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# Antithrombin-III Reduces Intestinal Ischemia/Reperfusion Deleterious Effects on Kidney: A Study in Rats

**Theodore Tsachalis[1*], Emmanouil Christoforidis[2], Kokkona Kouzi-Koliakou[3], Elena Kostidou[4], Konstantinos Vasiliadis[5], Konstantinos Blouhos[2], Louiza Andriopoulou-Oikonomou[3] and Charalampos N. Lazaridis[2]**

*[1]Second Department of Surgery, 424 General Military Hospital, Thessaloniki, Greece.*
*[2]Fourth Department of Surgery, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece.*
*[3]Laboratory of Histology-Embryology-Anthropology, Medical School, Aristotle University of Thessaloniki, Greece.*
*[4]Department of Zoology, Laboratory of Animal Physiology, School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece.*
*[5]First Department of Surgery, Papageorgiou Hospital, Thessaloniki, Greece.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors TT and EC designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors KV, KB and CNL managed the literature searches, and analyses of the study. Authors EC and KV designed the figures and contributed to the correction of the draft. Author EK performed the spectroscopy analysis and authors KKK and LAO managed the experimental process and completed the electron microscopy examination of the specimens. All authors read and approved the final manuscript.*

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## ABSTRACT

**Background:** Mesenteric ischemia-reperfusion (I/R) is a well-known cause for both local and remote organ injuries. A natural inhibitor of serine proteases, Antithrombin-III, was previously shown to attenuate the tissue damage after local I/R in several organ systems. Here, we examined

**Corresponding author: Email: emmch@auth.gr, e-tsachalis@hotmail.com;*

the effects of Antithrombin-III on renal injury, after mesenteric I/R.
**Methods:** Female Wistar Albino rats weighing 250-350g underwent median laparotomy and were randomized into 3 groups: (1) sham-operated group, with no mesenteric I/R, (group I, n=12), (2) control group, with mesenteric I/R, (group II, n=12), and (3) experimental group, with mesenteric I/R but Antithrombin-III treated (group III, n=12). At the end of a six hours reperfusion period animals were killed and renal tissue samples were examined for myeloperoxidase (MPO) activity levels, and for the presence of certain types of mitochondrial lesions, both of which have been proved to be reliable indicators of the severity of local and systematic post-perfusion deleterious effects.
**Results:** There was a significant increase in observed renal tissue mitochondrial defects (P<0.001) and MPO activity in the I/R control group when compared with the sham operated group (P<0.001).
The treatment of animals with Antithrombin-III significantly decreased the amount of mitochondrial damage (P<0.001) and MPO activity (P=0.018) compared with the control group.
**Conclusion:** The results of the present study suggest that mesenteric ischemia and reperfusion induce renal injury. A significant attenuation of intestinal I/R-related renal injury with the use of Antithrombin-III concentrate, warrants further studies to elucidate the potential role of this natural serine protease inhibitor in clinical settings.

*Keywords: Intestinal ischemia; Reperfusion; Renal injury; Antithrombin III.*

## 1. INTRODUCTION

Mesenteric ischemia-reperfusion (I/R) has been recognized as having the potential for inducing local and remote organ injuries. Many vital organs like the heart, the lungs, the liver and the kidneys, are highly vulnerable to such indirect harmful effects. On the other hand, Antithrombine-III (AT-III), mainly through discrete antihrombotic and antiinflamatory actions, has been shown to attenuate the deleterious effects of I/R injury[1]

It is now widely recognized that blood flow restoration in viable post-ischemic tissues, could have a further injurious effect, and thus aggravate any pre-existing ischemic injury [2,3]. Furthermore, it is also commonly known, that I/R injury can produce a systemic inflammatory reaction, which can be potentially more detrimental than its local effects. Renal injury is one of the most important manifestations of this systemic response [4]. Activated neutrophils, the complement components, coagulation factors, and proinflammatory and vasoactive mediators, such as eicosanoids, nitric oxide, cytokines, and oxygen-free radicals are some of the factors believed to have a role in the development of this I/R-induced renal injury [3,5-7].

Even though the exact mechanisms are not defined clearly, endothelial cell-activated leukocyte interactions seems to result in increased renal microvascular permeability resulting in extensive polymorphonuclear infiltrations, tubular dysfunction and renal failure [8-12].

AT-III which is a β2-globulin, is the physiologic inhibitor of thrombin and other serine proteases such as IXa, Xa, XIa, and XIIa. By contrast, thrombin was shown to play an important role in I/R-induced leukocyte rolling and adhesion. Hence, AT-III might be therapeutically efficacious in I/R injury through its capability to attenuate the thrombin-mediated effects on neutrophil-endothelial interactions. [1] In other experimental studies it has been shown that treatment with AT-III significantly abates neutrophil sequestration, thus attenuating tissue damage after local I/R injury in several organs such as the intestine, liver, and kidney [13-15].

In the present study, we aimed to investigate whether AT-III prevents harmful systemic effects of intestinal I/R injury on renal tissue in rats.

## 2. MATERIALS AND METHODS

### 2.1 Surgical Procedure and Experimental Design

For the performance of our study 36 female Wistar Albino rats weighing between 250 and 350g were used. The animals were housed in wire-bottom cages at 25°C (room temperature) with a 12-hour light-dark cycle and were fed standard rat chow and water during the 12 hours prior to surgery.

The use of anesthesia during the surgical procedure and subsequent postoperative care were consistent with the guidelines in the Directive 86/609/EEC and Animal Care and Use Training Manual of UCLA.[16,17]

All animals were fasted overnight and anesthetized by an intraperitoneal injection of PENTOTHAL* (Thiopentone Sodium) 50 mg/kg. With the help of a heating pad and a heating lamp the animals were kept warm. A subcutaneous dose of Ringer's lactated solution (30 ml/kg) was given to prevent the rats from dehydrating. The rats were randomized into 3 groups, using computer-generated random numbers, and a midline abdominal incision was performed to expose the superior mesenteric artery (SMA). In group I (sham-operated, n = 12), the SMA and collateral branches were isolated but not occluded, then the abdominal incision was closed, and the animals were followed-up for seven hours, to simulate the I/R interval in the other groups. In group II (intestinal I/R control-group, n = 12), the SMA was exposed carefully and occluded immediately distal to the aorta for 60 minutes, with an atraumatic micro-vessel arterial clamp (VASCU-STATT®, mini Straight, Single-Use Bulldog Clamp,10-15 gr, Scanlan ,USA).[18] Mesenteric ischemia was confirmed by measuring large and small intestinal wall perfusion with the use of laser Doppler (PeriFlux System 5000, Perimed, Stockholm, Sweden). By this procedure, a large portion of the distal part of the small bowel, and right colon were rendered ischemic. After 60 minutes of ischemia, the clamp was removed and a six hours reperfusion period began. Reperfusion was confirmed also by the use of the same technique i.e. laser Doppler flowmetry.

In group III (Antithrombine III-treated group, n = 12), the rats received 250 U/kg of Kybernin P** (Antithrombine III) intravenously, immediately after clamping the SMA, and the same procedures as described for group II were performed. The dose of AT III was determined according to similar experimental studies which resulted in improved outcome.[1,15]

## 2.2 Transmission Electron Microscopy (TEM) Examination of Renal Tissue

The use of a good scoring system is undoubtedly invaluable in assessing experimental data, especially in biological science. Unfortunately, our extensive search of the literature proved unhelpful in finding any scoring system correlating the amount of ultrastructural damage to that of the reperfused renal tissue. However, according to several previous studies, certain types of mitochondrial changes have proved to be of a great value in illustrating the viability and functional capacity of a postischemic tissue [18-28]. Hence, we decided to carry out this comparative study by putting our TEM examination data under statistical analysis, focusing on: 1. Mitochondrial swelling, 2. Cristae destruction, 3. Matrix condensation, 4. Mitochondrial membrane disruption and 5. Complete organellular destruction or degeneration [29-32].

The following procedure was carried out in order to examine the specimens under TEM. At first, subsequent to the indicated reperfusion time, the animals were anesthetized, and the median laparotomy incision was reopened and extended cephalad by performing a median sternotomy. Subsequently renal tissue blood washout was performed by means of a left ventricular puncture using a 12-gauge needle and a 50cm of $H_2O$ constant pressure perfusion assembly and after that the right atrium was cut to ensure outflow. The washout medium was 0.9% saline at 37°C. When the fluid flowing out of the right atrium was clear, perfusion was stopped and the left kidney was removed and halved. Many small (≈1 $mm^3$) tissue specimens were taken randomly from the cortical region of the one half of each harvested kidney. Thereafter, they were immediately fixed with 2.5% glutaraldehyde and 2% paraformaldehyde solution in 0.1 M sodium caccodylate buffer for 1 night and then post-fixed with 0.1% osmium tetraoxide solution in the same buffer. After routine dehydration in a graded alcohol series and propylene oxide, specimens were embedded in LR White embedding media. Semithin (1 μm) and thin (0.5 μm) cross-sections of tissue samples were cut by ultrami-crotome (Reichert-Jung, type 701701). The ultrastructural differences between sham, control and treated groups were evaluated in ultrathin sections with a JEOL JEM 2000 FX II microscope.

## 2.3 Renal Tissue Myeloperoxidase (MPO) Activity

Polymorphonuclear leukocyte (PMN) infiltration into tissue is a hallmark of acute inflammation.

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The degree of inflammation can be quantified by the identification and enumeration of PMNs histologically or by some other means.

Measure of MPO activity provides a very reliable alternative method for a quantitative assessment of neutrophil infiltration in postischemic tissue [33-36].

After the blood washout was completed, kidney samples for MPO measurements were immediately frozen in liquid nitrogen and stored at –70°C. Renal tissue MPO activity was determined by the method of Lopez-Neblina et al. [37], after which renal tissue samples were homogenized in 0.05 M potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HETAB) and 0.146% ethylenediaminetetra-acetate (EDTA); then the samples were sonicated in ice ten times for 5 s. Homogenates were centrifuged for 30 min at 12,500 *g* at 4°C. The supernatants were used for MPO assay. The MPO activity was assessed by measuring the $H_2O_2$-dependent oxidation of O-dianisidin. One unit of enzyme activity was defined as the amount of MPO that caused a change in absorbance of 1.0/min at 410 nm and 37°C [38].

### 2.4 Statistical Analysis

The continuous variable MPO was summarized using means with standard deviations (SD) and medians with ranges (minimum, maximum). Firstly, its normality was assessed using the Shapiro-Wilk's test. MPO was normally distributed; therefore, the ANOVA was used for the comparison of the groups. Post-hoc pairwise comparisons were assessed with the unpaired Student's t-test. The Bonferroni correction was used to adjust for multiple comparisons and the Bonferroni-adjusted p-values were reported. The chi-square test was used to test for ultrastuctural abnormalities among groups. All reported p-values were two-tailed with p <0.05 considered as significant. All analyses were conducted using SPSS 17 (SPSS, Inc., Chicago, IL).

## 3. RESULTS

### 3.1 TEM Examination

The ultrastructure of cells in the kidney was examined under TEM. The same number of randomly selected samples from each kidney (Table 1) were studied at a primary magnification of × 4.000 – × 20.000.

Sham operated group renal tissue specimens showed the characteristic filtration apparatus of the glomerulus, including fenestrated endothelium, uninterrupted basement membrane, and healthy podocytes. Organelles remained mostly intact. The overwhelming majority of mitochondria were arranged in an orderly fashion in the form of a circle or ellipse without swelling. With only a very few exceptions, mitochondrial membrane was intact and cristae were arranged in the form of concentric rings or vertical lines, with normal spacing and with uniform density.

By contrast, in the control group (only I/R) there were numerous ultrustructural morphological changes such as an increased number of lysosomes, effacement of the podocytic foot processes, flattening of the brush borders, and destruction of mitochondrial membranes, cristae, and matrix.

By further contrast, kidneys from AT III treated animals showed amelioration of these changes, with viable podocytes and preservation of foot processes. Whilst in control group specimens, loss of microvilli, extensive vacuolization, and mitochondrial breakdown in tubular epithelial cells indicated extensive damage, in AT-III treated animals, less frequent vacuolization and maintenance of internal cellular architecture was detected.

As indicated above, we decided to assess tissue injury severity by comparing some particular mitochondrial structural changes (Fig. 1). To this end a qualitative microscopic study of the selected specimens was performed and statistical analysis was done with a chi-square test and with $p$ <0.05 considered as significant.

The observed mitochondrial changes differed significantly among groups (p<0.001). In particular, the observed mitochondrial changes were significantly more in group II than in the other two groups (p<0.001, in both comparisons). Furthermore, the abnormalities were significantly more in group III than in group I (p<0.001) (Table 1).

### 3.2 Myeloperoxidase (MPO) Activity

There was a statistically significant difference between groups (Table 2) in levels of MPO activity (p<0.001). In particular, the levels of MPO activity were found to be significantly higher in group II in comparison to the other two groups (p<0.001 and p=0.018, respectively). The level of MPO activity was also higher in group III in comparison with group I (p=0.001).

**Table 1. Comparisons between groups according to the appearance frequency of significant mitochondrial changes**

| Groups | I | II | III |
|---|---|---|---|
| No. of specimens studied for each group* | 72 | 72 | 72 |
| No. of specimens containing significant mitochondrial changes (%) | 4 (5.6) | 59 (81.9) | 38 (52.8) |
| *Twelve specimens per group, 6 samples per specimen (n=72) | *(p-value <0.001)* | | |

*Group I: sham-operated group, no ischemia; Group II: control group (only I/R), 60 minutes of mesenteric ischemia and 6 hours of reperfusion; Group III: treated group, 60 minutes of mesenteric ischemia and 6 hours of reperfusion Antithrombine III-treated group*

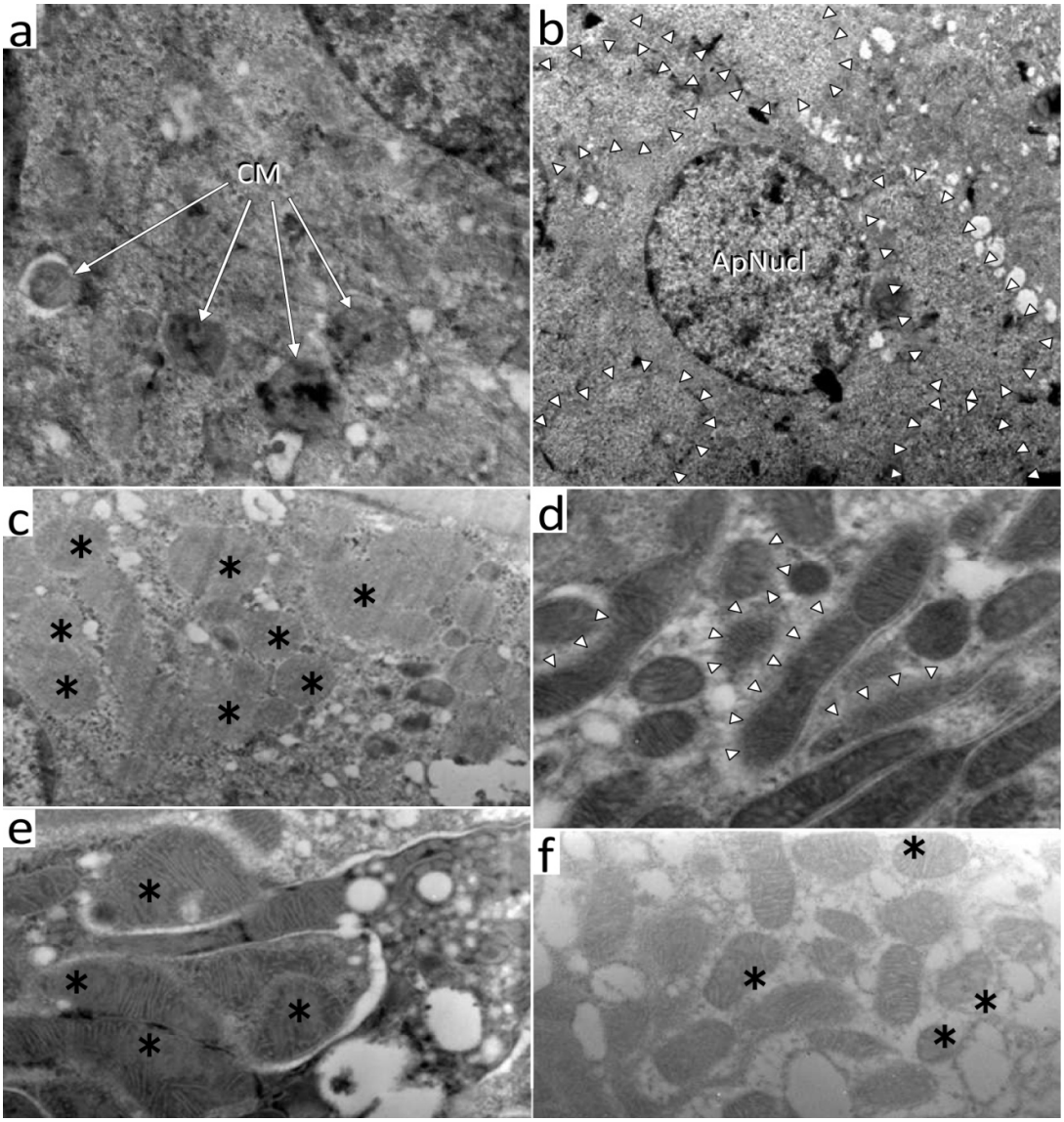


**Fig. 1. (images a to f). Typical mitochondrial lesions indicative of irreversible reperfusion cellular damage. a. condensed matrix (arrows), b. complete mitochondrial destruction (arrow heads), apoptotic nuclei (ApNucl), c. destroyed mitochondria (asterisks), d. mitochondrial membrane disruption (arrow heads), e. cristae destruction (asterisks), f. swollen mitochondria (asterisks)**

**Table 2. Comparisons of renal tissue MPO U/mg among groups**

| Group | N | Mean | Standard deviation | Median | Minimum | Maximum |
|---|---|---|---|---|---|---|
| I | 12 | 0.35 | 0.08 | 0.36 | 0.20 | 0.47 |
| II | 12 | 0.57 | 0.08 | 0.58 | 0.45 | 0.69 |
| III | 12 | 0.44 | 0.08 | 0.44 | 0.28 | 0.55 |
| ***p*-value** <0.001 | | | | | | |

** Overall comparison with ANOVA*

*Post-hoc pairwise comparisons (unpaired Student's t-test) with Bonferroni-adjusted p-values: I vs. II p<0.001. I vs. III p=0.001. II vs. II p=0.018 Group I: sham-operated group, no ischemia; Group II: control group (only I/R), 60 minutes of mesenteric ischemia and 6 hours of reperfusion; Group III: treated group, 60 minutes of mesenteric ischemia and 6 hours of reperfusion Antithrombine III-treated group*

## 4. DISCUSSION

In this study, reperfusion after 60 minutes of mesenteric ischemia led to neutrophil accumulation, reflected in elevated MPO activity, and extended ultrastructural changes in renal tissue. Intestinal mucosa is highly vulnerable to ischemic injury; moreover, reperfusion of this ischemic intestine may result in not only local but distant organ injury. In particular situations, intestinal ischemia accompanies some surgical procedures, such as thoracoabdominal aortic aneurysm repair, embolectomy for acute mesenteric arterial occlusion, or small-bowel transplantation. Renal tissue damage and kidney failure as a consequence of I/R-induced remote organ injury may complicate such surgical procedures.

Systemic neutrophil activation and infiltration after mesenteric I/R appears to be secondary to various mediators (eicosanoids, complements, cytokines, and oxygen-free radicals) derived from the revascularized tissues. [3-5,7-9,12,39-43] The renal tissue, along with the lungs, heart and liver, are some of the primary distant sites susceptible to such mediators and the neutrophil activation secondary to these mediators was shown to play an important role in the development of kidney injury. Up-regulation of the neutrophil and the renal endothelial adhesion molecules in a mesenteric I/R model has been reported previously as a cause of neutrophil accumulation and infiltration, inducing acute renal dysfunction. Some other studies have emphasized the relationship between I/R injury and the coagulation system.[1,44]

As an effect of I/R on the endothelium, coagulation and fibrin deposition at the vascular wall occurs, along with vasoconstriction and thrombin formation. Subsequently to the endothelial injury endothelial cells are "activated" getting in a prothrombotic and proinflammatory state, producing certain cell adhesion molecules promoting platelet and leukocyte adhesion and activation [45].

It has been shown that, in such conditions and during some inflammatory states, Thrombin creates the ability to increase the expression of endothelial P-selectin, E-selectin, and intercellular adhesion molecule-1, and the production of endothelial platelet activating factor (a potent agonist for neutrophils), leading to a parallel increase of neutrophil adhesion and recruitment. [43,46] As a natural inhibitor of thrombin and of other serine proteases, AT-III has been shown to decrease neutrophil rolling and adhesion to the endothelium [1]. In our study, decreased leukosequestration reflecting significantly lower MPO activity in the AT-III treated group can be attributed to the effect of AT-III concentrate on these neutrophil-endothelial interactions.

I/R induced subcellular morphological changes have been studied from a predictive point of view regarding the severity of cell injury. Mitochondrial abnormalities have been proposed as a safe indicator for the estimation of the irreversibility of a postischemic tissue injury. According to I/R induced cellular injury, structural mitochondrial damage seems to be of greater significance than other ultrastructural alterations. It has been proved that mitochondrial dysfunction affects cell viability through a wide array of events. Loss of ATP synthesis and increase in ATP hydrolysis, impairment in ionic homeostasis, ($Ca^{2+}$ in particular), formation of reactive oxygen species (ROS) and release of proapoptotic proteins are

all recognized as key factors in the generation of irreversible damage. This series of events explains why mitochondria are involved in both necrosis and apoptosis following post-ischemic reperfusion. [18-28]. There is evidence that significant changes of mitochondria such as swelling, pyknosis and disruption of the outer-membrane could take place in many apoptotic models [19,29-31]. Cristae destruction, matrix condensation, membrane fuzzification or disruption, presence of osmiophilic densities, [32] prominent degeneration and loss of normal organellular structure, [29] are also considered to be indicators of major and possibly irreversible cellular damage.

Decker and Wildenthal [32], in their very early experimental work on rabbit myocardial tissue after I/R, classified the severe mitochondrial swelling as being of great importance for cellular viability, as well as the presence of mitochondrial densities and matrix degeneration. Nakao et al, investigating the protective role of carbon monoxide on rat intestinal grafts, employed intestinal epithelial cell vacuolizations, consequent to mitochondrial breakdown and degeneration, as a benchmark for the severity of tissue injury. Di Lisa and Bernardi [45], in their review on mitochondria and ischemia–reperfusion injury of the heart, mentioned that matrix swelling may cause the rupture of the outer membrane, resulting in release of cytochrome c, which is able to trigger apoptotic cascade. [19,47,48] They also considered that mitochondrial swelling is generally synonymous with Mitochondrial Permeability Transition Pores opening, which in turn results in major modifications of mitochondrial function and structure that eventually jeopardize the maintenance of cell viability. Neto et al. [49], in their experimental work on the protective role of carbon monoxide during renal ischemia-reperfusion injury in rats, mentioned vacuolization, and mitochondrial breakdown in tubular epithelial cells as indicative of extensive damage. Sun et al. [50], in their study on the protective effect of ischemic postconditioning during hepatic ischemia-reperfusion injury, mentioned that mitochondrial membrane fuzzification or rupture, and cristae loosening and destruction are representative of severe postischemic injury.

AT-III also has been widely investigated in the clinical trials. Fourrier et al. [51], have shown that AT-III levels are decreased in patients with severe sepsis and these low levels correlate to the development of renal and liver failure, neurologic, and cardiac disorders, acute lung injury and poor outcome. Replacement of AT-III in such patients has been used in several clinical trials; [52-54] however, the improvement observed in animals treated with AT-III during sepsis or shock is, at the present time, unconfirmed in humans. Although it is not clear yet, there may be several possible reasons for this contradiction. One of the main differences between clinical and experimental studies seems to be inadequate plasma levels and the post-injury administration of AT-III in humans [55].

Apart from sepsis and shock states, there are some surgical conditions such as solid organ transplantation in which one can find a window of opportunity to manage I/R injury before ischemia. Furthermore, in some surgical procedures other than transplantation, like embolectomy for acute mesenteric artery occlusion or thoracoabdominal aortic aneurysm repair, one has time, at least before reperfusion, for administration of AT-III.

In summary, the data presented in this study suggest that mesenteric ischemia and reperfusion induce renal injury characterized by activated neutrophil sequestration, and increased frequency of ultramicroscopical damage. A significant attenuation of intestinal I/R-related renal injury with the use of AT-III concentrate warrants further study to elucidate the potential role of this natural serine protease inhibitor in clinical settings.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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