

**Evening Primrose Oil/ Vitamin E Combination Treatment Has Renoprotective Effect On
Gentamicin-Induced Nephrotoxicity By Suppressing Renal Tumor Necrosis Factor α (TNF- α) /
Nuclear Factor $\kappa\beta$ (NF- $\kappa\beta$) Pathway And Inhibiting Renal Oxidative Stress**

Dalia M. Abd-Elhalim¹, Mayada Saad Farrag², Mona A. Hussain^{3*}

¹Department of Medical Physiology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt,
²Department of Pathology, Faculty of Medicine, Port Said University, Port Said, Egypt, ³Department of
Medical Physiology, Faculty of Medicine Port Said University, Port Said, Egypt.

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Abstract

Gentamicin is an effective aminoglycoside antibiotic drug but unfortunately up to 30% of gentamicin-treated patients may develop nephrotoxicity. Suggested mechanisms of gentamicin-induced nephrotoxicity included inflammation and oxidative stress injury. Evening primrose oil (EPO) has anti-inflammatory and antioxidant effects. The aim of current study was to assess the possible renoprotective effect of EPO/vitamin E combination treatment on gentamicin-induced nephrotoxicity in rats. Additionally, to address the responsible mechanism(s) of this effect. Eighteen adult male albino Sprague-Dawley rats were equally and randomly divided into three groups; normal control, gentamicin control and EPO/vitamin E treated groups. Gentamicin control group and EPO/vitamin E treated group received IP gentamicin injections for 5 days (100 mg/kg). EPO/vitamin E treated group received EPO/vitamin E (10 g and 200 IU /kg/day respectively orally). Renal function, oxidative stress and histopathological changes were assessed. Renal tissue expressions of tumor necrosis factor α (TNF α) and nuclear Factor $\kappa\beta$ (NF- $\kappa\beta$) were assayed. Significant improvements of kidney function markers and tubular necrosis in EPO/vitamin E treated group were observed. EPO/vitamin E treatment significantly ameliorated the gentamicin-induced increase of renal lipid peroxidation and renal tissue expression of TNF α and NF- $\kappa\beta$. EPO/vitamin E treatment significantly ameliorated the gentamicin-induced decrease of renal glutathione and superoxide dismutase concentrations. Conclusion of current study is EPO/vitamin E combination treatment has renoprotective effect on gentamicin-induced nephrotoxicity by inhibiting renal TNF α /NF- $\kappa\beta$ signaling pathway and the oxidative stress. Further researches for addressing the renoprotective effect of EPO treatment only and to determine other possible responsible mechanisms are needed

INTRODUCTION

Gentamicin is an aminoglycoside antibiotic drug, used for the treatment of Gram-negative bacterial infections (1). Gentamicin has many advantages including its low cost, low potential for resistance and hypersensitivity and high potency (2). However, it was found that blood urea nitrogen (BUN) and serum creatinine concentrations characteristically increase 7–10 days after initiation of aminoglycoside therapy. This increase in BUN and serum creatinine indicates significant kidney damage. About 30% of patients treated with gentamicin develop nephrotoxicity (3). Hence the clinical use of aminoglycoside drugs is limited due to its nephrotoxic effect (4).

Several studies suggested that the underlying mechanisms of gentamicin-induced nephrotoxicity included inflammation and oxidative stress injury that resulted in proximal renal tubular necrosis (5), (6), (7).

One of the multiple signaling pathways that are thought to mediate the inflammatory process in gentamicin induced nephrotoxicity is the tumor necrosis factor α (TNF- α) / nuclear factor κ B (NF- κ B) signaling pathway. Gentamicin activation of TNF- α / NF- κ B pathway mediates inflammation by regulating the gene expression of cytokines, chemokines and adhesion molecules. These inflammatory molecules participate in the pathogenesis of tubulointerstitial impairment via the promotion of leukocyte attraction and adhesion to inflamed renal tubular cells. (8), (9), (10)

In addition to inflammation, several studies suggested that oxidative stress is directly involved in gentamicin-induced nephrotoxicity.

This suggestion was supported by findings of an increase in malondialdehyde (MDA) level, an index of lipid peroxidation (LPO), and decrease of kidney glutathione (GSH) content and antioxidant enzyme activities such as superoxide dismutase (SOD) (11).

Oenothera biennis, commonly known as evening primrose, is a native North American traditional medicinal herb (12). Evening primrose oil (EPO) is a rich source of polyunsaturated fatty acids mainly the essential fatty acid the linoleic acid (LA) (70–74%) and γ -linolenic acid (GLA) (8–10%), which are precursors of anti-inflammatory eicosanoids. In addition, GLA suppresses inflammation mediators such as interleukin 1 β , interleukin 6 and TNF- α (13). EPO is traditionally used in the treatment of, asthma, breast problems, eczema, premenstrual and menopausal syndrome and rheumatoid arthritis as all have an inflammatory component (14).

Several studies suggested that EPO had an inhibitory effect on lipid peroxidation, and a strengthening effect on the glutathione dependent antioxidant defense system and both effects resulted in decreasing the oxidative stress injury (15), (16).

On the other hand several researches suggested that vitamin E treatment alone or in combination with other antioxidants had renoprotective effect on gentamicin induced nephrotoxicity (17), (18) (19).

Taken together, this study was designed to assess the possible renoprotective effect of EPO/vitamin E combination treatment on gentamicin-induced nephrotoxicity in rats. Additionally, to address whether this effect is

mediated by changing renal TNF- α / NF- κ B signaling pathway and oxidative stress.

Materials & Methods

This work was performed at Medical Physiology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

Animals:

Eighteen adult male albino Sprague-Dawley rats, body weight 130 - 145 g were purchased from Egyptian Organization for Biological Products and Vaccines (Giza, Egypt). All rats were left to acclimatize for one week prior to the experiment and were housed in plastic cages maintained at controlled room temperature (22-24 °C) with 12 hour diurnal (day and night change) with free access to standard pellet animal diet and tap water. Rats were equally and randomly allocated into three groups; normal control, gentamicin control and EPO/vitamin E treated groups. This study was performed In accordance with the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda.

Methods:

Gentamicin induced nephrotoxicity: Rats in gentamicin control group and EPO/vitamin E treated group were treated with intraperitoneal injections of gentamicin (100 mg/kg) (Epigent, EIPICO, Egypt) for 7 days started from the 6th day to the 12th day (20).

EPO and vitamin E treatment: Rats in EPO/vitamin E treated group received EPO/vitamin E combined preparation (Primaleve capsules, Glaxo Smith Kline, Egypt) 10 g and 200 IU /kg/day respectively by gastric gavage (21) for the whole period of the study.

Urine collection and blood sampling: At the end of the study, rats were individually distributed in

metabolic cages to collect 24 hours urine samples for urine volume, albumin and creatinine assay. At the end of the experiment, rats were anaesthetized with ether and retrobulbar blood samples were collected for serum separation and storage at -80 °C until subsequent use for BUN and creatinine assay. Then anesthetized rats were sacrificed by decapitation and laparotomy was done for kidneys collection.

Assay of biochemical markers in blood and urine: BUN, serum creatinine, and urine albumin and creatinine were assayed by colorimetric method with specific kits. Creatinine clearance was subsequently calculated using the standard conventional formula. (urinary creatinine concentration (U) (mg/ml) x urine volume (V) (ml/min)/ serum creatinine concentration (P) (mg/ml) (3).

Oxidative stress markers assay in kidney homogenate: The left kidneys were kept in – 80 °C degree for subsequent homogenization and measuring the concentration of MDA, SOD and reduced glutathione by spectrophotometric method using specific kits supplied by Biodiagnostic, Egypt.

Quantitative real time Polymerase chain reaction (qPCR) for TNF α and NF κ β expressions in kidney tissues homogenate:

Total RNA was isolated from left kidney homogenate using Qiagen tissue extraction kit (**Qiagen, USA**) according to instructions of manufacture. The total RNA (0.5–2 μ g) was used for cDNA conversion using high capacity cDNA reverse transcription kit (**Fermentas, USA**). Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The

qPCR assay with the primer sets were optimized at the annealing temperature. The primer sequence for TNF α : forward ATGAGAAGTTCCCAAATGGC and reverse CTCCACTTGGTGGTTTGCTA. The primer sequence for NF κ B: forward CAGCTCTTCTCAAAGCAGCA and reverse TCCAGGTCATAGAGAGGCTCA (22). The relative expression was calculated according to Applied Biosystem software.

Histopathological evaluation of the kidney:

Right kidneys were dissected and immediately transferred to 10 % formalin for subsequent paraffin embedding. 4 μ m sections were cut from paraffin-embedded tissue blocks and were stained with Hematoxylin & Eosin (H&E), Periodic acid schiff (PAS) and Masson's trichrome (MT) stains. Kidney damage indices included the following histopathological alterations: mononuclear cell infiltration, tubular necrosis and tubular casts. These alterations were evaluated and graded as follows: 0 for no detectable lesions, 1 for mild changes, 2 for moderate changes, and 3 for severe changes (23).

Statistical analysis:

All data were expressed as mean \pm standard error of mean (SEM) and analyzed using Statistical Package for Social Sciences (SPSS) program version 20. Comparisons among groups were carried out using one way Analysis of Variance (ANOVA) followed by Bonferroni post hoc test. Exact Fisher test was used for assessment of the significance of histopathological changes. Data were considered statistically significant with $P \leq 0.05$.

Results:

Effect of gentamicin and EPO/Vitamin E treatment on kidney function biochemical markers:

Gentamicin treatment caused significant increase in serum creatinine, BUN concentrations and urine ACR in the gentamicin control group vs. control and EPO/Vitamin E treated groups. Urine albumin concentrations significantly increased in both groups that were treated with gentamicin vs the control group but EPO/ Vitamin E treatment significantly decreased urine albumin concentrations vs. the gentamicin control group. Urine creatinine concentrations significantly decreased in gentamicin control group vs control and EPO/Vitamin E treated groups. Creatinine clearance rate results showed significant decrease in both groups treated with gentamicin vs. the control group but there was an observed increase in creatinine clearance rate in EPO/Vitamin E treated group vs. gentamicin control group but this difference was statistically insignificant (**0.19 \pm 0.05 and 0.045 \pm 0.009 ml/min respectively**).

Table 1.

Effect of gentamicin and EPO/Vitamin E treatment on renal oxidative stress markers:

Gentamicin control group and EPO/Vitamin E treated group had significant increase in MDA concentration in renal tissue vs. the control group. We observed MDA decrease in renal tissues of EPO/Vitamin E treated group vs. gentamicin control group (**26.08 \pm 3.84 and 60.42 \pm 8.35 nmol/mg tissue**) but this difference was statistically insignificant. Reduced glutathione and SOD assay in renal tissues showed significant decrease in gentamicin control group and EPO/Vitamin E treated group vs. control group. But EPO/Vitamin E treatment significantly

ameliorated this effect vs. gentamicin control group. **Table 2.**

Effect of gentamicin and EPO/Vitamin E treatment on TNF α and NF κ B mRNA expression in renal tissues of the study groups:

Gentamicin treatment caused significant increase in mRNA expression of both TNF α and NF κ B in renal tissues of gentamicin control group vs control and EPO/Vitamin E treated groups.

Figure 1.

Effect of gentamicin and EPO/Vitamin E treatment on kidney histopathological changes:

Table 1: Kidney function biochemical markers: BUN and Serum creatinine concentrations, urine albumin and creatinine concentrations, urine albumin creatinine ratio (ACR) and creatinine clearance (Mean \pm SEM) in the study groups:

	Normal control group	Gentamicin control group	EPO/vitamin E treated group
BUN (mg/dl)	45.98 \pm 5.50	97.35 \pm 7.20 ^{a*}	53.50 \pm 3.07
Serum creatinine (mg/dl)	0.22 \pm 0.06	1.36 \pm 0.19 ^{b*}	0.53 \pm 0.08
Urine albumin(mg/dl)	30.88 \pm 3.92	91.27 \pm 6.08 ^{c*}	50.42 \pm 4.60 ^{c,d *}
Urine creatinine (mg/dl)	51.18 \pm 4.56	21.27 \pm 2.76 ^{e*}	37.90 \pm 3.30
ACR	6.11 \pm 0.71	46.54 \pm 6.82 ^{a*}	13.64 \pm 1.25
Creatinine clearance (ml/min)	0.83 \pm 0.18	0.045 \pm 0.009 ^{f*}	0.19 \pm 0.05 ^{f*}

^{a*} Significant increase in BUN concentrations and ACR in gentamicin control group vs. normal control and EPO/vitamin E treated groups (P= 0.000 in all comparisons).

^{b*} Significant increase in serum creatinine concentrations in gentamicin control group vs. normal control and EPO/vitamin E treated groups (P= 0.000 and 0.001 respectively).

^{c*} Significant increase in urine albumin concentrations in gentamicin control group and EPO/vitamin E treated groups vs. normal control group (P= 0.000 and 0.04 respectively).

^{d*} Significant decrease in urine albumin concentrations in EPO/vitamin E treated group vs. gentamicin control group (P= 0.000).

^{e*} Significant decrease in urine creatinine concentrations in gentamicin control group vs. normal control and EPO/vitamin E treated groups (P= 0.000 and 0.016 respectively).

^{f*} Significant decrease in creatinine clearance in gentamicin control and EPO/vitamin E treated groups vs. normal control group (P= 0.001 and 0.021 respectively).

Table 2: Oxidative stress markers concentrations (Mean \pm SEM) in the renal tissues in the study groups:

	Normal control group	Gentamicin control group	EPO/vitamin E treated group
MDA (nmol/g tissue)	7.47 \pm 1.08	60.42 \pm 8.35 ^{a*}	26.08 \pm 3.84
Reduced glutathione (nmol/mg tissue)	61.48 \pm 4.32	27.50 \pm 3.06 ^{b*}	47.73 \pm 1.49 ^{b*,c*}
SOD (U/g tissue)	3.06 \pm 0.09	1.05 \pm 0.07 ^{d*}	2.49 \pm 0.17 ^{d*,e*}

^{a*} Significant increase in MDA concentrations in gentamicin control group vs. normal control and EPO/vitamin E treated groups (P= 0.000 and 0.001 respectively).

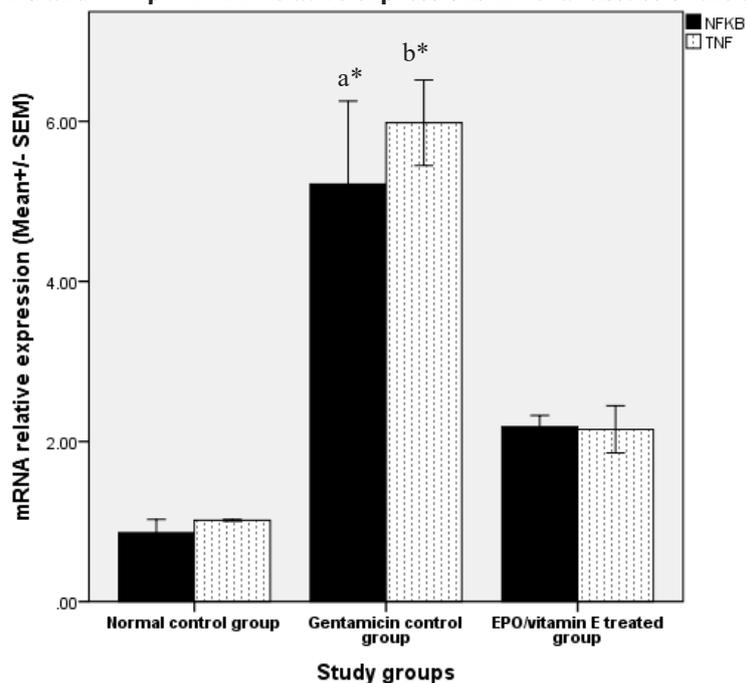
^{b*} Significant decrease in reduced glutathione concentrations in gentamicin control and EPO/vitamin E treated groups vs. normal control group (P= 0.000 and 0.024 respectively).

^{c*} Significant increase in reduced glutathione concentrations in EPO/vitamin E treated group vs. gentamicin control group (P= 0.001).

^{d*} Significant decrease in SOD concentrations in gentamicin control and EPO/vitamin E treated groups vs. normal control group (P= 0.000 and 0.01 respectively).

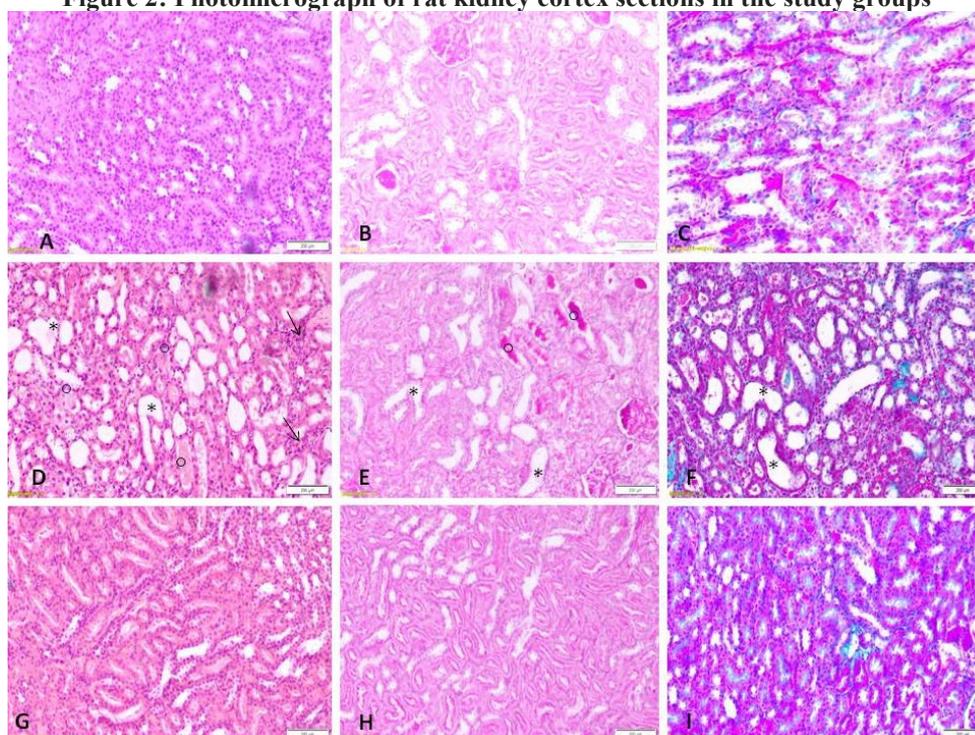
^{e*} Significant increase in SOD concentrations in EPO/vitamin E treated vs. gentamicin control group (P= 0.000).

Normal histology of kidney tissues in normal control group was observed. Kidney sections from gentamicin control group showed significant marked tubular necrosis and an increase in tubular casts and also variable degrees of mononuclear cells infiltration were observed. Kidney sections from EPO/vitamin E treated group showed almost complete prevention of these tubular histopathological alterations (H&E, PAS and MT respectively X 200). **Table 3 and figure 2.**

Figure 1: TNF α and NF κ B mRNA relative expressions in renal tissues of the study groups:

a* Significant increase in NF κ B mRNA expressions in renal tissues in gentamicin control group vs. normal control and EPO/vitamin E treated groups (P= 0.000 and 0.009 respectively).

b* Significant increase in TNF α mRNA expressions in renal tissues in gentamicin control group vs. normal control and EPO/vitamin E treated groups (P= 0.000 in both comparisons).

Figure 2: Photomicrograph of rat kidney cortex sections in the study groups

(A, B and C): Normal histology of kidney tissue in normal control group (H&E, PAS and MT respectively X 200). (D, E and F) Kidney sections from gentamicin control group showed marked tubular necrosis (asterisk), casts (circle), and mononuclear cell infiltration (arrow) (H&E, PAS and MT respectively X 200). (G, H and I) Kidney sections from EPO/vitamin E treated group showed almost complete prevention of tubular histopathological alterations (H&E, PAS and MT respectively X 200).

Table 3: Kidney histopathological changes (mononuclear cells infiltration, tubular necrosis and tubular casts) in the study groups.

	Mononuclear cells infiltration				Tubular necrosis				Tubular casts			
	-	+	++	+++	-	+	++	+++	-	+	++	+++
Normal control group	4	1	0	1	5	1	0	0	3	3	0	0
Gentamicin control group	0	4	1	1	0	1	0	5	0	3	2	1
EPO/vitamin E treated group	2	2	2	0	0	4	2	0	5	1	0	0
P value	0.204				0.000*				0.065			

- absent, + mild, ++ moderate and +++ severe. *Significant difference among the study groups.

Discussion:

In the current study, gentamicin treatment caused impairment in the renal function that resulted in an increase in BUN, serum creatinine and albumin creatinine ratio, and a decrease of urine creatinine concentrations and creatinine clearance rate. Histopathological changes showed significant tubular necrosis that was induced by gentamicin. These results were consisted with other previous researches (1), (4).

In the current study renal TNF- α / NF- κ B signaling pathway was evaluated to investigate the underlying mechanism of current results. It was found that gentamicin caused significant increase in renal tissue expression of TNF α and NF- κ B. The current finding is consisted with Kalayarasan et al., who found that after 6 days of gentamicin treatment, NF- κ B and TNF- α positive cells occupied most of the kidney tissues (8). NF- κ B is a transcription factor that is kept in an inactive state in the cytosol while bound to the inhibitory kappa β (Ik β) protein. NF- κ B positively regulates the expression of a number of genes including those of cytokines, cell adhesion molecules, complement factors and immunoreactants. Several proteins and molecules that activate NF- κ B

signaling have been described. TNF- α is one of the principal cytokines that promote IkB α degradation and NF- κ B activation. TNF- α induces the phosphorylation and subsequent degradation of Ik β . This, in turn, results in the activation and relocation of NF- κ B to the nucleus, leading to NF- κ B-mediated transcription of responsive genes (9), (24). Interestingly, NF- κ B activation stimulates expression of TNF- α thus establishing a positive auto regulatory loop that can amplify the inflammatory response (8).

Currently, it was observed that EPO/vitamin E combination treatment significantly improved the biochemical, morphological, oxidative and inflammatory profiles of the kidney and decreased the renal tissue expression of TNF- α and NF- κ B. The current finding may be attributed to the biological activity of chemical components of EPO that included polyunsaturated fatty acids (PUFAs), mainly linoleic acid (LA) and γ -linolenic acid (GLA) which belong to the group of omega-6 acids. Linoleic acid and γ -linolenic acid are precursors of compounds that lead to the generation of anti-inflammatory eicosanoids, such as the series 1 prostaglandins and 15-

hydroxyeicosatrienoic acid (15-HETrE) which suppress the inflammatory reaction and decrease inflammatory mediators such as TNF- α . On the other hand, linoleic acid can be converted to arachidonic acid that forms pro-inflammatory compounds, such as series 2 prostaglandins and series 4 leukotrienes. It has been shown that GLA supplementation causes a modest increase in the prostaglandin E1 level in tissues in relation to PGE2, but the biological properties of PGE1 are about 20 times stronger in comparison to PGE2 (13). Additionally we suggested that vitamin E may suppress the arachidonic acid conversion to proinflammatory mediators by 5 lipoxygenase and cyclooxygenase 2 as it had been demonstrated that specific forms of vitamin E and their metabolites have anti-inflammatory effects by inhibiting 5 lipoxygenase and cyclooxygenase 2 mediated eicosanoids, in addition to suppressing of NF- κ B (25), (26).

In the current study, it was observed that gentamicin induced a significant increase in renal MDA levels. Concurrently gentamicin caused significant decrease of both the activity of SOD and glutathione. Similar findings were reported by previous researches (16), (27), (3). It was suggested that gentamicin caused an overproduction of reactive oxygen species in renal tissue and this change resulted in oxidant-antioxidant imbalance and disrupt the membrane lipid composition through lipid peroxidation and subsequently increase the MDA (27). SOD catalyze the dismutation of superoxide anion free radical (O_2^-) into molecular oxygen and hydrogen peroxide (H_2O_2) and decrease O_2^- level which damages the cells at excessive concentration (28), hence gentamicin induced decrease of SOD may

be attributed to the overproduction of superoxide anions and hydrogen peroxide. Glutathione has a non-enzymatic antioxidant effect and acts as a free radical scavenger for the cells protection. Decrease of glutathione by gentamicin might be a result of excess generation of free radicals and increased consumption of glutathione in the protection of -SH group-containing proteins (16), (27).

In the present study, it was observed that EPO/vitamin E combination treatment significantly ameliorated gentamicin induced oxidative stress effect. The current finding may be attributed to the earlier documented antioxidant effect of vitamin E (25). The antioxidant property of vitamin E at level of renal tubules is probably mediated by enhancing superoxide dismutase and suppressing lipid peroxidation pathway (18). In regard to EPO antioxidant effect, it was reported that when tissue oxidative stress increased, EPO reduced lipid peroxidation and favored tissue antioxidant defense mediated by the glutathione system (15), (29).

It was suggested that, following the use of gentamicin, the increase in ROS production results in the destruction of I κ B and the release of NF- κ B, which after translocating into the nucleus, activates transcription of proinflammatory cytokines such as TNF- α that results in renal functional disturbances via vasoconstriction, decreased blood flow and infiltration of leukocytes (10). Hence, inflammation and oxidative stress are closely related pathophysiological events that are tightly linked with one another and with gentamicin induced nephrotoxicity. A number of reactive oxygen/ nitrogen species can initiate intracellular signaling cascade that enhances proinflammatory gene expression. On the other hand, inflammatory

cells liberate a number of reactive species at the site of inflammation leading to exaggerated oxidative stress (30) . So we suggest that combination treatment of EPO/vitamin E has antioxidant and anti-inflammatory effects that efficiently protect the kidneys against gentamicin induced toxicity.

In conclusion, EPO/vitamin E combined treatment had renoprotective effect against gentamicin induced nephrotoxicity in rats. The underlying mechanism of this effect included suppression of renal TNF- α / NF- κ β signaling pathway and inhibition of renal oxidative stress. Further researches for addressing the renoprotective effect of EPO treatment only and to determine other possible responsible mechanisms are needed.

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