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LABORATORY STUDY

## Amikacin induced renal damage and the role of the antioxidants on neonatal rats

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### ABSTRACT

Amikacin (AK) is frequently used on the treatment of Gram-negative infections on neonates, but its usage is restricted because of nephrotoxicity. In this study, on neonatal rats, we aimed to investigate the effects of erythropoietin and vitamin E on AK induced nephrotoxicity. A total of 35 newborn Wistar Albino rats were divided into four groups: (1) injected with saline (serum physiological was administered to placebo controls), (2) injected with AK (1200 mg/kg), (3) injected with AK + vitamin E (150 mg/kg), (4) injected with AK + erythropoietin (EPO) (300 IU/kg/day). In renal tissue, AK levels were significantly high in all groups except the control. Tissue malondialdehyde (MDA) and nitric oxide (NO) levels were statistically higher in AK-treated group than the control. MDA and NO levels were significantly decreased with the administration of vitamin E and EPO. Glutathione peroxidase (GPX) levels were statistically low in AK group compared with the controls. The levels of GPX, in vitamin E group, were increased significantly. However, superoxide dismutase and catalase levels were not significantly different in none of the groups. Insulin-like growth factor-1 values in AK, EPO and vitamin E groups were significantly higher than the control group. Histomorphological changes such as tubular epithelial necrosis were seen in AK treated group. Histopathological improvements observed with EPO and vitamin E administration. AK nephrotoxicity is related to oxidative stress and is supported with biochemical and histopathological findings. Vitamin E and EPO, as antioxidants, can be useful renoprotective agents for ameliorating AK induced nephrotoxicity in neonates.

### ARTICLE HISTORY

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### KEYWORDS

Amikacin; erythropoietin; neonatal rats; oxidative injury; vitamin E

### Introduction

Aminoglycoside antibiotics, especially amikacin (AK), are widely used in neonatal intensive care units for the treatment of severe, life-threatening Gram-negative bacterial infections. AK, which is frequently used on the treatment of Gram-negative infections on neonates, has high antibacterial efficacy, rapid onset of action, synergy with beta-lactam antibiotics, low resistance and low costs. Despite all its benefits, the clinical usage is restricted because of nephrotoxicity. Renal dysfunction mechanism of AK is not well known. The nephrotoxic side effects of AK have been documented in numerous species of studies.<sup>1–8</sup> Experimental studies had suggested that free oxygen radicals play a role in the pathogenesis of nephropathy and it has also been shown that toxicity might be prevented with several antioxidants.<sup>9–13</sup>

Both vitamin E and erythropoietin (EPO) frequently use in neonatal intensive care units for supplementation.

It has been shown that dietary supplementation of vitamin E suppresses oxidative stress and glomerulosclerosis in rat kidney.<sup>14–16</sup> Hematopoietic growth factor, EPO, is widely used in the treatment of anemia various of clinical conditions. Recombinant human EPO (rhEPO) has been shown to stimulate endothelial cell proliferation and angiogenesis.<sup>17,18</sup> EPO and its receptor are expressed in multiple tissues, such as in glomerular, mesangial and tubular epithelial cells in human, rat and mouse kidney.<sup>19,20</sup> The antiapoptotic effects of EPO have been shown in different models of nephrotoxicity but there is not enough evidence regarding the antioxidant effects of EPO on renal injury induced by AK.<sup>21–23</sup>

In this study, we aimed to investigate the role of oxidative tubular damage on AK induced nephrotoxicity and to evaluate the effects of antioxidants on biochemical and histopathological changes on neonatal rats.

## Materials and methods

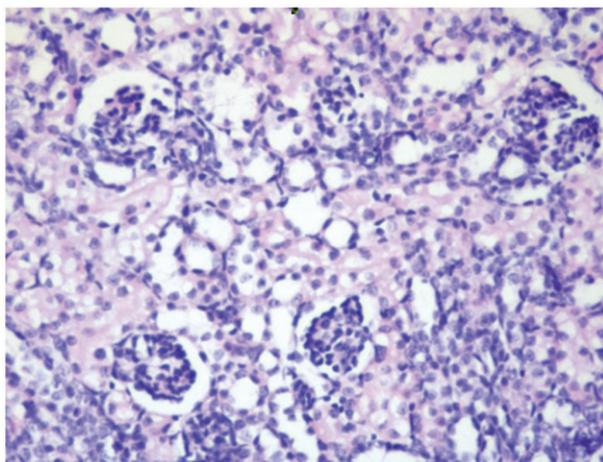
### Animal protocol

The present study was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of our institution. Thirty-five healthy newborn Wistar albino rats (age 3–7 days, birth weight 4–7 g) were used. Rats were kept with their mothers, fed with breast milk, in a temperature-controlled (22–24 °C) environment, with 55–60% humidity under a 12-h light–dark cycle for 7 days through experimentation.

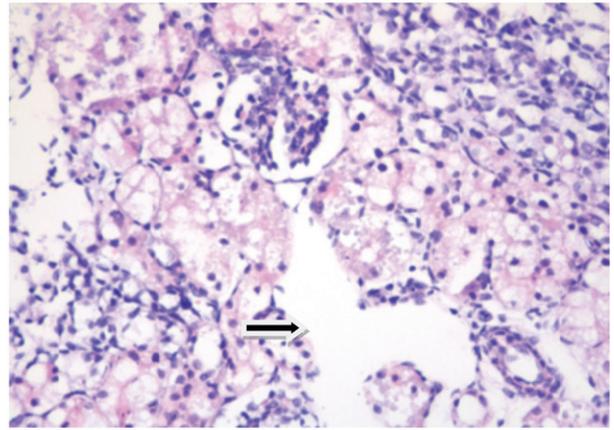
### Experimental protocol

A preliminary experiment was performed to determine the appropriate dosage to make nephrotoxicity to the newborn rats. After determining doses, rats were randomly allocated into four groups as follows: (i) Group 1, control ( $n=8$ ); (ii) Group 2, AK treated ( $n=8$ ); (iii) Group 3, AK+Vitamin E treated ( $n=9$ ) and Group 4, AK+EPO treated ( $n=10$ ). Group 1 injected with saline intraperitoneally (i.p.) for 3 days (4th, 5th, 6th days). AK (Amikozit flacon 0.5 g/2 mL; Eczacıbaşı, Istanbul, Turkey) was dissolved in distilled water and administered to Groups 2, 3 and 4 with a single dose of 1200 mg/kg, i.p., for 3 days. Vitamin E (Evigen ampul 300 IU/2 mL; Aksu-Farma, Istanbul, Turkey) was given at a dose of 150 mg/kg/day, i.p. 30 min before AK administration for 3 days.<sup>24</sup> Recombinant human EPO $\alpha$  (Eprex; 2000 UI/mL; Santa-Farma-Gurel, Istanbul, Turkey) was given at a dose of 300 UI/kg/day, i.p. 30 min before AK administration for 3 days.<sup>23</sup>

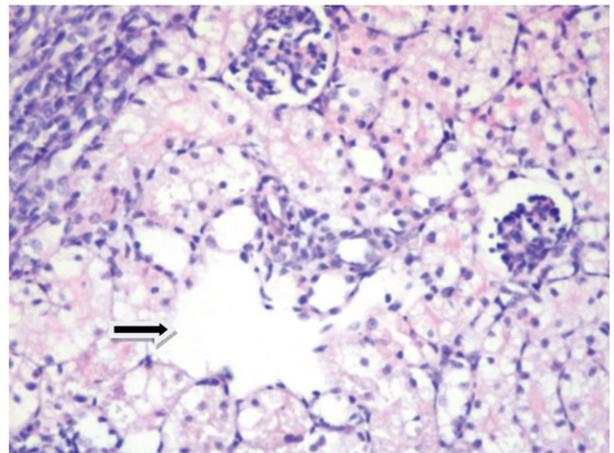
The daily dose of AK was administered 1/2 h after vitamin E and EPO treatment in the morning (i.e., vitamin E



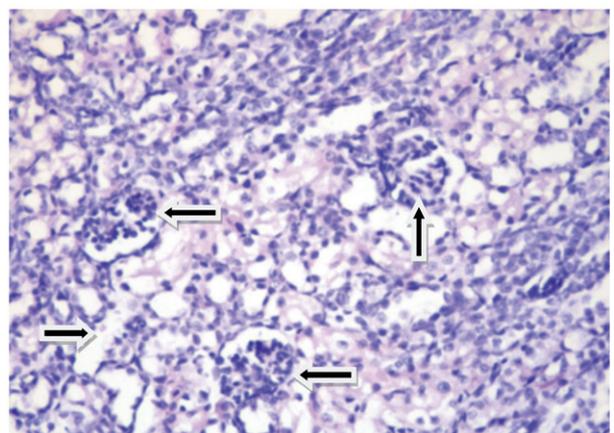
**Figure 1.** Control: Photomicrograph of the cortex of a kidney from Group 1 (control) showing normal renal histology (HE, 40 $\times$ ).



**Figure 2.** AK: Tubular necrosis and significant tubular dilatation (HE, 40 $\times$ ).



**Figure 3.** AK+vitamin E: Dilatation of tubules, vacuolization in the proximal tubules and the normal structure of glomeruli are seen. There is no inflammation in the interstitial space (HE, 40 $\times$ ).



**Figure 4.** AK+EPO: Mild dilatation of the tubules and the normal structure of glomeruli (HE, 40 $\times$ ).

and EPO was given at 08:30 h and AK was given at 09:00 h). Each injection was administered at the same time of the day. Isotonic saline solution (an equal volume to AK) was administered to Group 1 by i.p. injection. In addition, distilled water (an equal volume to vitamin E/EPO) was given i.p. to Groups 1 and 2.

### Specimen collection and methods

Rats were anesthetized with ether and killed 24 h after the last injection. Both kidneys were removed, weighed, decapsulated and divided equally into two longitudinal pieces. One-half of the right kidney was placed in formaldehyde solution for routine histopathological examination by light microscopy. The entire left kidney and the other half of the right kidney were washed with physiological saline for analysis of malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), nitric oxide (NO), glutathione peroxidase (GPX) and insulin-like growth factor (IGF-1) and determination of tissue concentrations of AK. Tissue samples were suspended in 3 mL Tris-HCl buffer, pH 7.3, that contained 0.25 mol/L sucrose and were stored at  $-80^{\circ}\text{C}$  until further analysis.

Renal tissue was homogenized in a motordriven tissue homogenizer (IKA Ultra-Turrax T25 Basic; Labortechnik, Staufen, Germany) with phosphate buffer (pH 7.4). Unbroken cells, cell debris and nuclei were sedimented by centrifugation at 5000g for 10 min at  $+4^{\circ}\text{C}$ . After homogenization, biochemical analysis was performed immediately.

Renal AK accumulation in kidney tissue supernatant was measured using commercially available kits by fluorescence polarization immunoassay by using Aoroset automatic analyzer by standard spectrophotometric methods (Max Planck-Ring, Wiesbaden, Germany).

Tissue MDA levels were determined from the homogenate by the double heating method of Draper and Hadley.<sup>25</sup> The principle of this method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. The prepared solution was cooled under tap water and its absorbance was measured using a spectrophotometer (Shimadzu UV-1601; Shimadzu, Kyoto, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA-TBA complex (absorbance coefficient =  $1.56 \times 10^5$  L/mol/cm) and is expressed as nmol/g protein in the kidney.

NO was assayed colorimetrically using the Griess reaction (Sigma Co., St. Louis, MO). The Griess reagent reacted with nitrite to form a pink to dark pink color after incubation for 10 min. The absorbance of the

samples was read at 540 nm and levels, expressed as micromolar of protein, were determined using a standard  $\text{NaNO}_2$  solution.

SOD activity was estimated in the supernatant according to the method described by Sun et al.<sup>26</sup> The measurement of SOD activity is based 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 at 15,000g for 3 min. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of reduction of NBT reduction. Activity is expressed as U/g protein.

GPX activity was determined spectrophotometrically at 340 nm by the method of Paglia and Valentine.<sup>27</sup>

CAT activity was measured using the method described by Aebi.<sup>28</sup> The principle of the assay is based on the determination of the rate constant  $k$  ( $\text{s}^{-1}$ ) of hydrogen peroxide decomposition. By measuring the change in absorbance per minute, the rate constant for the enzyme was determined. Activity is expressed as  $k$  (rate constant)/g protein.

Protein levels in the homogenate and supernatant were determined according to the method of Lowry et al.<sup>29</sup>

IGF-I concentrations were determined by a hydrochloride acid-ethanol extraction radioimmunoassay (RIA), using human IGF-I for labeling (Nichols Institute Diagnostics, San Juan Capistrano, CA) according to the method of Sjöberg et al.<sup>30</sup>

### Histopathological evaluation

Half of the right kidneys of the rats were fixed in 10% buffered formalin and then placed in fresh fixative solution and embedded in paraffin, sectioned at  $5\ \mu\text{m}$  and stained with hematoxylin-eosin. A pathologist, blinded to sample identity, examined all samples by using light microscopy. Light microscopy (Olympus BH-2; Olympus, Tokyo, Japan) was used to semiquantitatively evaluate kidney sections (Figures 1-4).

Tissues were examined for tubular epithelial alterations (dilatation, desquamation, vacuolization, necrosis, atrophy, casts), interstitial inflammatory cell infiltration, edema and glomerular changes. All histopathological parameters were graded as follows<sup>23</sup>:

- Level - (normal) = no changes.
- Level + (mild) = single cell necrosis, slight degenerative changes, few foci of dilatation, casts, inflammatory infiltration and edema.

**Table 1.** Biochemical data in the four experimental groups (mean  $\pm$  SD).

	Control	AK	AK + vitamin E	AK + Erythropoietin
MDA (nmol/mg protein)	1.28 $\pm$ 0.13	2.90 $\pm$ 0.76 <sup>a</sup>	1.90 $\pm$ 0.70 <sup>c</sup>	1.87 $\pm$ 0.49 <sup>d</sup>
NO ( $\mu$ mol/protein)	0.01 $\pm$ 0.008	0.09 $\pm$ 0.07 <sup>a</sup>	0.02 $\pm$ 0.01 <sup>c</sup>	0.02 $\pm$ 0.02 <sup>c</sup>
SOD (U/g protein)	6.51 $\pm$ 1.08	7.31 $\pm$ 1.28	6.03 $\pm$ 0.74	7.69 $\pm$ 1.76
GPX (U/g protein)	5.96 $\pm$ 1.29	3.71 $\pm$ 1.22 <sup>b</sup>	4.80 $\pm$ 0.27 <sup>c</sup>	3.89 $\pm$ 0.69 <sup>b</sup>
CAT (k/g protein)	1.67 $\pm$ 0.91	1.78 $\pm$ 0.54	1.95 $\pm$ 1.02	1.73 $\pm$ 0.98
IGF-1 (ng/mL protein)	1.09 $\pm$ 0.07	1.39 $\pm$ 0.13 <sup>b</sup>	1.30 $\pm$ 0.10 <sup>b</sup>	1.25 $\pm$ 0.25 <sup>b</sup>
AK	0.1 $\pm$ 0.000	60.01 $\pm$ 3.72 <sup>a</sup>	56.65 $\pm$ 3.57 <sup>a</sup>	58.60 $\pm$ 3.10 <sup>a</sup>

Notes: Data are the mean  $\pm$  SD. Compared with control group

<sup>a</sup> $p < 0.001$ ;

<sup>b</sup> $p < 0.05$ .

Compared with AK-treated group

<sup>c</sup> $p < 0.05$ ;

<sup>d</sup> $p < 0.001$ .

MDA, malondialdehyde; NO, nitric oxide; SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; IGF-1, insulin like growth factor-1.

- Level ++ (moderate) = changes at different foci throughout the kidney.
- Level +++ (severe) = severe damage or necrosis.

### Statistical analysis

The data were analyzed using statistical package for social sciences (SPSS) 11.0 software program (SPSS, Chicago, IL). One-way analysis of variance and *post hoc* multiple comparison tests (least-squares difference) were performed on biochemical variables to examine differences among the four groups. All data are expressed as the mean  $\pm$  standard deviation and  $p$  values of  $< 0.05$  were considered statistically significant.

### Results

In renal tissue, AK levels were significantly high in all groups except the control group. AK induced acute renal toxicity, manifested by a significant increase in kidney lipid peroxides measured as MDA and NO (Table 1). Tissue MDA and NO levels were statistically higher ( $p < 0.05$ ) in AK-treated group than the control. MDA and NO levels were decreased with the administration of vitamin E and EPO and the difference was statistically significant.

GPX levels were statistically low in AK group compared with the controls. The levels of GPX, in vitamin E group, were increased significantly. However, SOD and CAT levels were not significantly different in none of the groups.

IGF-1 values in AK, EPO and vitamin E groups were significantly higher than the control group.

The grading of histological changes is summarized in Table 2. By histopathological investigations, clear histomorphological changes such as tubular epithelial necrosis, tubular dilatation, vacuolar degeneration and edema were seen in AK treated group. Histopathological

**Table 2.** Histopathological changes in renal tissues.

Histopathological parameters	Control	AK	AK + vitamin E	AK + EPO
Tubular necrosis	-	+	-	-
Tubular dilatation	-	+++	++	+
Tubular epithelial desquamation	-	+	-	-
Tubular vacuolization	-	+++	++	+
Tubular atrophy	-	-	-	-
Tubular cylinder	-	+	-	-
Interstitial inflammation	-	-	-	-
Interstitial edema	-	+++	++	+

improvements observed with EPO administration were evident, but imperceptible with vitamin E.

### Discussion

Aminoglycosides appear to generate their nephrotoxic side effects by three general mechanisms: renal tubular toxicity, reduced glomerular filtration and reduction in renal blood flow<sup>31</sup>. AK is an aminoglycoside antibiotic, which is used in clinical practice to treat severe Gram-negative infections. However, its nephrotoxicity has limited the extend use of it. Proximal renal tubular cells are the primer site of damage in patients treated with the antibiotic AK. The nephrotoxic side effects of aminoglycoside antibiotics have been documented in numerous species of experimental animals<sup>2,32</sup>. Over the production of free radicals induces the lipid peroxidation (MDA), by destroying unsaturated fatty acids in the cell membrane.

Other scientists have used different methods of assessment of renal damage in an attempt to determine the relative sensitivity of the kidney to AK as well as the effect of age and dose<sup>3-7,10,33</sup>. One mechanism of this toxicity is believed to involve reactive oxygen radical species generation (ROS). Also, it has been shown that aminoglycoside antibiotics exert their adverse renal effects by generating of ROS. Some studies demonstrated that antioxidant administration has ameliorated AK induced nephrotoxicity<sup>9,11,12</sup>. The aim of the present study was to evaluate the effects of vitamin E and EPO

on antioxidative state of AK induced nephrotoxicity in neonatal rats. Before starting the study, a preliminary experiment was performed to determine the appropriate dosage to make nephrotoxicity to the newborn rats.

In this study, AK administration to rats increased MDA levels and vitamin E and EPO administration significantly decreased MDA production in the AK rats' kidneys compared with the only AK treated rats. In the present study, we created that marked nephrotoxicity by intraperitoneal administration of AK were high levels of AK and marked histological changes in the renal tissue were observed in Groups 2–4 rats. It is also evaluated AK induced renal damage and investigated the protective role of vitamin E and EPO using biochemical and oxidative markers (MDA, NO, SOD, GPX, CAT, IGF-1) and the morphology of the kidney as examined under light microscopy, commonly used to monitor the development of renal damage. The histopathological examination of kidneys revealed that there was a significant tubular damage indicated by tubular dilatation, tubular vacuolization and interstitial edema. Also, histopathological findings were parallel with the biochemical findings in AK treated group and this shows that free radicals may have critical role in renal nephrotoxicity and EPO or vitamin E is protective via their antioxidant properties.

In the kidney, the proximal tubule is responsible for the excretory transport from blood to urine of xenobiotics, xenobiotic metabolites and waste products of metabolism<sup>34</sup>. Aminoglycosides are known to be nephrotoxic and make their endothelial injury by reducing the structure and density of fenestrated endothelium<sup>1</sup>. NO is the part of endothelin-signaling cascade in the kidney and is produced by NO synthase (NOS). Both NOS and endothelin were found to be localized to the same nephron segments, including the proximal tubule<sup>35,36</sup>. Studies emphasize the increase of MDA and NO levels, which are the end product of lipid peroxidation in oxidative stress damage<sup>11,12</sup>. In literature, NO has been implicated in cyclosporin A induced nephrotoxicity in renal proximal tubules<sup>37</sup>. Another study indicates that, when proximal tubules were exposed to radiocontrast agents, aminoglycoside antibiotics or heavy-metal salts, NO production increased<sup>34</sup>. NO production has been implicated in renal diseases and the action of several nephrotoxic compounds<sup>38</sup>. In this study, renal tissue MDA and NO levels were statistically high in AK treated group. Another study has shown that plasma NO levels and the nephrotoxicity was statistically high in gentamicin treated rats<sup>39</sup>.

Although CAT and GPX using the same substrate for their activities, in this study, a significant difference between the groups for GPX activity was found but CAT

activity was not affected. GPX is the first step enzyme defense against H<sub>2</sub>O<sub>2</sub>; subsequently, CAT takes part in enzymatic reaction. It could be explained by the short time of the study. This study made on newborn rats and the tissue samples were taken within 3 days. We thought that if the time was much longer we could see the decline of CAT activity.

EPO acts by binding to its specific receptor on the surface of erythroid progenitor cells to stimulate cell survival, proliferation and differentiation<sup>40</sup>. In animal models, EPO treatment in rodents was also observed to stimulate NO production<sup>41–43</sup>. EPO is the primary erythroid cytokine that provides for up-regulation of mature red blood cell production in response to hypoxic or ischemic stress<sup>44</sup>.

In the study, renal tissue MDA and NO levels were statistically high in AK treated group and these levels were significantly decreased with the administration of EPO. Similarly, Kaynar et al.<sup>45</sup> emphasize the role of EPO in the prevention of AK induced nephropathy and they found that serum urea levels were significantly improved in rats treated with EPO. In this study, we could not able to take serum specimen for urea and creatinine because our study groups were newborn rats and when we finish the study the rats were only 7 g.

Several studies have shown that rhEPO may play an important role also in the prevention of cisplatin-induced nephrotoxicity<sup>22,46</sup>. Renoprotective effect of rhEPO also evaluated in vancomycin induced nephrotoxicity in rats and it has been reported to play a role in injury recovery<sup>23</sup>. rhEPO administration has been shown to make an important cytoprotective effect against cisplatin-induced oxidative damage and renal injury. They showed that rhEPO reduced MDA and protein carbonyl levels. rhEPO also prevented glutathione depletion and ameliorated the increased CAT activity induced by cisplatin treatment<sup>47</sup>.

Recently, it was shown that rhEPO decreased the ROS levels, the MDA levels and ameliorated glutathione modulation induced by cisplatin and mitomycin C in cultured Vero cells<sup>48</sup>.

Vitamin E is an essential nutrient that functions as a non-enzymatic antioxidant. It is an important biological free radical scavenger, which can exert its effect both on cells and membranes by protecting low-density lipoproteins and polyunsaturated fats in membranes from oxidation<sup>49</sup>. Authors have shown the effect of vitamin E against gentamicin<sup>14,16,50</sup>, vancomycin<sup>51</sup>, cisplatin<sup>52</sup> and colistin<sup>53</sup>. In the literature, there was no investigation about the effect of vitamin E against AK induced nephrotoxicity in rats.

In this study, renal tissue MDA and NO levels were decreased with the administration of vitamin E. Also, the

levels of GPX in vitamin E group were increased significantly. An increase of SOD levels in AK-treated group was detected and also in AK+vitamin E treated group the SOD levels were decreased; but the results were not statistically significant.

Abdel-Naim et al.<sup>16</sup> made a toxicity study with gentamicin and found that vitamin E ameliorated the rise in renal content of MDA and enhanced the renal SOD activity. Patel Manali et al.<sup>50</sup> also showed the gentamicin toxicity and reported that vitamin E and NAC significantly restored renal functions, reduced lipid peroxidation, enhanced reduced glutathione level. These results of gentamicin and vitamin E were similar with our study with AK and vitamin E.

In conclusion, one of the important mechanisms of AK nephrotoxicity is tubulointerstitial injury related to oxidative stress. This injury is supported with biochemical and renal histopathological findings. In the group treated with AK a significant increase of the MDA and NO levels as well as the decline of antioxidant enzyme GPX was shown to be statistically significant compared to the control group. Vitamin E and EPO, as antioxidants, can be useful renoprotective agents for ameliorating AK induced nephrotoxicity in neonates. To the best of our knowledge, this is the first study investigating the protective effect of EPO and vitamin E in case of AK induced nephrotoxicity on neonatal rats. In this regard, to better understand the preventive properties of EPO and vitamin E in neonates, more experimental or clinical studies are suggested.

## Acknowledgments

This study has the approval of the animal ethics committee of the institution and conducted in accordance with standards such as the NIH Guide to the Care and Use of Laboratory Animals. All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

## Disclosure statement

The authors declare that there are no conflicts of interest.

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