Pharmacological Research, Vol. 46, No. 4, 2002
doi:10.1016/S1043-6618(02)00168-8, available online at http://www.idealibrary.com on IDEAL®

THE EFFECTS OF ANTITHROMBIN-III ON INDUCIBLE NITRIC OXIDE SYNTHESIS IN EXPERIMENTAL OBSTRUCTIVE JAUNDICE AN IMMUNOHISTOCHEMICAL STUDY

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Accepted 12 July 2002

The absence of bile in the gastrointestinal tract stimulates bacterial overgrowth and bacterial translocation. In the response to endotoxin and LPS-induced endotoxemia which may be prevented by antithrombin-III (AT-III); endothelial cells; and various cells release cytokines, nitric oxide (NO) and other mediators. The purpose of this study was to examine blood NO levels and renal inducible NO synthase (iNOS) expression and determine whether AT-III has an inhibiting effect on renal injury and iNOS expression in obstructive jaundice (OJ). Forty rats were randomized into four groups: group A (Sham), group B (Sham + AT-III, 250 IU kg^{-1}), group C (OJ), group D (OJ + AT-III, 250 IU kg^{-1}). All animals were sacrificed on the 10th day and blood samples were taken for bilirubin and NO level determination. In addition, iNOS expression of the renal tissues was evaluated immunohistochemically. Blood NO levels were found to be 32.99 μmol l^{-1} in group A, 32.26 μmol l^{-1} in group B, 46.33 μmol l^{-1} in group C, and 34.71 μmol l^{-1} in group D. The intensity of iNOS staining in the OJ + AT-III group was less than the intensity of iNOS staining in the OJ group in the renal tissue. This study shows that OJ causes increased production of NO in blood and increased iNOS expression in the kidney. AT-III inhibits iNOS expression and reduces the level of blood NO. Thus, our findings indicate that under conditions of OJ, AT-III limits renal cellular injury by inhibiting LPS-induced iNOS expression.

KEY WORDS: obstructive jaundice (OJ), nitric oxide (NO), inducible NO synthase (iNOS), antithrombin-III (AT-III).

INTRODUCTION

Obstructive jaundice (OJ) is an important clinical problem that may lead to serious sequelae. The systemic consequences of OJ and hyperbilirubinemia are wound breakdown, sepsis, coagulopathy, gastrointestinal hemorrhage, cardiovascular problems, immunodepression, and renal failure [1]. The absence of bile in the gastrointestinal tract stimulates bacterial overgrowth and bacterial translocation. Increased concentrations of bacteria and endotoxin in the portal blood lead to systemic endotoxemia [2, 3]. As a response to endotoxin in LPS-induced endotoxemia; tumor necrosing factor-α (TNF-α), nitric oxide (NO) and other mediators were released from endothelial and Kupffer cells [4]. LPS activates the Kupffer cells and macrophages in the liver and promotes cytokine release. The increased expression of inducible NO synthase (iNOS) is induced by injection of LPS [5].

Antithrombin-III (AT-III) acts as a physiological inhibitor for thrombin and other proteases generated in the coagulation cascade [6]. It is reported that AT-III levels are decreased in sepsis, major injury, and OJ. AT-III inhibits proinflammatory mediators such as TNF-α, interleukin-1 (IL-1), IL-6, and intercellular adhesion molecules (ICAM) [7, 8]. In addition, it has a reducing effect in the adhesion of neutrophils and leukocytes to endothelial cells and it also acts as an anti-inflammatory mediator in LPS-induced endotoxemia by inhibiting leukocyte activation [9]. AT-III has a dose-related effect, inhibits coagulation cascade at doses of 50–100 IU kg^{-1} and prevents endotoxemia at a dose of 250 IU kg^{-1} [10]. We investigated the effect of

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AT-III on the blood NO level and kidney iNOS expression in the rats with OJ.

MATERIAL AND METHODS

Forty male Wistar–Albino rats weighting 200–250 g were housed under standard laboratory conditions and were allowed free access to food and water. Institutional guidelines for animal care were followed throughout the experimental study. The rats were anesthetized by intramuscular 1 cc kg^{-1} ketamine hydrochloride. After abdominal shaving, all were covered with surgical drapes. An abdominal incision was made and bile duct ligation (BDL) carried out by the technique described in detail by Ball *et al.* [11]. The Sham operation was consisted of laparotomy and mobilization of the bile duct only.

The rats were divided into four groups: group A ($n = 10$), Sham operation; group B ($n = 10$), Sham operation and s.c. administration of 250 IU kg^{-1} AT-III (Kybernin) (immediately and for 5 days p.o.); group C ($n = 10$), BDL (no administration of AT-III); group D ($n = 10$), BDL and s.c. administration of 250 IU kg^{-1} AT-III (starting 5 days after BDL and continuing to 10th day). The animals were scarified on the 10th day. Blood samples were taken for bilirubin and NO levels.

Nitric oxide level determination

NO is deactivated very rapidly by oxidation to stable end products like nitrite and nitrate in biological fluids. Measurement of nitrite and nitrate were making by using a procedure based on the Griess reaction [12]. Blood samples were separated and stored at −80 °C until they were used for assay. Nitrates were quantitatively converted to nitrites for analyses. Enzymatic reduction of nitrate to nitrite was carried out by using coenzymes (NADPH and FAD). *N*-1-(naphytanyl)-ethylenediamine dihydrochloride, sulfanilamide and incubation solutions were mixed. These mixtures were incubated for 5 min at room temperature in dimmed light and measured at 550 nm. A total of 1.00 mM sodium nitrite was used as standard for determination of nitrite and 80 mM potassium nitrate was used as standard for determination of nitrate (Nitric Oxide Colorometric Assay; Boehringer Mannheim, Cat. No. 1756281, Mannheim, Germany).

Histopathology and immunohistochemistry

Histopathological studies were performed on 5-μm slides. Tissue sections were formaline-fixed by using standard procedures with uniform conditions of fixation, and stained by hematoxylin and eosin, and PAS. iNOS was determined immunohistochemically by a standardized streptavidin–biotin–peroxidase method on formaline-fixed paraffin-embedded renal tissues. After deparaffinization, sections were treated with 10% hydrogen peroxidase in filtered water to block endogenous peroxidase activity. To retrieve antigen, slides were boiled with 10 mmol l^{-1} citrate buffer (pH 6.8) for 10 min. After preincubation with UV block (Lab Vision) for 20 min, sections were incubated with primary antibody to iNOS (1.1500; Transduction Laboratories, No. 32020) overnight at 4 °C, followed sequentially with biotinylated goat antipolyvalent (Lab Vision) for 20 min, and streptavidin–peroxidase complex (Lab Vision) for 30 min. 3-Amino-9-ethylcarbazole

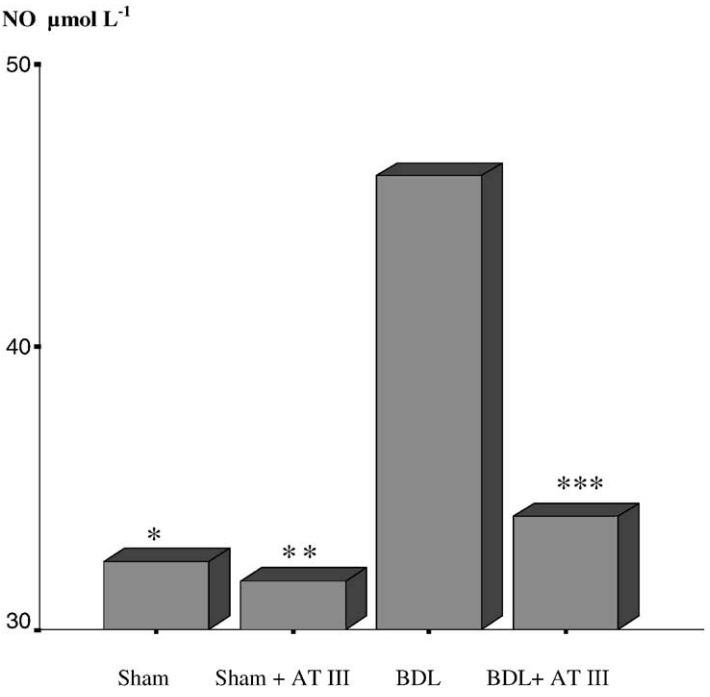

Fig. 1. The blood NO levels in the groups. The asterisks indicate statistical significance versus BDL group ($^{*}P = 0.014$, $^{**}P = 0.004$, $^{***}P = 0.022$).

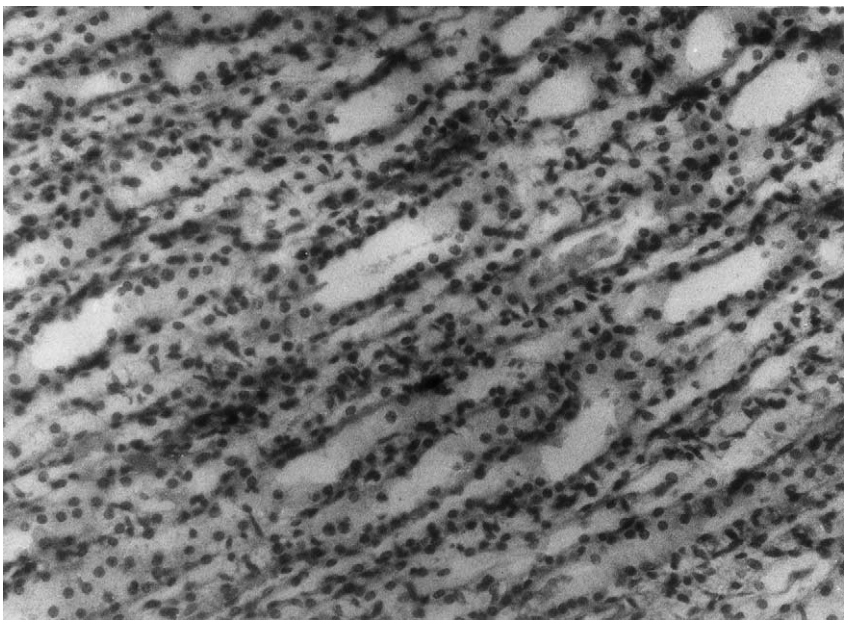

Fig. 2. The renal histopathological changes in OJ group, H–E ×20.

(AEC) was used as the chromagen and hematoxylin was used as a nuclear counterstain. Semiquantitative method was used for immunohistochemical evaluation. The staining score variable was used for iNOS staining only in the epithelial component. This variable was obtained as the product of the points of two variables (staining pattern and staning intensity) staining type; no staining (0 point), focal and weak staining (1 point), diffuse and moderate staining (2 points), diffuse and intense staining (3 points).

Statistical analysis

The differences between the groups were analyzed by the one-way ANOVA test, followed by the Mann–Whitney *U*-test. Data were presented as mean ± SD. *P* values of less than 0.05 were considered to be significant.

RESULTS

Animals underwent BDL and transection became markedly jaundiced within 72 h. Mean direct bilirubin levels were 9.2 and 8.9 mg dl^{-1} in groups 3 and 4, respectively.

Blood NO levels

Blood NO levels determined by the Nitric Oxide Colorometric Assay were 32.99 ± 3.50 μmol l^{-1} in group A, 32.26 ± 1.64 μmol l^{-1} in group B, 46.33 ± 2.51 μmol l^{-1} in group C, and 34.71 ± 2.85 μmol l^{-1} in group D (Fig. 1). While there were significant differences between group A (Sham) and group C (OJ) ($P = 0.014$), group B (Sham + AT-III), and group C (OJ) ($P = 0.004$), and group C (OJ) and group D (OJ + AT-III) ($P = 0.022$), no significant differences were observed between the other groups.

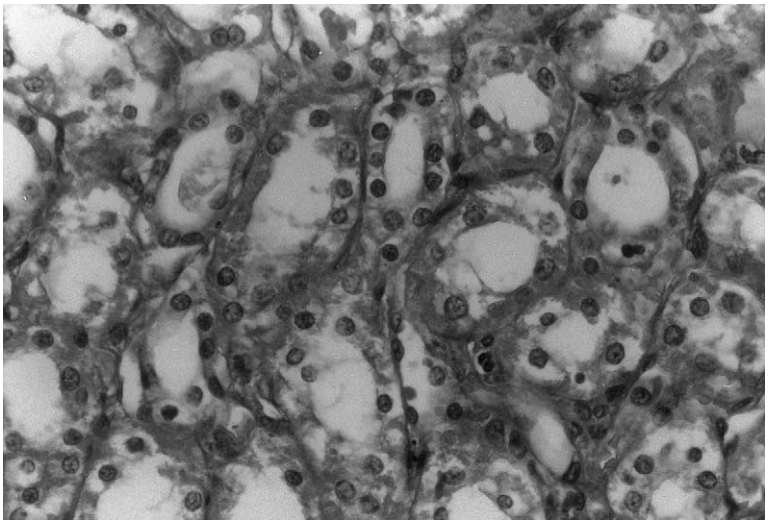

Fig. 3. The renal histopathological changes in Sham group, H–E ×20.

Histopathology

There were minimal differences between the OJ and OJ + AT-III groups in hematoxylin–eosin (H–E) and PAS-stained sections. It was determined by swelling and vacuolization in the cytoplasm of tubular epithelium. Loss of cilia and blebbing in the apical cytoplasm of the proximal tubular epithelium was noticeable. In addition, accumulation of bilirubin pigment was observed in the cytoplasm of the epithelial cells (Fig. 2). Peritubular interstitial edema in the medulla was determined. These findings were less evident in the Sham and OJ + AT-III group than in the OJ group (Figs 3 and 4).

Immunohistochemistry

Immunohistochemical staining was evaluated only in the tubular epithelium with cytoplasmic pattern. In all groups, iNOS staining was present in the glomeruli, the capillary bed, the interstisium, the peritubular capillary endothelium and the transitional epithelium covering the papilla. In the control group, iNOS staining was observed most evidently in the tubular epithelia in the medulla (Fig. 5). In the same areas of the OJ group, the staining intensity increased with iNOS (Fig. 6), but in the OJ + AT-III group, iNOS staining was less than in the OJ group (Fig. 7). Tissue staining score of iNOS were 0.6 ± 0.16 in group A, 0.7 ± 0.21 in group B, 2.8 ± 0.13 in group C, and 1.1 ± 0.17 in group D. While there were significant differences between group A (Sham) and group C (OJ) ($P = 0.001$), group B (Sham + AT-III) and group C (OJ) ($P = 0.01$) and group C (OJ) and group D (OJ + AT-III) ($P = 0.001$), no significant differences were observed between the other groups.

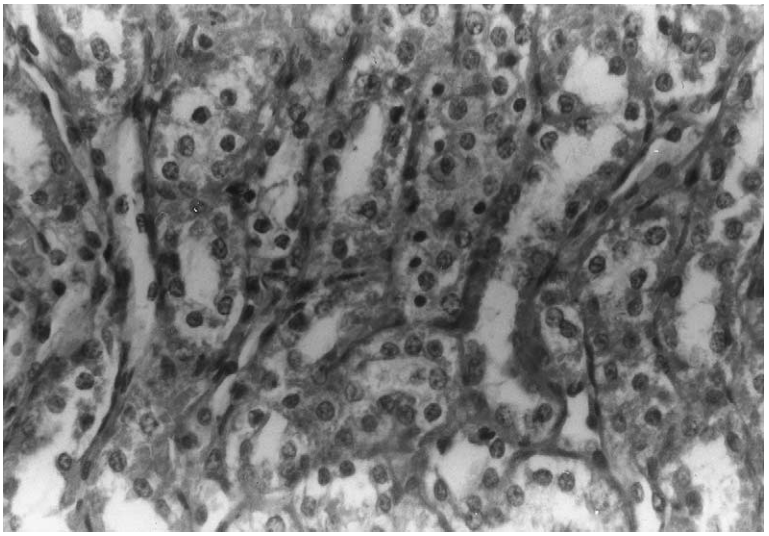

Fig. 4. The renal histopathological changes in OJ + AT-III group, H–E ×20.

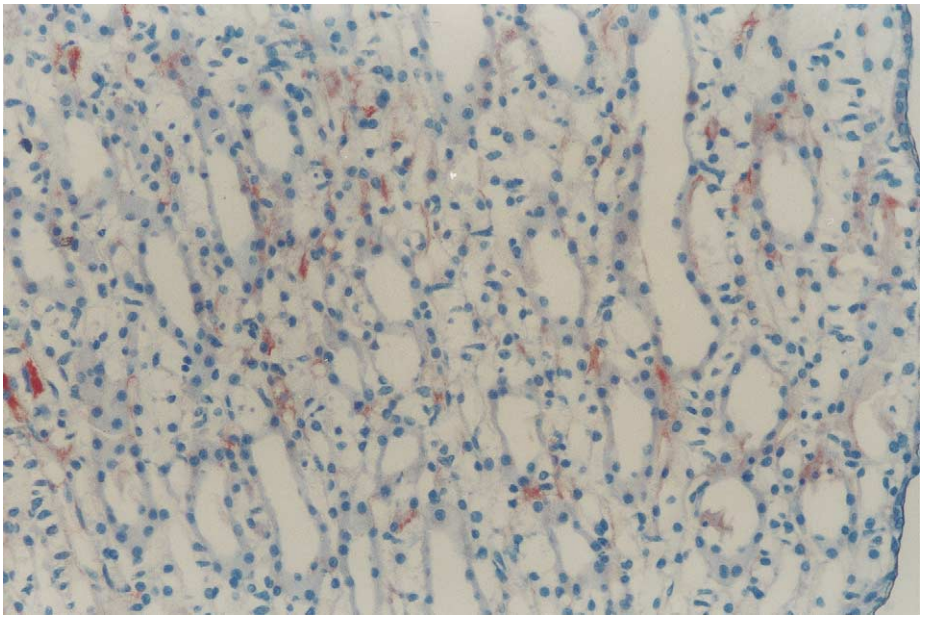

Fig. 5. iNOS staining in tubule epithelium in the Sham-operated group, ×10.

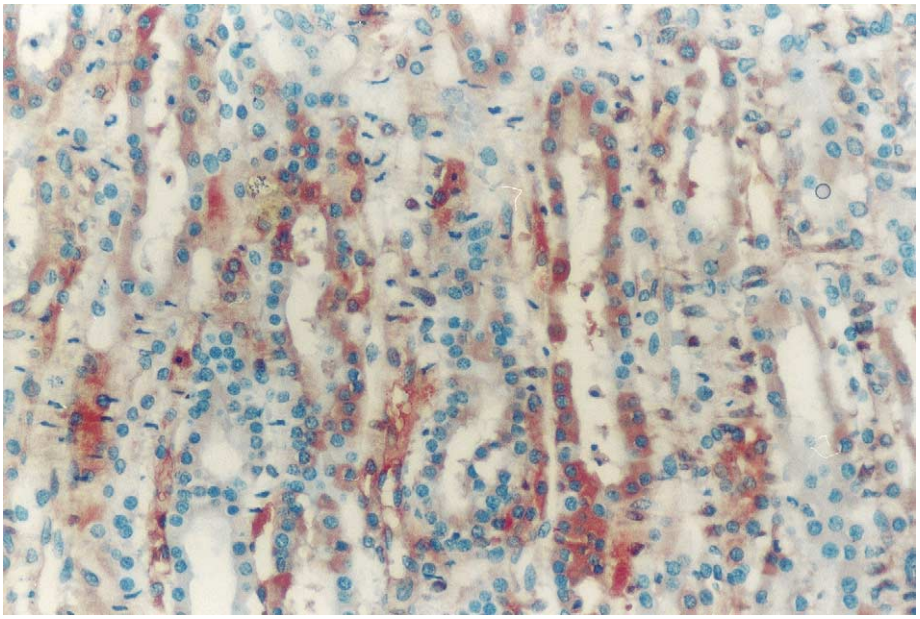

Fig. 6. Intensive iNOS staining in OJ group, ×10.

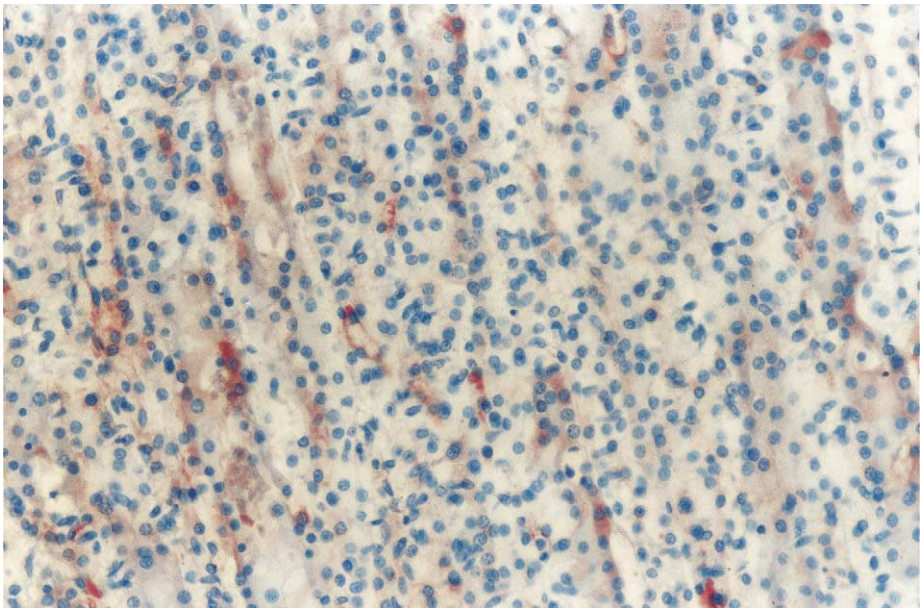

Fig. 7. In OJ with AT-III group iNOS showing less staining than the OJ group, ×10.

DISCUSSION

OJ leads to deficiency of bile in the intestinal lumen. In the absence of bile in the intestinal lumen; the bile salts, bile pigments, phospholipids cannot be used by the mucosa. This event promotes intestinal absorption of endotoxin and results in portal endotoxemia and systemic endotoxemia [2, 3]. LPS, which are cleared by Kupffer cells in the liver in healthy people, cannot be cleared in patients with OJ, resulting in systemic endotoxemia and multiorgan failure [11]. At the initial phase of endotoxemia with LPS; complement systems, neutrophils, monocytes, endothelial cells and coagulation cascades are activated. As the neutrophils, monocytes and endothelial cells are activated, proinflammatory cytokines are secreted and, at the same time, these cytokines reactivate these cells. On the other hand, the activation of the complement system activates chemotaxis, resulting in an increase of free radicals and lysosomal enzymes. Activation of neutrophils, monocytes, and endothelial cells results in the overexpression and synthesis of adhesion molecules like ICAM, VICAM, E-selectin, platelet activating factor, leukotrienes, lipid mediators such as arachidonic acid metabolites and mediators originated from endothelial cells. As a result of these mediators and adhesion of cells; proteases and free radicals increase, and at the same time a coagulation

cascade is activated, resulting in impairment of microcirculation, both of which lead to multiorgan failure [13].

iNOS, which is one of the enzyme synthesis of NO is not expressed under normal conditions, but is induced by cytokines and endotoxin [14]. It is known that, in OJ, iNOS is synthesized in macrophages, neutrophils and endothelial cells as a result of LPS-induced endotoxemia, thus leading to the overproduction of NO [15]. It is also thought that cellular damage is triggered by endotoxemia with LPS and increased lipid peroxidation and, leads to hepatic and renal damage [16]. Kawamira *et al.* [17] have shown that increased hepatic lipid peroxidation and liver damage via excessive NO synthesis occurs in OJ. Shiomi *et al.* [18] have stated that there is excessive production of NO leading to impairment in mitochondrial function in hepatic tissue of rats with OJ via lipid peroxidation. On the other hand, renal failure is a common problem in OJ patients. The exact reason of renal impairment in OJ is unknown, but many factors have been implicated; including renal and hemodynamic changes, increased atrial natriuretic peptide (ANP), renin angiotensin, prostoglandins, the toxic effect of biliurubin in the tubulus and extracellular fluid changes. Moreover, it is mentioned that a humoral mediator called ANP increases in OJ, leading to natriuresis, kaliuresis, peripheral vasodilatation and hypotension. NO is a mediator potentially responsible for these changes [19]. At present, it is thought that in endotoxemia, increases in cytokine levels and especially mediators such as NO play a major role in the settlement of renal failure [20, 21]. Various investigators have shown that overexpression of iNOS in tissue and increased level of NO in blood enhances the renal damage [22, 23]. Also, Schwartz *et al.* [24] have demonstrated that inhibition of iNOS expression atenues the renal damage that is caused by LPS-induced endotoxemia. As mentioned here, in OJ, increased NO levels lead to deleterious effects in tissue and it is very obvious that inhibition of the overproduction of this molecule plays a major role in preventing cellular damage.

In our experimental study, we found increased iNOS expression in renal tissue and increased NO levels in blood. These results are consistent with the hypothesis that portal and systemic endotoxemia induces the production of blood NO and the expression of tissue iNOS. In the present study, AT-III is administered to rats in OJ for the first time. Blood NO levels and iNOS staining in the kidney tissue were determined and these were found significantly lower than in those not administered AT-III. We attributed this difference to AT-III's reducing effect of endotoxemia that probably stems from LPS in jaundiced subjects. There is not any study, which has been done before to investigate the relation between AT-III and iNOS and NO. Similarly, Opal [25] think that AT-III may be used as therapeutically rational drug in sepsis caused by LPS or of other causes. Also, the study, which has been done by Murakami *et al.* [26], has demonstrated that fatal effect of LPS in rats can be deleted by the use of some pharmacological agents, which increase the level of AT-III, is similar to the result of our experimental study.

Our findings suggest that AT-III limits renal cellular injury by the inhibition of LPS-induced iNOS expression under conditions of OJ. Since AT-III limits the hematologic and renal complications, which are developed in OJ and in endotoxemia, related to OJ, this drug may be a candidate of being an important alternative in the treatment of complications related to OJ and in the treatment of renal impairment developed in OJ. On the other hand, AT-III may be useful agent in preventing renal injury in the sepsis, which caused other etiologic agent such as G negative bacteria, because the pathophysiologic mechanism of renal injury is similar to OJ.

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