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UROLOGY - ORIGINAL PAPER

Combination antioxidant effect of α-tocoferol and erdosteine in ischemia–reperfusion injury in rat model

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Abstract
Introduction Renal ischemia/reperfusion (I/R) which is an important cause of renal dysfunction is inevitable in renal transplantation, surgical revascularization of the renal artery, partial nephrectomy and treatment of suprarenal aortic aneurysms.
Aim The purpose of this study was to investigate the efficacy of α-tocopherol and erdosteine combination in the reduction in injury induced by ROS in a rat model of renal ischemia–reperfusion.
Materials and methods Thirty-six- male Wistar albino rats weighing 200–250 g were utilized for this study. Rats were divided into six groups, and each group was consistent of six rats: (1) sham-operated (control), (2) ischemia group (3) I/R group, (4) I/R/α-tocoferol group (5) I/erdosteine group (6). I/R/α-tocoferol and erdosteine group. Biochemically tissue MDA, XO and SOD activities, light and electron microscopic findings were evaluated.
Results The erdosteine and α-tocoferol significantly reversed the effect of protein oxidation and lipid peroxidation induced by I/R shown by the decreased levels of MDA and XO activities. Both MDA and XO levels were found to be lower in group 6 compared to single agent treatment groups, and this was significantly different. All treatment groups showed increased SOD activity, which accounts for their oxidative properties. The mean Paller score of the combination treatment group (group 6) was lower than all groups except the sham group (3.67 ± 1.2), and this finding was statistically significant (0.05). Our results showed that the antioxidant pretreatment with α-tocopherol and erdosteine combination reduced lipid peroxidation of renal cellular membranes in a model of normothermic renal ischemia–reperfusion in rats. Combination of erdosteine and α-tocopherol has a synergistic effect of protection against oxidative processes. Long-term use of α-tocopherol seems to have a greater effect on the prevention of IR injury. However, further investigations are needed for the clinical applications of our findings.

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Keywords Ischemia–reperfusion injury · Antioxidants · Erdosteine · α-Tocopherol · Kidney

Introduction

Renal ischemia/reperfusion (I/R), which is an important cause of renal dysfunction is inevitable in renal transplantation, surgical revascularization of the renal artery, partial nephrectomy and treatment of suprarenal aortic aneurysms [1, 2].

Although the exact mechanisms involved in the pathogenesis of renal I/R injury have not been fully understood, it is generally believed that reactive oxygen and nitrogen species (ROS, RNS) are key mediators of I/R induced damage to the kidney [3–5]. Restoration of blood flow to ischemic tissues can result in recovery of cells if the injury is not permanent. However, depending on the intensity and the duration of the ischemic insult, variable number of cells may still die after blood flow is reconstituted, by necrosis, as well as by early.

The prognosis is complicated by the fact that reperfusion, although essential for the survival of ischemic renal tissue, may lead to additional damage (reperfusion injury), contributing to renal dysfunction. Two important mechanisms have been proposed to explain this reperfusion injury. One of the theory is the detrimental effect of generated reactive oxygen radicals during reintroduction of molecular oxygen to the previously ischemic tissue, and renal inflammation, involving cytokine/adhesion molecule cascades with recruitment, activation, and diapedesis of circulating leukocytes is also implicated [6–10].

A number of drugs or chemicals have been used to prevent I/R injury in kidneys. TNF-inhibitors, edaravone, geranylgeranylacetone, catechin, glibenclamide, tempol, midkine, simvastatin and L-arginine were among some of the compounds found to be effective in the prevention of lipid peroxidation and general renal damage [11].

α-Tocopherol (vitamin E) is localized in the cell membranes and contributes to their stability and seems to protect the membrane lipids against oxidative damage [12]. In a previous study, we have demonstrated the effectiveness of α-tocopherol in a similar ischemia–reperfusion injury model in rats [13].

Erdosteine [*N*-(carboxymethylthioacetyl)-homosysteine thiolactone] as a thiol derivate, contains two blocked sulfhydryl groups, which become free only after hepatic metabolization. Experimental and clinical studies have demonstrated the free radical scavenging properties of erdosteine. The reducing potential of these sulfhydryl groups accounts for free radical scavenging and antioxidant activity of erdosteine [14–16]. In a previous study, effect of erdosteine in the I/R injury rat model has been proven to be effective [17].

The purpose of this study was to investigate the efficacy of α-tocopherol and erdosteine combination in the reduction in injury induced by ROS in a rat model of renal ischemia–reperfusion. Our theory was that combination of increased cellular resistance to oxidative process with the use of α-tocopherol and free radical scavenging with erdosteine would prove a higher tissue protection in the I/R injury of the renal cells than used alone since they involve different antioxidant pathways.

Materials and methods

Animals and procedure

Thirty-six- male Wistar albino rats weighing 200–250 g were utilized for this study. They were purchased from the Selcuk University Experimental Research Center (Konya, Turkey) and housed in animal laboratory of our university. The animals were fed with a standard diet and kept on a physiological day–night rhythm.

Rats were divided into six groups, and each group was consistent of six rats: (1) sham-operated (control), (2) ischemia group (3) I/R group, (4) I/R/α-tocoferol group (5) I/R erdosteine group (6). I/R/α-tocoferol and erdosteine group.

Immediately before surgical intervention, the rats were anesthetized with ketamine (50 mg kg^{-1}). The body temperature of the rats was kept constant during the operation. A laparotomy incision was performed in the midline. The left kidney was identified, 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 micro clamps except in the control group. Intraperitoneal serum was physiologically

maintained during the operation. Heparin was not given to prevent arterial occlusion. The clamp was then released following 45 min of ischemia. 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 all groups except the sham group. After removal of the clamps, the existence of pulsations was observed, and pencil doppler ultrasound (Multi Dopplex) was used to confirm the of the arteries. Additionally, 10 mg kg^{-1} 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 unilateral nephrectomies were performed. Erdosteine was given orally 10 mg kg^{-1} day^{-1} for a week before intervention.

Histological and ultrastructural examination

For light microscopy, the tissue specimens were fixed in 10% formaldehyde, processed in an autotechnicon and embedded in paraffin. Sections of 5-μm thickness were cut with a microtome and stained with hematoxylin–eosin (H–E). Stained specimens were investigated by a Nicon Eclipse E400 light microscope. The microscopic findings were scored using the semi-quantitative scores developed by Paller et al.[18] 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.

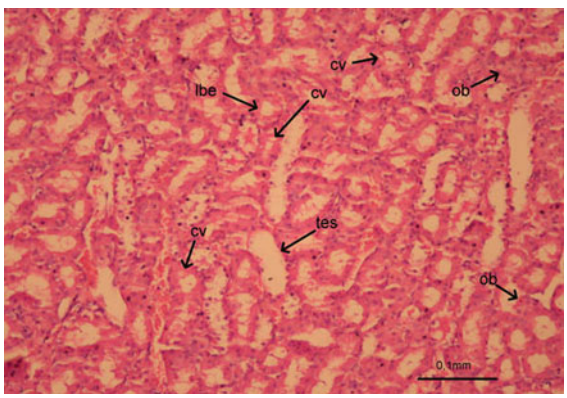

Fig. 1 Tubular epithelial smoothness (*tes*), loosing of brush-like edge (*lbe*), cytoplasmic vacuolization (*cv*) and obstruction of tubular lumen (*ob*) were seen

Then for each specimen, the same microscopic area was photographed by using a Nikon Coolpix 5000 photograph attachment. The photograph of Nikon micrometer microscope slide was also taken during the procedure. All photographs were then transferred into PC environment and analyzed by using Clemex Vision Lite 3.5 Image Analysis Program. The length was calibrated by comparing the photograph of specimen with the photograph of Nikon micrometer microscope slide. Tubular epithelial cells length and tubular epithelial lumens were measured with using the same Image Analysis Program (Fig. 1).

At least ten cortical tubules for each photograph were evaluated, and the mean values were calculated automatically with Clemex Image Analysis Program. 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% glutaraldehyde in 0.1 M PBS (pH 7.4) at 4°C for one day. They were postfixed 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 washed twice with cold saline solution, placed into plastic tubes, labeled and stored at −70°C until biochemical analyses. Renal tissue malondialdehyde (MDA) level, xanthine oxidase (XO) and superoxide dysmutase (SOD) activities were also performed in all groups.

Preparation of kidney tissue for biochemical analyses

The kidney tissues were weighed and homogenized in a four volumes of ice-cold Tris–HCl buffer (50 mmol l^{-1}, pH 7.4) using homogenizer (Ultra Turrax IKA T18 Basic) after cutting of the kidneys into small pieces with a scissors (for 3 min at 16,000 rpm). MDA analyze was measured at this homogenate stage. The homogenate was then centrifuged at 5,000 × *g* for 60 min to remove debris, and the activities of XO were measured at this homogenate stage. The clear supernatant solution was

extracted with an equal volume of a chloroform/ethanol mixture (3/5, volume per volume [v/v]). After centrifugation at 5,000×*g* for 30 min, the clear upper layer (the ethanol phase) was taken and used in the SOD activity and protein assays. All preparation procedures were performed at 4°C.

Kidney tissue malondialdehyde analysis

Malondialdehyde (MDA) levels were estimated by the double heating method of Draper and Hadley [19]. The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 ml of 100 g l^{-1} trichloroacetic acid solution was added to 0.5 ml supernatant in each centrifuge tube, and the tubes were placed in a boiling water bath for 15 min. After cooling in tap water, the tubes were centrifuged at 1,000*g* for 10 min, and 2 ml of the supernatant was added to 1 ml of 6.7 g l^{-1} TBA solution in a test tube, and the tube was placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance was measured using a spectrophotometer (Shimadzu UV-1601, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex (absorbance coefficient of 1.56×105 cm^{-1} M^{-1}) and is expressed as nanomoles/gram wet tissue.

Kidney tissue xanthine oxidase activity

Xanthine oxidase (XO) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm (M2). One unit of activity was defined as 1 mmol uric acid formed per min at 37°C, pH 7.5. Results were expressed in units per gram protein for tissue.

Kidney tissue superoxide dismutase activity

Total (Cu–Zn and Mn) superoxide dismutase (SOD) activity was determined according to the method of Sun et al. [20] with a slight modification by Durak et al. [21]. The principle of the method is based briefly on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Activity was expressed as units per gram protein.

Statistical analyses of biochemical analysis

Statistical analysis of data was performed by using one-way analysis of variance (ANOVA), the homogeneity of the groups were evaluated with Levene test. Tukey's HSD test was used for intra-group comparisons. *P* value of <0.05 was accepted as statistically significant.

Results

Biochemical results

The renal MDA and XO levels were higher in both of the nontreatment groups compared to the α-tocopherol treatment group (group 4), the erdosteine treatment group (group 5) and the combination treatment group with α-tocopherol plus erdosteine (group 6). The erdosteine and α-tocopherol significantly reversed the effect of protein oxidation and lipid peroxidation induced by I/R shown by the decreased levels of MDA and XO activities. Both MDA and XO levels were found to be lower in the combination treatment group compared to single agent treatment groups, and this was significantly different (Fig. 2). SOD was lowest in the untreated I/R group (group 2). All treatment groups showed increased SOD activity, which accounts for their oxidative properties.

Light microscopy results

When the specimens were analyzed using the Paller scoring system, the highest score (6.33 ± 0.52) was obtained in the I/R-nontreatment group (group 3), as expected. The lowest Paller Score was in the sham group (group 1) (0.33 ± 0.52; $P < 0.05$). Pure ischemia group (group 2) had a lower score (5.00 ± 1.2) than the I/R group (group 3). However, the difference in the mean Paller scores from the pure ischemia group and the I/R group was not statistically significant. Scores obtained in the AT treated groups

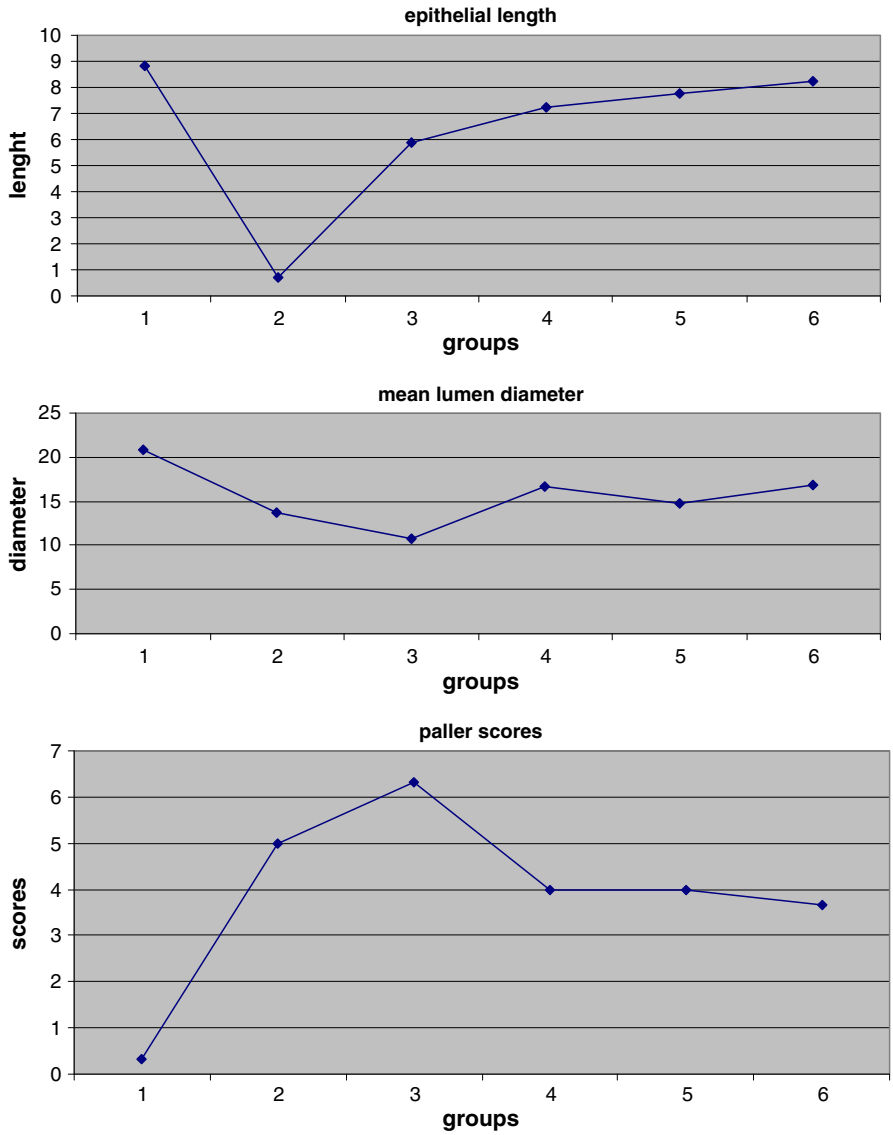

Fig. 2 The graphical presentation of epithelial lengths, mean lumen diameters and the Paller scores of the groups

and the erdosteine treated groups were similar (4 ± 0.63 and 4 ± 1.1, respectively; $P > 0.05$). The mean Paller score of the combination treatment group (group 6) was lower than all groups except the sham group (3.67 ± 1.2), and this finding was statistically significant ($P < 0.05$).

Epithelial cell length measurements using the image analysis program revealed the shortest measurements in the I/R group (group 3) (5.87 ± 0.63 μm). The pure ischemia group (group 2) measurements showed a longer mean cell length (6.73 ± 0.62 μm) than the I/R group. The epithelial cell length was found to be longer in the combination treatment group (group 6) than the single α-tocopherol (group 4) and single erdostain (group 5) groups (8.53 ± 1.38, 7.30 ± 1.12, 7.74 ± 0.90 μm, respectively; $P < 0.05$).When the pure ischemia group (group 2) was compared to the treatment groups there was no statistical difference for the single agent treatment groups (groups 4 and 5; $P > 0.05$).Again when the pure ischemia, the I/R and the α-tocopherol groups had statistically compatible mean epithelial cell lengths.

The tubular luminal width again assessed by the image analysis system showed the smallest mean diameters in the I/R group (10.70 ± 2.04 μm). The mean width in the pure ischemia group (group 2;

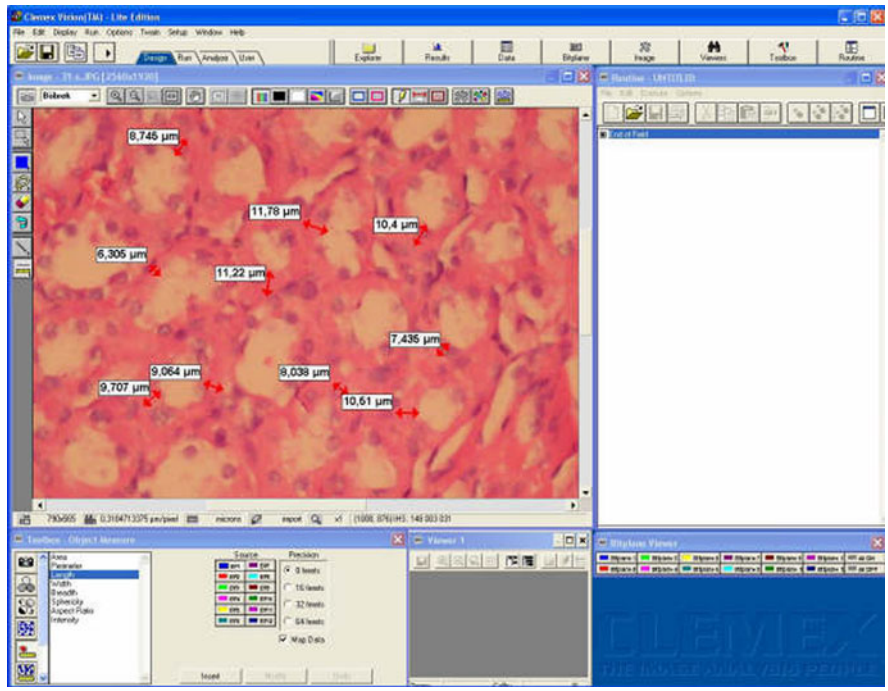

Fig. 3 Tubular epithelial length was measured with image analysis system

13.77 ± 1.82 µm) was less than all the treatment groups (groups 4, 5 and 6; 16.59 ± 2.60, 14.80 ± 1.43, 16.92 ± 2.31 µm, respectively). The sham group (group 1) had statistically significant wider luminal width than the rest groups ($P < 0.05$). The mean width measurements were similar in between all groups except the sham group (2, 3, 4, 5 and 6) (Figs. 1, 3, 4)

Electron microscopy results

The ultra-structural electron microscopy evaluation of the cells, IR group showed the most striking pathological findings such as the occlusion of the proximal convoluted tubules lumens by apoptotic epithelial cells and electron dense proteinous material. Epithelial cells of the proximal tubules were flattened, and their microvilli were diminished in some areas. Mitochondrial degeneration, increased number of lysosomes and cytoplasmic vacuoles were significant features. The other pathologic group is the pure ischemia group. In this group, lumens of the tubules were also obstructed with electron dense material but apoptosis was not evident as IR group. Some epithelial cells had rare microvilli, and there were no obvious mitochondrial degeneration or vacuoles in the cytoplasms. Increased in number of lysosomes were observed. In the AT treated group (group 4), lumens of proximal convoluted tubules were occluded and microvilli on the apical parts of the cells were lost but cellular organization, height of the epithelial cells, baso-lateral folding and mitochondrial organizations were healthy. Necrosis and apoptosis, increased number of vacuolization and lysosomes were not observed. Results of the erdosteine treated group (group 5) were similar to the AT group but sloughed apoptotic cells in the lumen were also noted in this group. In the combination treatment group (group 6), lumens of proximal convoluted tubules were also occluded by apoptotic and necrotic epithelial cells. Number of big vacuoles and lysosomes were increased, but microvilli of some the cells were preserved.

Discussion

The IR injury is an important pathological process in urological procedures such as partial nephrectomy and renal transplantation, as it is considered as the major determinant of early graft dysfunction. Ischemia is caused by loss of blood supply to the organ and results in rapid damage of the tissues with high metabolic activity, such as the renal cells [15].

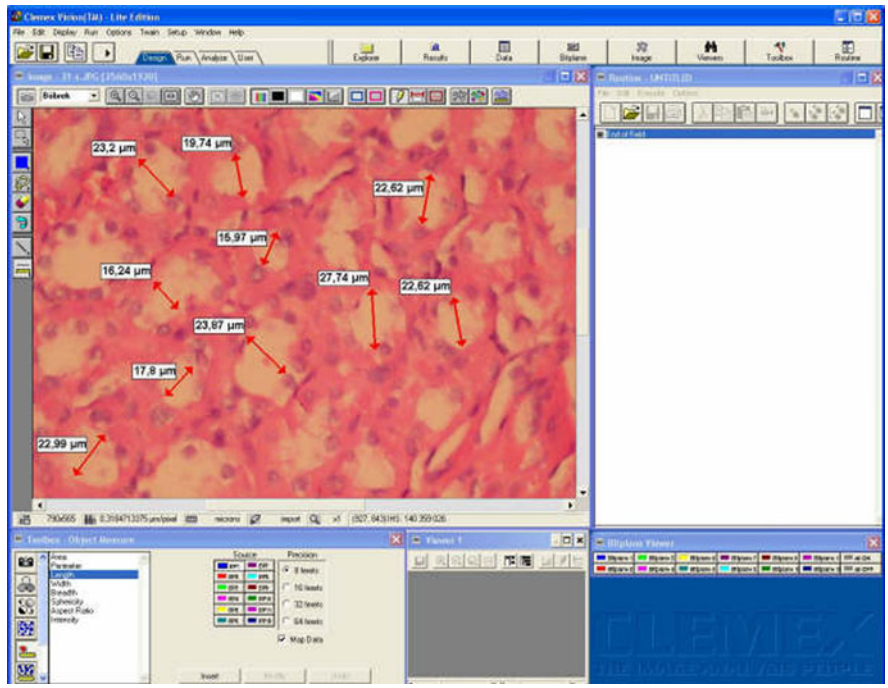

Fig. 4 Tubular lumen wideness was measured with image analysis system

In reperfusion after a transient ischemia, significant reactive oxygen species (ROS) production occurs because of hypoxanthine oxidation (XO). The ROS produced by XO can induce cell injury through lipid peroxidation of the mitochondrial, the lysosomal and the outer cell membranes, and thus cell functions [13]. Our results were consistent with this information, and IR group had the highest XO activity compared to the sham and pure ischemia groups. Blocking the detrimental ROS activities will theoretically improve outcomes in renal transplants and renal surgeries with transient iatrogenic ischemia. Malondialdehyde is a product of lipid peroxidation that polymerizes and connects to the proteins and causes deterioration of the proteins. MDA is another by-product that we can monitor for oxidative processes.

The aim of our study was to evaluate the combined antioxidant effects of erdosteine and α-tocopherol, whose effects have previously been proven in the I/R injury [13, 17]. Combination therapy with two agents that interfere at different levels of oxidative processes would provide a synergistic protective measure. We have evaluated biochemical, light microscopic and electron microscopic parameters.

Our results showed that both single agents provided protection as shown by the statistically significant differences of MDA levels in the I/R group versus both of the single agent groups. Combination group showed improved MDA levels against single agent treatments, and this was statistically significant.

Vitamin E (α-tocopherol) reacts with hydroxyl radicals and hydrogen peroxide and thus prevents lipid peroxidation in the membranes against lipid peroxidation [3]. It has been demonstrated that α-tocopherol administration reduced the ischemia–reperfusion injury in the kidney in rat model [13].The injection of α-tocopherol before renal ischemia had a mild effect on the XO content in rats. The tissue XO levels were also mildly affected in our study as Kirpatovskii [22] also reported. On the other hand, Rhoden [23] showed significant decrease in tissue MDA levels in IR injury in a rat study. Dietary enrichment of rats with α-tocopherol was effective in suppressing the renal epithelial lipid peroxidation whereas dietary deficiency of vitamin E lead to greater structural and functional renal impairment because of increased lipid peroxidation following renal ischemia [24, 25]. So it's logical to think that the action of the α-tocopherol is not rapid on set. This may explain why we did not demonstrate significant differences in XO levels in the IR group and the α-tocopherol group.

Erdosteine itself, does not posses a free thiol and thus cannot act as ROS scavenger, but gains antioxidant activity after hepatic metabolization [26, 27]. Erdosteine inhibits the XO activity and by this way might decrease the I/R injury. Beneficial effect of erdosteine as an antioxidant against the I/R toxicity in rats has been shown [28, 29]. The statistically significant reduced XO and MDA levels have been noted in our study groups with erdosteine compared to the I/R group. The synergistic effect of α-tocopherol and erdosteine is more evident as shown by the MDA levels since erdosteine effect may be more rapid onset.

Superoxide dysmutase is an endogenic enzymatic antioxidant against the oxidative stress. The reperfusion decreases the plasma SOD levels compared to pure ischemia [30]. This finding was consistent with our results, and SOD level was lower in the I/R group compared to that of the pure ischemia. On the other hand, the increase in SOD with the use of antioxidant therapies shows that damage can be prevented in reperfused tissues. Our study revealed that the use of α-tocopherol, erdosteine or combinations effectively prevent tissue damage in rat kidneys with the increased SOD levels. Combination therapy again showed a higher SOD activity compared to single treatment modalities especially when compared to the α-tocopherol group.

Light microscopic findings did not reveal major differences between the pure ischemia and I/R groups. However, they both were higher than any of the treatment groups. Single agents again proved to have similar structural effects in terms of light microscopy Paller scores; however, the combination group had a low Paller score with statistical significance; thus signaling the beneficial effect of combination therapy.

Ultra structurally, all groups except the sham specimens showed occlusion of the convoluted tubules. Flattened proximal tubules and loss of their microvilli, mitochondrial degeneration, increased number of lysosomes and cytoplasmic vacuoles are other pathological findings associated with ischemia and reperfusion injury. All of these findings were evident in the I/R group but except the occlusion of the tubules ultrastructure was preserved in all of the antioxidant treatment groups. In the combination group, microvilli were more preserved than the other groups.

Our results showed that the antioxidant pretreatment with α-tocopherol and erdosteine combination reduced lipid peroxidation of renal cellular membranes in a model of normothermic renal ischemia–reperfusion in rats. Combination of erdosteine and α-tocopherol have a synergistic effect of protection against oxidative processes. Long-term use of α-tocopherol seems to have a greater effect on the prevention of IR injury. However, further investigations are needed for the clinical applications of our findings.

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