours resulted in local intestinal thrombin formation, fibrinogen to fibrin conversion, and the suppression of the activity of the plasminogen activator. They concluded that intestinal I/R had led to local intravascular coagulation and fibrin deposition [5]. Regarding the association of thrombosis and inflammation, Engelmann and Massberg introduced the term “immunothrombosis” describing the physiological role of thrombosis in immune defense. However, they suggested that when not controlled, the formation of microvascular immunothrombosis particularly can lead to various states of pathological thrombosis, even though its mechanism of occurrence is similar to benign, immune defense-enhancing thrombosis [13].

Thrombosis can also enhance inflammation through platelets. First, leukocytes accumulate within the microvascular circulation, and then they are transferred to the perivascular tissues; thus, the reperfusion is not achieved adequately [14]. When platelets are relocalized together with leukocytes particularly in I/R injury sites, tissue factors, adhesion molecules, and proinflammatory cytokines are released, worsening the severity of the tissue injury [15].

tPA is an enzyme secreted mainly from cells located within the vascular endothelium. By binding to an already formed clot, it catalyzes the conversion of the zymogen form of plasminogen to plasmin. Plasmin initiates the fibrinolytic process, and eventually lysis of the clot occurs through the degradation of fibrin contained in the thrombus matrix. Currently, tPA is clinically used in various diseases involving thrombosis in their pathophysiologic processes such as acute ischemic stroke [16], acute myocardial infarction [17], and pulmonary thromboembolism [18]. tPA has also been the preferred agent for thrombolytic therapy in children [19-21]. It was even successfully used intravenously in two neonates with midgut volvulus and severe intestinal ischemia to restore perfusion following a derotation of the volvulus [22].

IL-6 and TNF-α were both shown to have significant roles in ischemia-reperfusion injury. They have been shown to interact to enhance the severity of injury [23]. The significant differences regarding intestinal I/R injury severity between the tPA and sham groups in the histopathological evaluation, including anti-IL-6 and anti-TNF-α staining, revealed that administering tPA before and during the reperfusion state in the post-ischemic intestine was effective in intestinal I/R injury. Our hypothesis was that by initiating the fibrinolytic process, tPA would attenuate the severity of I/R injury by avoiding the adverse effects of macro and particularly microvascular thrombosis. This hypothesis was proven according to our study results.

Although our study was one of the first studies in the medical literature on intestinal I/R injury,

tPA has been more extensively studied in I/R injuries of other organs with controversial results. Boettcher et al., in their recently published study on I/R injury in neonatal rats, compared therapeutic targeting of extracellular DNA with fibrin targeting in a neonatal midgut volvulus model in rats [4]. They used a special DNase (DNase1) for the elimination of extracellular DNA and a tPA-heparin combination for the elimination of fibrin. Even though the effects of DNase1 were more enhanced, both treatments served their purposes and reduced the severity of I/R injury. The first difference in our study was that tPA was used alone, not combined with heparin. The other difference was related to the experimental model; our model involved the occlusion of the superior mesenteric artery, and their study was performed using the midgut volvulus model.

Plasminogen activator inhibitor (PAI-1) is a molecule that acts as the primary limiter of fibrinolysis, preventing exaggerated fibrinolytic activity by inhibiting the function of the plasminogen activator. In a recently published study by Praetner et al., it was shown that the deficiency of PAI-1 lessened the severity of tissue injury, and additionally reversed microvascular dysfunction and leukocyte accumulation [24]. A 2009 study by Lau et al. [25] concluded that enhanced fibrinolysis serves to protect against I/R injury in the lungs. They did not use tPA, but they performed the experiment on mice lacking the PAI-1 gene; it was suggested the fibrinolytic process was enhanced since PAI-1 was absent in the environment. However, the same authors, in another study, hypothesized that impaired fibrin degradation, in other words, increased fibrin levels, would lead to an enhanced inflammatory response; they concluded that the depletion of tPA attenuates I/R injury of the lungs by inhibiting the extravasation of neutrophils [26].

To conclude, we suggest that the fibrinolytic cascade and particularly the tissue plasminogen activator have important pathophysiological roles in I/R injury as proven by the significant reducing effect of tPA on intestinal I/R injury demonstrated histopathologically in our study. Further experimental studies should be conducted on the effects of fibrinolytic management on intestinal I/R injury. Since tPA is a clinically used agent in conditions with thrombosis involved in their pathophysiology, if experimental studies with tPA on I/R injury yield positive results, its clinical use in I/R should be further investigated.

Disclosure of conflict of interest

None.

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