The Effect of Alpha Lipoic Acid on Pathogenesis of Experimental Nephrolithiasis and Epithelial Mesenchymal Transition

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The Effect of Alpha Lipoic Acid on Pathogenesis of Experimental Nephrolithiasis and Epithelial Mesenchymal Transition

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ABSTRACT: In the present study, it was aimed to examine the effects of early calcium oxalate (CaOx) crystal formation and deposition on the kidneys and the effects of alpha-lipoic acid (ALA) in the prevention and early treatment of CaOx deposition in rats. Sixty rats were divided into six groups (n=10 per group). Ethylene glycol (EG) and ammonium chloride (AC) (0.75% EG + 0.75% AC) were added to the drinking water of different groups for 7 or 14 days to induce nephrolithiasis. The effects of the CaOx and ALA (100 mg/kg per day orally) on kidney were investigated via histopathological, immunohistochemical, and biochemical methods. EG+AC application for both 7 and 14 days caused crystal accumulation in the tubule lumens, cystically dilated tubules, and hydropic degeneration in the tubular epithelium. However, inflammatory cell infiltration was observed merely in 14 days. When EG+AC administration was applied for 14 days only, it caused expression of ED1, alpha smooth muscle actin (α-SMA), and vimentin in the tubulointerstitial areas. However, α-SMA and vimentin expression was not observed in tubular epithelial cells. Transforming growth factor beta-1 (TGF-β1) expression was also detected in the tubules and intertubular cells at 14 days. It was determined that ALA administration with EG+AC reduced the crystal accumulation in the tubule lumens (p<0.001), the degeneration of the tubular epithelium (p<0.001), and the expression of TGF-β1. In addition, it was detected that ALA caused an increase in glutathione peroxidase (GPx) (p<0.001) and Catalase (CAT) (p>0.05) activities, which decreased with EG+AC application. This study suggests that ALA may be an effective strategy for reducing acute kidney injury caused by CaOx.

Keywords: Alpha lipoic acid; Calcium oxalate; Epithelial Mesenchymal Transition; Nephrolithiasis
INTRODUCTION

Nephrolithiasis is a common urinary system disease and its occurrence is associated with oxidative stress, inflammation, apoptosis, and epithelial mesenchymal transition (Ding et al., 2021).

Ethylene glycol (EG) is a substance that causes oxidative stress and then stone formation in the kidneys, which is used in experimental kidney calcium oxalate (CaOx) crystal formation and deposition studies in rats (Fan et al., 1999; Bahadoran et al., 2016). Ethylene glycol has been widely used in combination with ammonium chloride (AC) to achieve uniformly high rate of kidney crystal deposition (Fan et al., 1999).

Calcium oxalate crystals can lead to acute and chronic tubular injury, tubulointerstitial inflammation, interstitial fibrosis and progressive kidney failure (Khan, 2004; Kanlaya et al., 2013; Liu et al., 2013; Hu et al., 2015; Convento et al., 2017).

Epithelial mesenchymal transition (EMT) is a cellular process which the epithelial cells acquire mesenchymal characteristics (Kanlaya et al., 2013; Liu et al., 2013; Convento et al., 2017). EMT plays a crucial role in the initiation and development of renal interstitial fibrosis which occurs in response to several stress conditions such as mechanical stretch, oxidative stress, calcium oxalate crystallization, and subsequently aggravates renal fibrosis in nephrolithiasis (Kanlaya et al., 2013; Liu et al., 2013; Convento et al., 2017; Ding et al., 2021).

The first objective of the nephrolithiasis treatment is to decelerate the development of renal fibrosis (Hu et al., 2015). Alpha-lipoic acid (ALA) is an antioxidant used in the prevention and treatment of diseases that may occur due to oxidative stress (Kowluru and Odenbach, 2004; Ergene, 2018; Wang et al., 2020). In various studies, it is stated that ALA reduces oxidative stress in kidneys, inflammation, and crystal accumulation in the tubules (Kowluru and Odenbach, 2004; Yu et al., 2012; Ergene, 2018; Wang et al., 2020; Canvdar et al., 2021).

The purpose of this study was to examine the effects of early CaOx crystal formation and accumulation on the kidneys and to investigate the effect of ALA on crystal deposition and on EMT in rats.

MATERIALS AND METHODS

Animals and experimental protocol

In the study, sixty male two months old Wistar Albino rats weighed 200-250 g were used. This study was conducted with Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee’s permission numbered 2019/03-2. Experimental applications were carried out in accordance with the conditions of care and use of laboratory animals (12 hours light: 12 hours dark and 24±3°C). During the experiment, standard commercial rat food (pellet feed) and tap water were provided ad libitum to the rats.

The rats were randomly divided into equal six groups (n = 10 per group). The study design was presented in Table 1. In order to induce nephrolithiasis, some groups were given 0.75% EG and 0.75% AC added tap water (Bahadoran et al., 2016; Fan et al., 1999; Khan, 1991). In addition, some groups were given ALA (100 mg/kg per day orally) (Gunes et al., 2016; Topsakal et al., 2019).

At the end of the study, anesthesia was produced by means of a cocktail prepared by using xylazine (10 mg/kg) and ketamine (50 mg/kg). Then, the rats were sacrificed, kidneys were removed and used for histopathological, immunohistochemical and biochemical examinations.

Histopathological examination

Kidneys were fixed in 10% neutral buffered formalin. After passing through alcohol and xylol series according to routine tissue follow-up methods, the tissues were embedded in paraffin and 4 μm transversal sections (within the cortex, medulla, and pelvis) were stained with Hematoxylin Eozin (HE) for morpholog-

### Table 1: Groups and applications

<table>
<thead>
<tr>
<th>Groups</th>
<th>Drinking water</th>
<th>ALA</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Water</td>
<td></td>
<td>14 day</td>
</tr>
<tr>
<td>Group 2</td>
<td>Water (% 0.75 EG + % 0.75AC)</td>
<td></td>
<td>7 day</td>
</tr>
<tr>
<td>Group 3</td>
<td>Water (% 0.75 EG + % 0.75AC)</td>
<td>ALA 100 mg/kg/bw/daily</td>
<td>14 day</td>
</tr>
<tr>
<td>Group 4</td>
<td>Water (% 0.75 EG + % 0.75AC)</td>
<td>ALA 100 mg/kg/bw/daily</td>
<td>7 day</td>
</tr>
<tr>
<td>Group 5</td>
<td>Water (% 0.75 EG + % 0.75AC)</td>
<td></td>
<td>14 day</td>
</tr>
<tr>
<td>Group 6</td>
<td>Water</td>
<td>ALA 100 mg/kg/bw/daily</td>
<td>14 day</td>
</tr>
</tbody>
</table>
ical changes and Pizzolato’s method for calcium oxalate crystal deposition (Pizzolato, 1964; Luna, 1968). Tubular crystal deposition, tubular dilatation, hydropic degeneration of tubular epithelial cells, and inflammatory cell infiltration were assessed and graded for each animal. Microphotographs were taken after examining them under a light microscope.

Histopathological changes were graded as no change (-), mild (+), moderate (++), and severe (+++) (Topsakal et al., 2019; Kutlu and Alcigir, 2019). Crystal accumulation in tubules (6 areas in total, 2 areas in the cortex, medulla, and pelvis), was graded as “-” no crystal deposits; “+” 5 or less crystal deposition; “++” 6-10 crystal deposition; “+++” more than 10 crystal deposits (using the mean value), after counting at the same magnification (x100) (Lee et al., 1992; Fan et al. 1999).

**Immunohistochemical examination**

In the immunohistochemical examination, SensiTek HRP (SensiTek HRP, ScyTek Laboratories, Logan, UT) kit was used according to the manufacturer’s instructions. Serial sections, 4 μm, were collected on poly-l-lysine coated slides and incubated overnight at 56°C. Tissue sections were deparaffinized in xylene and hydrated through a graded series of ethanol and blocked for endogenous peroxidase activity with 3% H2O2 in methanol for 30 minutes. An endogenous avidin/biotin blocking kit (ab64220) was used for endogenous biotin blocking. Proteinase K (Abcam, ab64220) was used as antigen retrieval. The sections were then incubated in a blocking serum (ScyTek laboratories) for 10 min in order to block non-specific binding. Subsequently, the sections were incubated with the following primary antibodies: ED1 (Abcam, ab64220 / 1:100 dilution, 45 min / 45˚), transforming growth factor beta-1 (TGF-β1) (Abcam, ab92486 / 1:100 dilution, 120 min / 45˚), alpha smooth muscle actin (α-SMA) (Abcam, ab5694 / 1:100 dilution, 45 min / 45˚), and vimentin (Thermoscientific, MA3-745 / 1:20 dilution, 60 min / 45˚). After washing with phosphate buffered saline (PBS) at room temperature, sequential incubations with biotinylated polyclonal antibodies (Scy Tek Laboratories) and peroxidase-labeled streptavidin (ScyTek Laboratories) were performed. For the control sections, PBS was used instead of primary antibody as the negative control. Positive reactions were visualized with 3,3’-diaminobenzidine tetrahydrochloride (DAB, ScyTek Laboratories, Logan, UT). The sections were counterstained lightly with hematoxylin.

Positive cells in renal epithelia or interstitial cells were assessed semiquantitatively as follows; “3+” if more than 50% of cells are positive; “2+” if 10-50% of cells are positive; “1+” if less than 10% of cells are positive; if there is no positivity “-“(Yamate et al., 2005).

**Biochemical analysis**

*Tissue Homogenization:* In order to homogenize the kidney tissue samples used in biochemical analyzes, approximately 1 g tissue sample was taken and homogenized in 10 ml PBS (pH: 7.4) using an ultrasonic homogenizer (Bandelin Electronic UW 2070, Germany). Then the homogenates were centrifuged at 5000 rpm, 4°C, for 30 minutes to obtain tissue homogenate supernatants (Kazak et al. 2020). The supernatant was used for spectrophotometrical (UV 2100 UV-VIS Recording Spectrophotometer Shimadzu, Japan) analysis of glutathione peroxidase (GPx) and catalase (CAT) activities.

*Determination of Glutathione Peroxidase Activities:* Glutathione peroxidase is an enzyme that catalyzes the conversion of hydrogen peroxide to water by using reduced glutathione. The method reported by Beutler (1975) was used to determine GPx activities in tissue supernatants. Glutathione peroxidase activity was presented as a U/g protein.

*Determination of Catalase Activity:* Catalase (CAT) decomposes hydrogen peroxide into water and oxygen with its catalytic activity. The determination of CAT enzyme activities in tissue supernatants was carried out according to the method described by Aebi (1984). CAT activity of tissues was presented as k/g protein.

**Statistical analysis**

In the statistical analysis of the findings, the IBM SPSS Statistics 22.0 computer program was used. One-way Anova analysis was used to determine whether there was a statistical difference between the groups for the determined characteristics. The Duncan test was used to compare multiple groups. Statistical significance was accepted as p<0.05.

**RESULTS**

Microscopically, histopathological findings and scoring observed in the groups’ were presented in Table 2. Kidneys in group 1 and group 6 were observed to have normal histological structures (Fig. 1a). In other groups, lesions with varying extents such as
crystal deposition within the tubules, cystically dilated tubules, hydropic degeneration of tubular epithelial cells, and inflammatory cell infiltration were observed.

In group 2, in rats that were given EG+AC for 7 days, mild crystal deposition in tubular lumens, dilated tubules, and hydropic degeneration of tubular epithelium were noted (Fig. 1b). In group 3, the group that was given EG+AC for 14 days, similar lesions were observed in moderate severity (Fig. 1c, Fig. 3a, Fig. 3b). However, inflammatory cells such as monocytes, macrophages and relatively few neutrophils in the interstitial region was detected in this group, which was not observed in group 2 (Fig. 1d).

In group 4, the group that was given EG+AC+ALA for 7 days, crystal deposition and hydropic degeneration in the tubules were significantly decreased compared to group 2 (P<0.001) (Fig. 2a). In group 5, ALA had a similar effect in rats that were given EG+AC+ALA for 14 days; compared to group 3 it

Table 2: Histopathological findings of experimental groups

<table>
<thead>
<tr>
<th>Groups/Parameters</th>
<th>Crystal deposition within the tubules</th>
<th>Inflammatory cell infiltration</th>
<th>Cystically dilated tubules</th>
<th>Hydropic degeneration of tubular epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>0.00±0.00b</td>
<td>0.00±0.00b</td>
<td>0.00±0.00b</td>
<td>0.00±0.00b</td>
</tr>
<tr>
<td>Group 2</td>
<td>1.00±0.22b</td>
<td>0.00±0.00b</td>
<td>0.71±0.18b</td>
<td>1.00±0.22b</td>
</tr>
<tr>
<td>Group 3</td>
<td>2.14±0.34a</td>
<td>1.43±0.30b</td>
<td>2.00±0.31a</td>
<td>2.14±0.34a</td>
</tr>
<tr>
<td>Group 4</td>
<td>0.57±0.30ac</td>
<td>0.43±0.20ab</td>
<td>0.71±0.29a</td>
<td>0.00±0.00c</td>
</tr>
<tr>
<td>Group 5</td>
<td>1.14±0.26a</td>
<td>1.14±0.40a</td>
<td>1.57±0.48a</td>
<td>1.00±0.22b</td>
</tr>
<tr>
<td>Group 6</td>
<td>0.00±0.00c</td>
<td>0.00±0.00b</td>
<td>0.00±0.00b</td>
<td>0.00±0.00c</td>
</tr>
<tr>
<td>p</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

* Different letters on the same column are statistically significant (p<0.001).

Figure 1: Histopathological changes in the kidneys with EG+AC. a) Kidney tissue has normal histological structure, Group 1; 100x, HE. b) Cystically dilated tubules (stars) and CaOx crystals (arrows), Group 2, 400x, HE. c) Moderate hydropic degeneration of tubular epithelium, cystically dilated tubules (stars) and CaOx crystals (arrows), Group 3, 400x, HE. d) Inflammatory cell infiltration in the intertubular region, Grup 3, 400x, HE.
significantly reduced crystal deposition (Fig. 3c) and hydropic degeneration (P<0.001); however, although it reduced inflammatory cell infiltration, it was found that it did not have a statistically significant effect on inflammatory cell infiltration (Fig. 2b).

Immunohistochemical findings are presented in table 3. Macrophage-specific antibody ED1 immunoexpression was observed in group 3 and group 5 on 14 days only (Fig. 4a, Fig. 4b). ED1 positivity was not significant in other groups. TGF-β1 expression was observed at moderate intensity in the tubular epithelium and interstitial cells in group 3, which was applied EG+AC for 14 days (Fig. 5a). In group 5, on the other hand, TGF-β1 expression in the epithelial and interstitial regions was milder than in group 3 (Fig. 5b). Almost no TGF-β1 positivity was detected in other groups.

In the staining performed for the detection of myofibroblasts, α-SMA immunoexpression was found in the interstitial cells of group 3 and group 5, which

**Table 3: Immunohistochemical findings of experimental groups**

<table>
<thead>
<tr>
<th>Groups/Antibodies</th>
<th>Interstitial ED1</th>
<th>Tubular TGF-β1</th>
<th>Intertstitial TGF-β1</th>
<th>Tubular α-SMA</th>
<th>Intertstitial α-SMA</th>
<th>Tubular Vimentin</th>
<th>Intertstitial Vimentin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 3</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
<td>-</td>
<td>1+</td>
<td>-</td>
<td>1+</td>
</tr>
<tr>
<td>Group 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 5</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>-</td>
<td>1+</td>
<td>-</td>
<td>1+</td>
</tr>
<tr>
<td>Group 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 2:** The effects of ALA on histopathological changes in the kidneys induced by CaOx crystal accumulation a) Kidney tissue has normal histological structure, Group 4, 100x, HE b) Inflammatory cell infiltration in the intertubular region (stars), Group 5, 100x, HE.

**Figure 3:** Calcium oxalate crystal deposition in kidney a) Moderate crystal deposition, Group 3, 100x, Pizzolato’s method. b) Crystal deposition, Group 3, 400x, Pizzolato’s method. c) Mild crystal deposition, Group 5, 100x, Pizzolato’s method.
underwent EG+AC application for 14 days. However, α-SMA immunoeexpression was not observed in the tubular epithelium (Fig. 6a). In other groups, α-SMA positivity was found only in the vessel walls. Similarly, vimentin expression was detected only mildly in the intertubular region in group 3 and group 5. No vimentin immunoeexpression was detected in the tubular epithelium (Fig 6b).

Figure 4: Immunohistochemical findings of ED1 in kidneys. DAP, ABC-P. a) ED1 positivity in the interstitial cells (arrows), Group 3, 100x, b) ED1 positivity in the interstitial cells (arrows), Group 5, 400x.

Figure 5: Immunohistochemical findings of TGF-β1 in kidneys. DAP, ABC-P. a) Moderate TGF-β1 positivity in tubular epithelium and interstitial cells, Group 3, b) Mild TGF-β1 positivity in tubular epithelium and interstitial cells, Group 5, 400x.

Figure 6: Immunohistochemical findings of α-SMA and vimentin in kidneys. DAP, ABC-P. a) α-SMA positivity in the vessel wall and in the interstitial cells, Group 3, 400x. b) Vimentin positivity in the interstitial cells, Group 5, 100x.
Renal tissue GPx and CAT activities of the groups were presented Table 4. Group 2 and group 3 GPx and CAT activities were found to be lower compared to group 1 and group 6 (p<0.001). It is determined that group 4 and group 5 GPx activities were higher compared to group 2 and group 3 (p<0.001). There was no difference in group 1 and group 6 GPx and CAT activities.

DISCUSSION

Nephrolithiasis is a common urinary system disease, characterized by secondary stone formation in both humans and animals (Kutlu and Alcigir, 2019; Ding et al., 2021). Several pathophysiological mechanisms are involved in nephrolithiasis including oxidative stress, inflammation, apoptosis, and EMT (Ding et al., 2021).

The renal CaOx model created in rats with ethylene glycol is frequently used to mimic CaOx accumulation in humans (Wang et al., 2020). In previous studies, an increase in urinary oxalate excretion occurs within 2 days in rats supplemented with 0.75-1% EG; hyperoxaluria in 3 days; CaOx crystalluria in 2 weeks; CaOx nephrolithiasis in 4-6 weeks (Khan, 1997). In this study, EG+AC induced crystal formation in the tubules in both 7 and 14 days.

Calcium oxalate crystals are the most common crystals in the kidneys and cause tubulointerstitial injury and inflammation (Khan, 2004). Wang et al. (2020) stated that ALA reduces crystal accumulation in the kidneys. In the present study, it was discovered that the application of ALA in the CaOx model significantly reduced the crystal formation and degenerative changes in the tubular epithelium in both 7 days and 14 days. In previous studies, it was stated that the adhesion and accumulation of CaOx crystals are easier when the cells are damaged. Therefore, it is assumed that there may be a link between ALA’s reduction of crystal deposition and damage to the tubular epithelium (Wiessner et al., 2001; Thamiselvan et al., 2003; Wang et al., 2020).

In previous studies, it was stated that macrophages accumulate around the tubules with CaOx stones in rats and they play a role in the phagocytosis of crystals (Kanlaya et al., 2013; De-Water et al., 2000). Most of these macrophages were found to be ED1 (monocyte and macrophage-specific antibody) positive (Jercan et al., 2012; De-Water et al., 2000). In histopathological and immunohistochemical examination conducted within our study, it was found that EG+AC application induced macrophage infiltration only in 14 days. Macrophages are also found in healthy kidneys and increase in the state of disease, causing kidney injury, inflammation, and fibrosis (Cao et al., 2015; Ding et al., 2021). In other studies, it was stated that because ALA significantly reduces macrophage infiltration in the tubulointerstitial region by suppressing nuclear factor-kappa B activity, it has an anti-inflammatory effect (Kang et al., 2009; Yu et al., 2012; Wang et al., 2020; Cavdar et al., 2021). However, in this study, it was observed that ALA did not cause a significant statistical change in inflammatory cell infiltration. It was concluded that ALA did not show an anti-inflammatory effect in a short period of 14 days.

The molecular pathogenesis of nephrolithiasis is very complex. The role of transforming growth factor beta-1 (TGF-β1), which is a profibrotic cytokine, in the initiation and development of fibrosis has been investigated many times, and in these studies, it was stated that TGF-β1 is the most important mediator in renal EMT (Khan, 2004; Hu et al., 2015, Convento et al., 2017). Macrophages are important in cytokine production. It has been determined that there is a strong correlation between the number of macrophages and TGF-β1 and that interstitial macrophages produce TGF-β1 (Muchaneta-Kubara and El Nahas,

Table 4: Renal GPx and CAT activities (Mean±SE) in groups

<table>
<thead>
<tr>
<th>Groups/Parameters</th>
<th>GPx U/g protein</th>
<th>CAT k/g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>488.68±23.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.92±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 2</td>
<td>328.04±7.90&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.51±0.03&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 3</td>
<td>276.21±10.74&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.41±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 4</td>
<td>432.67±14.09&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.63±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 5</td>
<td>398.44±8.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.55±0.06&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6</td>
<td>460.81±23.65&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.81±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>-<sup>e</sup> Different letters on the same column are statistically significant (p<0.001).

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In the present study, EG+AC application for 7 days neither initiate macrophage infiltration nor cause TGF-β1 expression. However, similar to previous studies, in animals treated with EG+AC for 14 days, TGF-β1 positivity was observed in the kidney tubules and intertubular region with macrophage infiltration. Although ALA did not affect macrophage infiltration in these groups, it was found that it reduced TGF-β1 positivity. It was stated that TGF-β1 did not show any immunoreactivity in healthy rat kidneys. Likewise in our study, TGF-β1 positivity was not observed in groups with no injury or had mild crystals and inflammation (Muchaneta-Kubara and El Nahas, 1997).

Alpha smooth muscle actin (α-SMA), a marker for myofibroblasts, is positive in the media layer of the renal arteries and arterioles in normal rat kidneys (Muchaneta-Kubara and El Nahas, 1997; Nakatsuji et al., 1998). It was reported that α-SMA expression in the interstitium was observed as of the 7th day in rats that underwent nephrectomy (5/6) (Muchaneta-Kubara and El Nahas, 1997). It is stated that there is a significant correlation between α-SMA positive tubular epithelial cells, interstitial α-SMA positive myofibroblasts, and tubulointerstitial fibrosis (Ng et al., 1998; Jercan et al., 2012). Nakatsuji et al. (1998) found that α-SMA positivity was observed in tubulointerstitial fibrotic regions, especially around the basal membrane of tubules. In the present study, there was positivity in vessels in all groups. It was detected in the tubulointerstitial areas only in the groups that underwent EG+AC for 14 days. However, α-SMA expression was not found in tubular epithelial cells in any group.

It was reported that vimentin expression was observed in cystic dilated tubules or collapsed in nephrosis induced by daunomycin in rats. In normal tubular epithelium, vimentin is negative (Gröne et al., 1987). Hu et al. (2015) found that glyoxalate increased the positivity of α-SMA and vimentin, which are interstitial markers in the kidneys of rats. Convento et al. (2017) reported that the mesenchymal marker vimentin became positive in proximal tubule epithelial cells in 48 and 72 hours in vitro. However, in our study, the tubular epithelium was vimentin negative in all groups. Mild positivity in the interstitial region was noted in group 3 and group 5, where EG+AC was applied for only 14 days.

Convento et al. (2017) in their in vitro study, determined that proximal tubular epithelium stimulated with CaOx produced TGF-β1, acquired mesenchymal features such as invasion and migration, and showed expression of α-SMA and vimentin. In this in vivo study, it is found that when immunohistochemical findings are evaluated together, 14 days of EG+AC application initiated TGF-β1 production in tubular epithelium however, the tubular epithelial cells did not acquire mesenchymal features (α-SMA, vimentin) such as invasion and migration during this period.

Panigrahi et al. (2017) reported a decrease in CAT activities on the 14th and 28th days during the application of 1% AC in the first 14 days and 0.75% EG with 1% AC in the last 14 days. Shirfule et al. (2013) reported a decrease in renal GPx and CAT activities in rats given 0.75% EG containing 1% ammonium chloride for 5 days, followed by only 0.75% EG for 16 days. Similar to previous studies, in the present study, it was determined that GPx and CAT activities decreased in the groups that were given drinking water with 0.75% EG and 0.75% AC for 7 days and 14 days (Shirfule et al., 2013; Panigrahi et al., 2017). Thus, it is considered that EG and AC added to the drinking water of rats may adversely affect renal antioxidant enzyme activities, even if applied in different amounts and at different times. In the present study, the fact that the GPx activities of group 4 and group 5 were significantly higher compared to group 2 and group 3, as well as the fact that the CAT activities were also high, although not statistically significant, shows that ALA may have an antioxidative effect. In addition, in this study, it was determined that there was no difference when group 1 and group 6 antioxidant enzyme activities were compared. Thus, it is thought that ALA does not have any negative effect on the renal enzymatic antioxidant system.

CONCLUSIONS

Consequently, in the experimental EG+AC model in rats, it was observed that CaOx crystals and degeneration were formed in 7 days, and the inflammatory cellular reaction occurred in 14 days. In this model, it was observed that EG+AC application for 14 days initiated TGF-β1 production in the tubules and intertubular region, however did not cause α-SMA and vimentin expression in tubular epithelium. Therefore, in this period, it was determined that the tubular epithelium did not acquire mesenchymal features such as invasion and migration, and although it triggered EMT, it did not cause EMT yet. On the other hand, it was observed that ALA reduced the amount of crystals formed and the degeneration formed in the tubular epithelium also attenuated TGF-β1 expression in
the epithelial and tubulointerstitial areas, which has an important initiating role in EMT. In addition, it was determined that EG and AC may cause oxidative stress in renal tissue by reducing GPx and CAT activities. Nevertheless, it is also considered that ALA can prevent oxidative stress by increasing decreased antioxidant enzyme activities, especially GPx activity.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

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