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ORIGINAL PAPER

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Prevention of renal damage by alpha tocopherol in ischemia and reperfusion models of rats

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Abstract Ischemia-reperfusion injury in rat kidneys most probably comes from oxidative stress, but the possible preventive effect of alpha-tocopherol (AT) treatment on this injury has not yet been established. Forty male Wistar rats were randomly divided into four groups. The left renal arteries of all rats except the controls were clamped to induce renal ischemia. The left kidneys of the rats in the ischemia group were removed following 40 min ischemia. The rats in the ischemia-reperfusion and ischemia-reperfusion-AT groups were treated similarly, but in these groups the renal arteries were re-perfused for 1 h following ischemia. The rats in the ischemia-reperfusion-AT group also received 10 mg/kg AT 3 h prior to ischemia. The specimens were examined histopathologically and ultrastructurally, and the tissue calcium levels were measured. Light microscope and ultrastructural examination showed that the greatest damage occurred in the ischemia-reperfusion group. The highest level of tissue calcium was also found in this group. In the ischemia-reperfusion-AT-treated group, less tissue damage and a lower tissue calcium concentration was found compared to both the ischemia and ischemia-reperfusion groups. Our results indicate that AT can reduce tissue damage after ischemia-reperfusion injury.

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Introduction

One of the four mechanisms responsible for tissue damage after ischemia involves reactive oxygen species (ROS) related damage [27]. Tissue damage after ischemia is not solely restricted to the ischemia period, but it can occur after reperfusion status is re-established. One of the possible explanations for this phenomenon is ROS related damage [26, 33], since ROS have a harmful effect on the cell membrane lipids, proteins, nucleic acids and carbohydrates [7, 14, 43]. Alpha-tocopherol (AT) is one of the well-known defense molecules against oxidative damage and is amply present in nature [8, 9, 16, 32]. It contains an aromatic ring, which constitutes the chemically active part of the compound. AT was reported to be a protective agent against tissue damage after ischemia in isolated organs [37]. AT treatment corrected the glomerular filtration deficiency [12] and proteinuria by enhancing ATP synthesis during reperfusion status following ischemia [40]. Furthermore, pretreatment with AT significantly reduced the lipid peroxidation of renal cells and renal dysfunction induced by renal ischemia-reperfusion (I/R) in rats [38]. The possible mechanism of the protective effect of AT on I/R injury seems to be mediated by a direct chain breaking effect of AT on lipid peroxidation (LPO) [42]. Although previous experiments showed the beneficial effect of AT treatment in I/R injury of animal kidneys [38, 42], this effect has not yet been confirmed by either histopathological or ultrastructural analyses. In the current study, we investigated whether the protective effect of AT on I/R damage in the kidney of rats could be correlated with the concurrent ultrastructural and histopathological findings. For this purpose, we examined the rat kidneys by light and electron microscopy and measured tissue calcium levels after ischemia, I/R, and I/R and AT treatment (I/R/AT).

Materials and methods

Forty 200–240 g, 5 month old, male Wistar rats were fed with standard rat chow and water ad libidum. Immediately before surgical intervention, the rats were anesthetized with ketamine (125 mg/kg). The body temperature of the rats was kept constant during the operation. A laparotomy incision was performed in the midline. The right kidney was removed as in previous studies [20, 21] and the left perirenal fat tissue was dissected. Under optical magnification (×10) (Zeiss Opmi Pico) renal vascular pedicle was isolated and the renal artery was clamped using appropriate microclamps except in the control group. Intraperitoneal serum was physiologically maintained during the operation [19]. Heparin was not given to prevent arterial occlusion. The clamp was then released following 40 min of ischemia. The rats were randomly divided into four groups (ten in each). In the ischemia group, a left nephrectomy was performed following the 40 min ischemia. Left nephrectomies were performed following 40 min ischemia and 1 h reperfusion in the I/R and I/R/AT groups. After removal of the clamps, the existence of pulsations was observed and pencil doppler ultrasound (Multi Dopplex) was used to confirm the patency of the arteries. Additionally, 10 mg/kg AT was given intraperitoneally to the I/R/AT group 3 h prior to ischemia. The control group was treated similarly, but only anesthesia, laparotomy, and bilateral nephrectomies were performed.

For light microscopy, the tissue specimens were fixed in 10% formaldehyde, processed in an autotechnicon and blocked into paraffin. Sections of 5-μm thickness were cut with a microtome, stained with hematoxylen-eosin (H-E) and examined with a light microscope. We used semi-quantitative scores developed by Paller et al. [35]. In this system, following scores are used: (1) tubular epithelial smoothness: 1, (2) loosing of brush-like edge: 2, (3) cytoplasmic vacuolization: 1, (4) cell necrosis: 1 or 2, and (5) obstruction of tubular lumen: 1 or 2.

The maximum attainable score in this system is seven. We examined at least 100 cortical tubules in ten different areas and for each animal the mean value ± SD was calculated. One way ANOVA with Tukey's post-hoc test was used for statistical analysis (with groups as the independent variable).

Electron microscopic specimens were fixed in 2% paraformaldehyde and 2.5% gluteraldehyde in 0.1 M PBS (pH 7.4) at 4°C for 1 day. They were post-fixed with 2% osmium tetroxide, and then dehydrated in graded ethanol and embedded into Araldite. Ultrathin sections were cut using a diamond knife and stained with both uranyl acetate and lead citrate. Tissue specimens were examined using a Leo 906E electron microscope with an accelerating voltage of 80 kV.

The tissue specimens obtained for biochemical analyses were weighed and homogenized (after adding 1 ml physiological serum). The specimens were then centrifuged and the clear supernatants divided into separate tubes. The calcium (Ca) concentration in the supernatants were measured with an auto-analyzer and expressed as mg/dl. However, the tissue calcium concentration was expressed as mg/wet tissue weight. One way ANOVA with Tukey's post-hoc analysis was again used for statistical comparison of Ca concentrations between groups.

Results

Normal arterial flows were observed in all reperfusion cases after removal of the clamps. The histopathological changes, such as tubular cell flattening, loosing of brush-like edges, cytoplasmic vacuolization, necrosis, and lumen obstruction of tubules, were observed in almost all of the specimens from the I/R group (Fig. 1). Similar changes were present in the control group, however only to a limited extent. The histopathological damage in the ischemia group was significantly less than for the (I/R) group (Fig. 2). The I/R/AT group showed the least histopathological damage among the study groups (Fig. 3). The histopathological features of the specimens belonging to the I/R/AT group were very similar to the specimens obtained from the controls (Fig. 4). We also noticed that there was an absence of necrosis and obstruction of tubular lumen in the I/R/AT group.

The greatest histopathological damage score occurred in the I/R group (4.92 ± 0.12) followed by the ischemia group (3.52 ± 0.1) and I/R/AT group (3.07 ± 0.18). The lowest score was observed in the control group (0.38 ± 0.09) (Fig 5). All statistical comparisons made between the four groups gave significant results in histological scoring ($P < 0.05$).

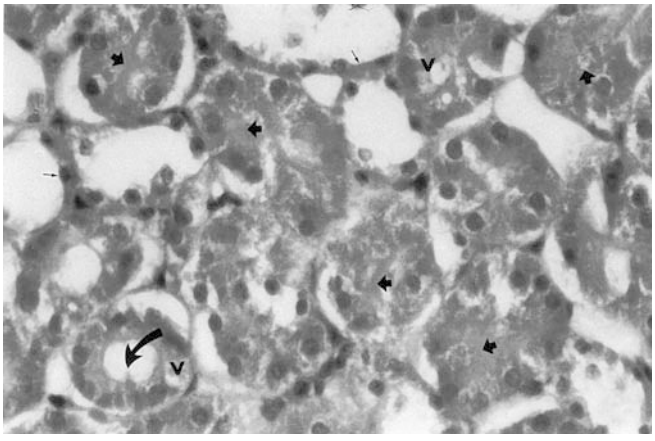

Fig. 1 A tissue specimen belong to the ischemia-reperfusion group. Tubular obstructions are seen in most of the tubular lumens (*thick arrows*). Some of the tubular epithelial cells are flattened compared to the normal ones (*thin arrows*). Occasionally, some epithelial cells show vacuolization (*v*). Brush-like edges are rarely observed (*big arrow*) (H-E, ×40)

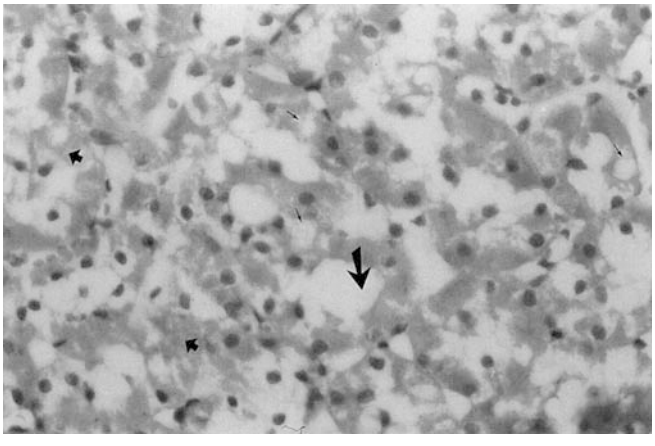

Fig. 2 Histopathological specimens belong to ischemia group reveal conspicuous cytoplasmic vacuolization (*thin arrows*). Because of necrotic changes, epithelial cells can be observed as silhouettes in many areas (*thick arrows*). Loosing of brush like edges of tubular cells are seen (*big arrow*). (H-E, ×40)

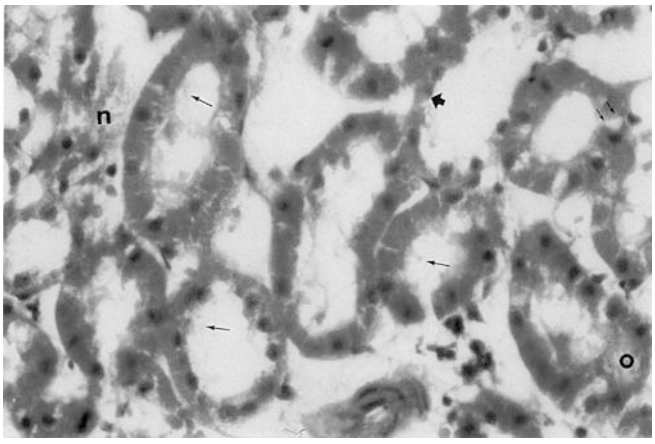

Fig. 3 An histopathological specimen belonging to the ischemia-reperfusion-alpha tocopherol group shows that although flattening of tubular epithelial cells is still seen in this group (*thick arrow*), these changes are less severe compared to the ischemia and ischemia-reperfusion groups. Brush-like edges of tubular epithelial cells can be observed in many areas (*thin arrows*). Cytoplasmic vacuolization (*double arrows*), lumen obstruction of tubular epithelial cells (*o*), and necrotic changes (*n*) are not extensively observed (H-E, ×40)

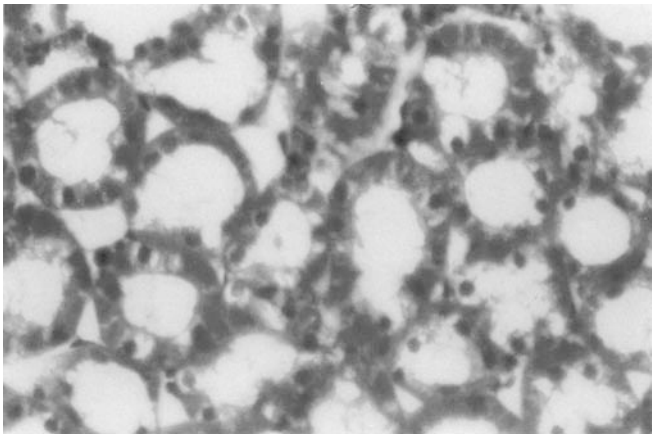

Fig. 4 An histopathological specimen that belongs to the control group is shown. The brush-like edges can be seen clearly. There is no tubular obstruction or necrotic change

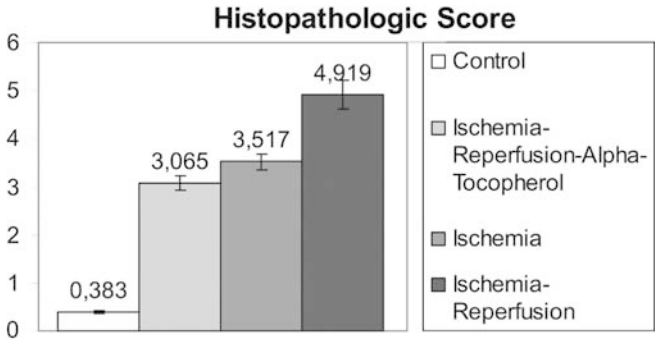

Fig. 5 The results of histopathological scoring to assess tissue damage following renal ischemia, renal ischemia/reperfusion and renal ischemia/reperfusion/alpha-tocopherol treatment in rats are seen. Control group rats have the least extensive damage. The ischemia/reperfusion group has the highest score and the ischemia/reperfusion/alpha-tocopherol group has less extensive damage compared both to the ischemia and ischemia/reperfusion groups

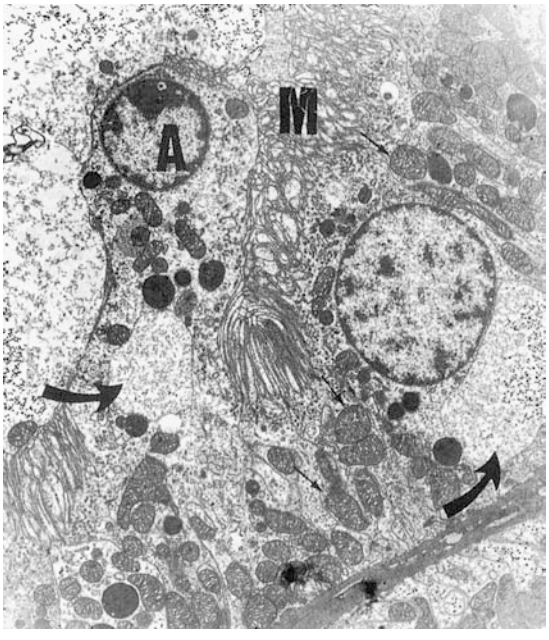

Fig. 6 In this micrograph of the ischemia group, an apoptotic cell (*A*) is next to the other cells. Tubular cells are seen with apical microvilli borders (*M*), long mitochondria (*arrows*), lysosomes, and big vacuoles (*big arrow*) (×3,597)

Ultrastructural examination revealed that epithelial cells of the proximal renal tubule in the ischemia group showed apoptotic changes and the sloughing of apoptotic cells into the lumen, but the microvilli of the epithelial cells were still intact. The cytoplasm of the epithelial cells had long mitochondria, lysosomal granules, and big vacuoles filled with flocculent material (Fig. 6). In the I/R group, histopathological damage was more pronounced than in the ischemia group. In this group, the proximal tubule epithelium showed mitochondrial damage, cytoplasmic condensation, and microvilli disappearance. The lumen of the proximal tubules was irregular and interstitial areas were invaded by plasma cells and macrophages (Fig. 7). In the I/R/AT group, damage was less pronounced than in the ischemia-reperfusion group. The loss of microvilli was not observed but some big vacuoles filled with flocculent material and lysosomal granules were detected in the cytoplasm of the tubular epithelial cells (Fig. 8). These features varied only minimally from the controls (Fig. 9).

The highest tissue calcium level was detected in the ischemia-perfusion group (14.81 ± 0.82 mg/wet tissue weight). A significantly lower calcium level was measured in the ischemia group (11.67 ± 0.48 mg/wet tissue weight) compared to the I/R group. In the I/R/AT group, a significantly lower level of calcium (9.92 ± 0.99 mg/wet tissue weight) was found compared to the other groups excluding the controls. The lowest tissue Ca level was detected in the control group (9.23 ± 1.12 mg/wet tissue

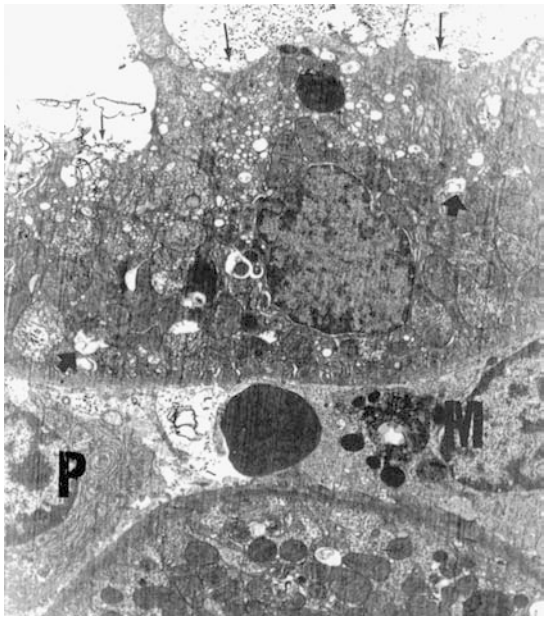

Fig. 7 In this micrograph of the ischemia-reperfusion group, there is vacuolization (*thick arrows*), cytoplasmic condensation, and loss of brush border (*thin arrows*) on the apices of proximal tubule cells. A plasma cell (*P*) and a macrophage (*M*) are seen in the interstitial area. (×3,597)

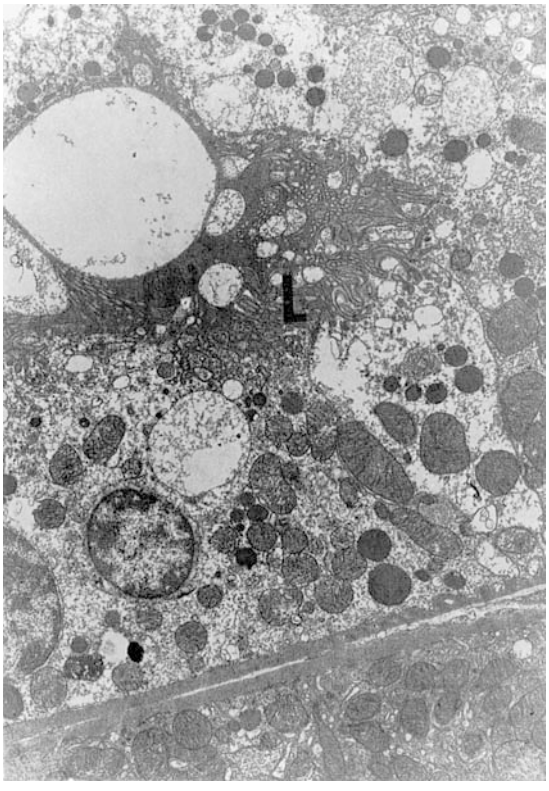

Fig. 8 In the ischemia-reperfusion-alpha-tocopherol group, necrosis and apoptosis are not seen. The luminary surface (*L*) of the proximal tubular cells has long microvilli. Cytoplasmic degeneration is less than in the other groups. (×3,597)

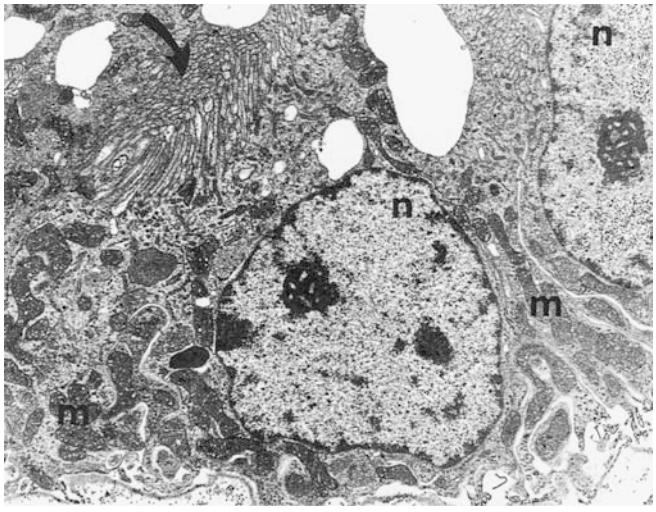

Fig. 9 In this micrograph of the normal group, the proximal tubule epithelium is in a normal relationship with the nucleus (*n*) and nucleolus. Apical borders of the epithelia contain normal appearing extensions of the microvillae (*arrow*). Elongated mitochondria (*m*) and basal invaginations are also seen (×4,646)

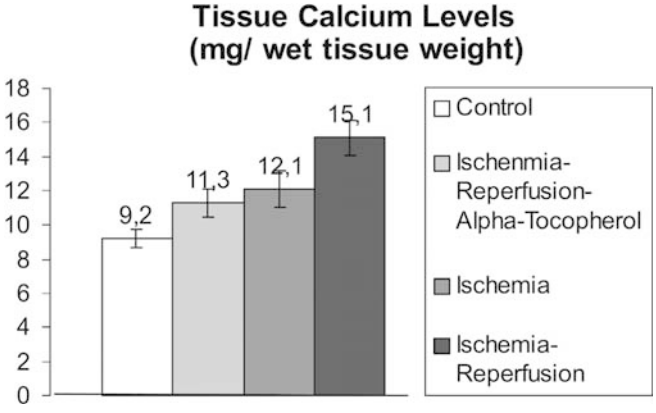

Fig. 10 Tissue calcium levels are at their highest level in the ischemia/reperfusion group of rats. In the ischemia/reperfusion/alpha-tocopherol group, although the tissue calcium level is higher than controls, it is lower compared both to the ischemia and ischemia/reperfusion groups

weight). There was no statistically significant difference between the control and I/R/AT groups in terms of tissue Ca levels ($P > 0.05$). When tissue calcium levels of ischemia and I/R groups were compared to the control group, significant differences were found ($P < 0.05$) (Fig. 10).

Discussion

Acute renal insufficiency induced by ischemia is a clinical and experimental entity. It is characterized by a severe reduction of glomerular filtration, widespread tubular damage, epithelial necrosis, and tubular obstruction [3]. The clinical entity of acute renal insufficiency can be induced by renal artery clamping for experimental purposes [33]. The flattening of tubular epithelial cells,

loosing of brush-like extensions, cytoplasmic vacuolization, epithelial cell necrosis, and tubular obstruction are histopathological features of acute renal insufficiency. Although a wide range of potential mechanisms are present to explain the pathogenic mechanism of tissue damage after renal ischemia, the most attractive one is the oxidative mechanism. Neutrophils, which are concentrated in the area of ischemia, produce superoxide via NAPDH oxidase, and the tissue damage is believed to come from ROS [18]. Thus, it is logical to think that tissue damage following ischemia can be prevented by concomitant AT treatment since AT provides good protection against oxidative damage [1, 2]. In this experiment, we showed that significantly less histopathological damage occurred in AT-treated rats compared to the untreated ones. This finding can also be accepted as indirect evidence supporting the mechanism of free radical damage.

The more extensive damage seen in the I/R group compared to the ischemia group could be caused by ROS, since ROS are produced mainly in the reperfusion phase and could cause more damage than ischemia itself [6, 12, 17, 31]. We observed that the tissue damage did not solely occur during ischemia but continued during reperfusion [26]. The entrance of oxygen into tissues during reperfusion can activate xanthine oxidase which produces huge amounts of superoxide anion [18]. A previous experimental study reported that tissue levels of ROS increased three- to tenfold during reperfusion compared to normal levels [34].

The I/R/AT group of rats showed the least tissue damage among all groups except the controls. We did not observe any lumen obstruction or cell necrosis in this group. The most likely explanation for this observation is the prevention of ROS-associated damage by the antioxidant properties of AT [11 23, 25, 41]. AT has also chain breaking capabilities and this feature can prevent the lipid peroxidation effect of free oxygen radicals on cell membranes [9]. The reducing effect of AT on lipid peroxidation products measured by malondialdehyde and chemiluminescence levels after I/R injury to rat kidneys has been reported previously [38]. Uysal and co-workers [42] found that during long-term reperfusion (72 h) following renal ischemia in rats, AT triggered a decrease in the MDA levels in the ischemic tissue but did not provoke a significant effect on superoxide dismutase or catalase activities. Their results showed that AT has a protective effect on I/R injury by a direct chain breaking effect on lipid peroxidation. AT treatment was also reported to have a beneficial effect on renal ischemia in rats without reperfusion [13, 45]. The most important difference between our study and previous studies is that the previous studies did not provide any histopathological or ultrastructural data on the preventive effect of AT treatment for either ischemia or I/R induced damage, however, our study confirmed this preventive effect both histopathologically and ultrastructurally.

The electron microscopic findings correlated well with the histopathological findings. The worst cell damage was observed in the I/R group. A lower degree of cell damage was found in the ischemia group. We found only slight cell damage in I/R/AT treated rats. In this group, we could even observe microvilli in the proximal tubule. Another interesting finding was that the mitochondrial damage was correlated with increasing cell damage. Although we observed mitochondrial swelling and damage in the I/R group, the I/R/AT group showed only mitochondrial swelling. This can be accepted as evidence for more pronounced damage in the I/R group [22]. Mitochondrial damage during reperfusion has been reported previously [15]. AT treatment prevented mitochondrial damage, since we did not observe any such damage in the I/R/AT group of rats.

The cellular calcium concentration increased along with the wasting of the cell fuel depot following ischemia [4, 28, 39]. In our study, the tissue calcium concentration was significantly higher in the ischemia group than in the controls. The increase in cellular calcium concentration indicates enhanced cell damage and the beginning of programmed cell death [5, 41]. It was reported that calcium played a primary role in cell death after ischemia and reperfusion [30]. Increased cell calcium concentration restricts mitochondrial functions and increases in mitochondrial calcium levels. This can cause cell death [44]. Previous reports indicate an increased calcium concentration in mitochondria after ischemia [10]. We measured parallel increases in tissue calcium levels along with the enhanced histopathological and ultrastructural evidence of tissue damage [24, 29]. Another demonstration of the preventive effect of AT was finding significantly lower tissue calcium levels in the I/R/AT group compared to the other groups. AT may have a preventive effect on permanent cell damage, since a massive calcium collection in the cell causes irreversible cell damage. Furthermore, we did not observe any statistically significant difference between the control and I/R/AT groups.

In conclusion, tissue damage can occur during the ischemia-reperfusion phase. We showed this damage using light microscopy, electron microscopy, and biochemical methods. Additionally, this damage can be significantly reduced using concomitant AT treatment. In daily practice, ischemia and the reperfusion that follows it are frequently encountered clinical problems. Thus, it is important to know that tissue damage can continue after reperfusion. There is a tiny gap between reversible and irreversible tissue damage. Our study clearly demonstrated that AT could prevent further tissue damage after reperfusion had been established. Thus, its use to prevent irreversible tissue damage after ischemia and reperfusion can save the organ under stress. Further studies are clearly needed to reveal the beneficial effects of AT therapy in renal surgery and renal clamping.

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