sciendo

ACTA FACULTATIS
MEDICAE NAISSENSIS

DOI: 10.2478/afmnai-2018-0018

UDC: 616.61-085.356:615.33.065

Original article

Correlation between Biochemical and Morphometric Parameters in Gentamicin-Induced Kidney Injury: The Role of Co-Supplementation with Vitamins C and E

Nenad Stojiljković[1], Sonja Ilić[1], Milica Veljković[1], Pavle Randjelović[1], Dragan Radovanović[2], Jovan Todorović[3], Nikola Stojanović[3]

[1]*University of Niš, Faculty of Medicine, Department of Physiology, Niš, Serbia*
[2]*University of Niš, Faculty of Sport and Physical Education, Department of Physiology, Niš, Serbia*
[3]*University of Niš, Faculty of Medicine, Niš, Serbia*

SUMMARY

The purpose of our study was to determine the effects of co-treatment with vitamins C and E on gentamicin-induced nephrotoxicity and to quantify renal structural changes through morphometric analysis. Experiments were done on 24 Wistar rats divided into three groups of 8 animals each. The GM group animals were treated with gentamicin in the dose of 100 mg/kg. The GMEC group received both vitamin E in the dose of 100 mg/kg and vitamin C in the dose of 200 mg/kg together with gentamicin. The control group received normal saline. We confirmed nephrotoxicity in the GM group of rats by increased concentrations of creatinine and urea, altered parameters of oxidative stress (CAT, MDA, AOPP) and histopathological analysis of renal sections. Morphometric analysis showed increased glomerular basement membrane thickness and significant changes of glomerular and tubular parameters in the GM group. Analysis of parameters of oxidative stress showed that vitamins C and E significantly attenuated nephrotoxic effect of gentamicin; in addition, histological and morphometric analysis showed reduced histopathological damages of renal structures in the GMCE group. This study indicated that vitamins C and E could provide a significant protective effect against gentamicin-induced morphological and functional kidney alterations.

Key words: **Gentamicin, vitamin C and E, nephrotoxicity, morphometry, oxidative stress**

Corresponding author:
Nenad Stojiljković
Email: nenstojiljkovic@gmail.com

INTRODUCTION

Gentamicin is an aminoglycoside antibiotic very effective in the treatment of Gram-negative bacterial infections (1). However, many investigations documented that it causes dose-dependent nephrotoxicity in 10-25% of treated patients (2). Some studies indicate the importance of reactive oxygen and nitrogen species in its nephrotoxicity. Together with increase in lipid peroxidation and decrease in antioxidative enzyme levels, they cause kidney damage (3).

GM-induced nephrotoxicity is functionally manifested as proteinuria, increased serum urea and creatinine levels, and decreased glomerular filtration rate which all lead to acute kidney failure (4).

Recent data implicate the development of inflammation which is characterized by mononuclear infiltrations with consecutive release of proinflammatory cytokines and activation of NF-kB as a response to oxidative stress, which mediates gentamicin-induced kidney damage (5, 6).

Target spot in GM-induced kidney impairment is the renal cortex, precisely glomerules and proximal tubules. Morphological alterations include necrosis and apoptosis of tubular cells (7-9), mitochondrial vacuolization and lysosomal swelling (10, 11).

Gentamicin-treated rat kidneys are very sensitive to reactive oxygen species (ROS) due to decreased activity of endogenous antioxidative enzymes such as manganese superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase (12). Enormous ROS production has a direct impact on certain macromolecules and causes cellular damage and necrosis through mechanisms which include membrane lipid peroxidation, protein denaturation and DNA damage. It is important to point out that oxidative stress (13) and mitochondrial cytochrome C leakage (14) is not the only mechanism of renal damage in GM-induced nephropathy; carbonyle stress also has a certain role in its pathophysiology (15).

Many studies investigated protective effects of different antioxidants in GM-mediated nephrotoxicity, including selenium, lipoic acid, melatonin, vitamin E, green tea, N-acetyl cysteine and other (16-18).

Vitamin E is a well-known endogenous antioxidant which attenuates cellular damage by protecting essential fatty acids against free radical-mediated oxidative deterioration. Vitamin C has a strong antioxidative activity. Its mechanism of action is through ROS neutralization, amelioration of DNA and plasma membrane dam-age and reducing mutations (19).

Some investigations documented better effect in co-treatment of these two antioxidants. Co-supplementation of vitamin C and E decreases lipid peroxidation and enhances antioxidative enzyme activity in diabetic rat kidneys (13, 20, 21). However, in this experimental model, alterations of glomerules, GBM and tubules were not quantified through morphometric analysis before nor were advanced oxidation protein products (AOPP) determined, which was the main goal in our study.

MATERIAL AND METHODS

Adult Wistar rats (n = 24; weighing 250–300 g) housed at the vivarium of the Institute for Biomedical Research, Faculty of Medicine, University of Nis were used in this study. Animals were kept in sterile (pathogen-free) polycarbonate cages under controlled conditions with a 12 hour day / night cycle at 20 ± 2 °C, with humidity around 60%, with food and water ("VET-FARM"-Subotica) available ad libitum. The animals were acclimated one week before the experiment and randomly divided into three groups. All experimental procedures were conducted in accordance with the principles for the care and use of laboratory animals in research, the Declaration of Helsinki and European Community guidelines for the ethical handling of laboratory animals (EU Directive of 2010; 2010/63/EU), and were approved by the local Ethical Committee (No. 01-2625-7).

EXPERIMENTAL PROTOCOL

All rats were randomly divided into three groups, each consisting of 8 animals. The treatment protocol was designed as follows:

Group I – control (C) group, received normal saline 1 ml/day intraperitoneally (i.p.)

Group II – the first experimental group (GM group) was treated with gentamicin (Galenika AD, Belgrade, Serbia) i.p. in the dose of 100 mg /kg body weight (BW)/24 h.

Group III – the second experimental group (GEC group) received oil solution of vitamin E in the dose of 100 mg/kg BW/24 h (Pharmamagist, Budapest, Hungary) and vitamin C (Galenika AD, Belgrade, Serbia) i.p. in the dose of 200 mg/kg BW/24 h together with the same dose of gentamicin as in the GM group.

All animals were treated for eight consecutive days and after the last treatment, i.e. 9 days after the beginning of the experiment; the animals were anaesthetized with 80 mg/kg of ketamine (10% Ketamidor,

Richter pharma AG, Wien, Austria). Blood samples were taken from the aorta and the serum for biochemical analysis was separated after centrifugation at 2000 rpm for 10 minutes. Both kidneys were dissected from animals where one was used for biochemical analysis, while the other was used for histopathological processing and further light microscopic examination.

HISTOLOGICAL ANALYSIS

Dissected kidneys were fixed in 10% buffered formaldehyde, dehydrated in different alcohol solutions and further processed for paraffin embedding. Kidney tissue species were cut at a thickness of 5 μm using a HistoRange microtome (model: LKB 2218, LKB-Produkter AB, Bromma, Sweden). Tissue samples were routinely stained with hematoxylin–eosin (HE), Periodic Acid Schiff (PAS) and Jones methenamine silver according to the conventional staining protocols. For histopathological examination of kidney tissue, the microscope (LEICA DM 2000 LED) and digital camera (LEICA DFC 450) were used.

BIOCHEMICAL ANALYSIS

Blood samples were analyzed for markers of kidney function impairment. Urea, creatinine, sodium and potassium concentrations in serum were measured by using an automatic biochemical analyzer (A25 Biosystems, Barcelona, Spain).

KIDNEY TISSUE HOMOGENIZATION AND DETERMINATION OF OXIDATIVE STRESS PARAMETERS

Kidney tissue was cut and homogenized in ice-water with homogenizer (IKA Works de Brasil Ltda Taquara, RJ 22, 713-00). Kidney homogenates were centrifuged at 4000 rpm for 10 minutes at 4 ^{0}C in order to obtain clear supernatants. The protein content was measured according to the Lowry's method (22) using bovine serum as standard. The amounts of AOPP in the renal tissue homogenates were determined using spectrophotometric method described by Witko-Sarsat et al. (23). Lipid peroxidation was measured by determining malondialdehyde (MDA) levels that in reaction with thiobarbituric acid (TBA) give colored product which absorbance was measured at 532 nm (24). Renal catalase (CAT) activity was determined using a standard method previously described by Goth (25).

MORPHOMETRIC ANALYSIS

For quantification of changes determined by the histological analysis of kidney tissue, morphometric analysis was used to examine the structures of kidneys (glomeruli, glomerular basement membrane, proximal and distal tubules and cell nuclei of kidneys interstitium). Morphometric analysis was performed using a light microscope Leica DMR (Leica Microsystems AG, Wetzlar, Germany) using the computer program Image J: (http://rsbweb.nih.gov/ij/). All experimental animals were analyzed. Spatial calibration, by object micrometer (1:100), as well as optical density calibration were performed before each analysis.

For the analysis of glomeruli, samples stained with HE method were used and photographed under the lens magnification of 10 times. The analysis included all observed glomeruli at magnification of 10x, according to the principle of random selection of selected visual fields per one case. In the observed glomeruli, the following morphometric parameters were determined: GBM thickness, cellularity, area, perimeter, Feret's diameter as well as circularity.

Morphometric analysis of the renal tubules was performed on the samples stained with PAS method and photographed under lens magnification of 10x. The analysis included 10 tubules, according to the principle of random selection of selected visual fields per one case. In each visual field five randomly selected proximal and five randomly selected distal tubules were analyzed. The following parameters were determined: area and nucleo-cytoplasmic ratio. The analysis included only those tubules whose contours were clearly visible.

Morphometric analysis of interstitial cells nuclei was performed on the samples stained with HE method and photographed under lens magnification of 40x, on a total of 10 randomly selected fields. Morphometric analysis included 10 randomly selected nuclei for each field of view (a total of 100 nuclei per case). We determined the following parameters: area, optical density, perimeter, Feret's diameter and circularity.

STATISTICAL ANALYSIS

The obtained parameters were expressed as the mean ± SD. Statistical significance between different groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparison (Graphpad Prism version 5.03, San Diego, CA, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

HISTOLOGICAL ANALYSIS

Control group of rats showed normal histological tissue features (Figure 1a and b). In some proximal tubules of the experimental group treated with gentamicin, coagulation-type necrosis was present, while in other tubules pre-necrotic changes with eosinophilia, condensation of cytoplasm and nuclei hyperhromasia were observed (Figure 1c). In some tubules, eosinophilic hyaline cylinders were found. Glomeruli in the GM group of rats were enlarged and edematous, with reduction of Bowman's space, while GBM was blurred. Epithelial cells of distal tubules showed signs of reversible damage, while mononuclear infiltrate was present in the interstitium. On the samples stained with Jones method, irregularly thickened GBM can be seen as well as the coagulation necrosis with the presence of dark inclusions and vacuolation of the cytoplasm of cells of proximal tubules that still have nuclei (Figure 1d). The kidney cortex of rats in GEC group had relatively preserved morphology. In the epithelium of particular proximal tubules, discrete signs of parenchymatous degeneration were present. In the interstitium, focal mononuclear inflammatory infiltrate was predominantly composed of lymphocytes and histiocytes (Figure 1e). Mild congestion was present in the capillary network of glomeruli. The distal tubules were of normal appearance. GBM was significantly thinner compared to the GM group (Figure 1f).

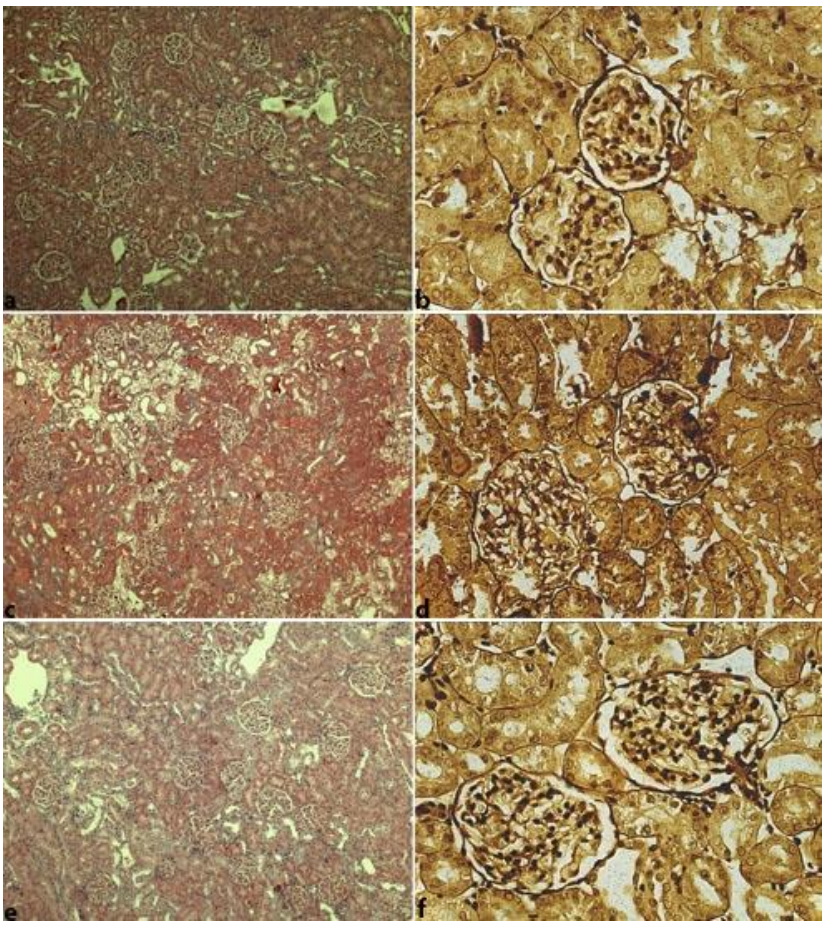

Figure 1. **Histopathology of renal glomeruli and tubules of (A) control group of rats showed normal histological tissue features (HE x 200); (B) control group of rats with normal structure of glomeruli (JONES x 400); (C) gentamicin-treated rats showed areas of proximal convoluted tubule epithelial necrosis and vacuolation of cytoplasm (HE x 200); (D) gentamicin-treated rats showed enlarged glomeruli with reduction of Bowman's space, while GBM was irregularly thickened (JONES x 400); (E) GMCE group with discrete signs of parenchymatous degeneration in proximal tubules (HE x 200); (F) GMCE group with thinner GBM than in the GM group.**

BIOCHEMICAL ANALYSIS

The analysis of biochemical parameters showed significant increase in the concentrations of urea and creatinine in the GM group when compared to the control group ($p < 0.001$). Creatinine and urea concentrations in rats treated with gentamicin and vitamins C and E were significantly lower compared to the GM group ($p < 0.001$). The concentration of potassium in the GM group was significantly decreased in comparison with the C group ($p < 0.001$), while the concentration of sodium was also decreased, but with no statistical significance. The concentration of potassium in the GMCE group was significantly higher than in the GM group ($p < 0.001$), while the concentration of sodium in this group was not significantly different from the other groups (Table 1).

ANALYSIS OF OXIDATIVE STRESS

Analysis of oxidative stress marker AOPP showed significantly elevated renal levels of AOPP in the GM group compared to the control group of rats ($p < 0.001$). Simultaneous administration of vitamin C and E with gentamicin reduced oxidative stress, which was evidenced by a significant decrease in renal AOPP levels compared to those in the GM group ($p < 0.001$) (Table 2).

Gentamicin administration to rats significantly increased the MDA levels in the kidney tissue compared to the C group ($p < 0.001$). Administration of vitamins C and E in the GMCE group reduced lipid peroxidation, which was evidenced by a significant decrease in MDA levels in this group compared to those obtained for the GM group ($p < 0.001$) (Table 2).

The determination of renal catalase activity showed significantly reduced activity of this parameter in the GM group compared to the control group ($p < 0.001$). The activity of CAT in the GMCE group was significantly lower than in the control group ($p < 0.01$), but was also significantly elevated compared to the GM group ($p < 0.001$) (Table 2).

Table 1. **Serum concentrations of creatinine, urea, sodium and potassium in experimental and control group of rats**

Biochemical parameter	C	GM	GMCE
CREATININE	56.78 ± 9.61	395.9 ± 82.17*	88.66 ± 18.04#
UREA (mmol/L)	6.72 ± 0.43	41.24 ± 7.28*	15.05 ± 3.82**#
Na (mmol/L)	145.9 ± 4.44	142.2 ± 2.22	145.1 ± 3.03
K (mmol/L)	5.58 ± 0.40	4.46 ± 0.19*	5.35 ± 0.26#

Data are presented as a mean ± SD. *p < 0.001 vs. C group; **p < 0.01 vs. C group; #p < 0.001 vs. GM group

Table 2. **Parameters of oxidative stress in kidney homogenates of experimental and control group of rats**

Biochemical parameter	C	GM	GMCE
AOPP (μmol/mg)	6.802 ± 0.7401	21.64 ± 5.575*	9.174 ± 1.194#
MDA (nmol/mg)	2.531 ± 0.06165	10.04 ± 1.903*	4.907 ± 0.4657#
CAT (kU/g)	13.37 ± 0.6352	2.885 ± 0.6582*	10.75 ± 0.4951**#

Data are presented as a mean ± SD. *p < 0.001 vs. C group; **p < 0.01 vs. C group; #p < 0.001 vs. GM group

MORPHOMETRIC ANALYSIS

The glomerular morphometric parameters of animals from the experimental and control group are presented in Table 3. Statistically significant differences were found between the experimental group of animals treated with gentamicin and the control group in glomerular size (glomerular area, perimeter and Feret´s diameter) ($p < 0.001$), which proved glomerular congestion. In animals treated with combination of vitamins C and E and gentamicin (GMCE group) glomerular area, perimeter and Feret´s diameter were significantly lower compared to the group of rats treated with gentamicin only ($p < 0.001$). The cellularity was significantly lower in the GM treated group compared to the control group of animals ($p < 0.001$). In the GMCE group, cellularity was significantly higher compared to the GM group ($p < 0.001$). Analysis of glomerular circularity showed statistically significant differences between the GM and control group ($p < 0.05$) and between the GMCE and control group ($p < 0.01$).

The mean glomerular membrane thickness was significantly higher in the GM group compared to the C group of rats ($p < 0.001$). In the GMCE group, the membrane thickness was decreased when compared to the GM group ($p < 0.001$) (Figure 2).

Table 3: **Glomerular morphometric parameters of the experimental and control group of rats**

Morphometric parameter	C	GM	GMCE
AREA ($\mu m^{2)}$)	7152 ± 424.4	9873 ± 1079*	7932 ± 331.4#
PERIMETER (μm)	315.6 ± 11.19	363.8 ± 20.85*	324.7 ± 7.98#
FERETS DIAMETER (μm)	112.5 ± 6.36	128.8 ± 6.44*	114.1 ± 4.42#
CELLULARITY (cells/$\mu m^{2)}$)	0.00742 ± 0.00187	0.00474 ± 0.00049*	0.00721 ± 0.00060#
CIRCULARITY	0.9019 ± 0.0275	0.9260 ± 0.0058***	0.9381 ± 0.0102**

Data are presented as a mean ± SD.
*p < 0.001 vs. C group; **p < 0.01 vs. C group; ***p < 0.05 vs. C group; #p < 0.001 vs. GM group

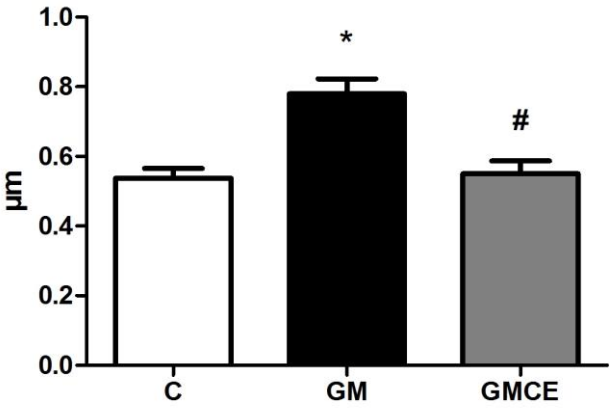

Figure 2. **Glomerular membrane thickness in experimental and the control group of rats. Data are presented as the mean ± SD. *p < 0.001 versus C group, #p < 0.001 versus GM group**

The measured parameters of proximal and distal tubules in each group are shown in Figure 3. The area of proximal tubules was statistically significantly decreased in the group of animals treated with gentamicin in comparison to the control group ($p < 0.05$). In the GMCE group, the area of proximal tubules was significantly increased compared to the GM group of animals ($p < 0.05$) (Figure 3A). The value of nuclear-cytoplasmic ratio in the epithelial cells of proximal tubules in the GM group of animals was statistically significantly larger when compared to the control and GMCE group ($p < 0.001$) (Figure 3B). The area of distal tubules and nuclear-cytoplasmic ratio in the epithelial cells of distal tubules showed no statistically significant difference between the control and experimental group of animals (Figure 3c and 3d).

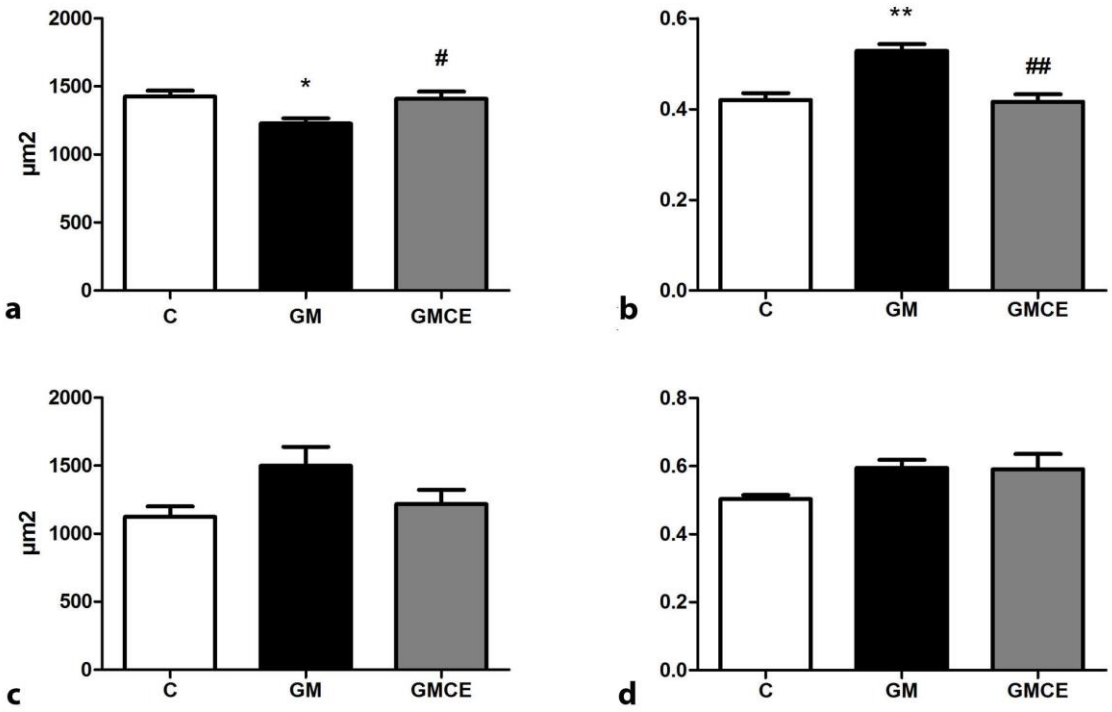

Figure 3. **Morphometric analysis of proximal and distal tubules in experimental and control group of rats showing (A) area of proximal tubules; (B) N/C ratio of epithelial cells of proximal tubules; (C) area of distal tubules; (D) N/C ratio of epithelial cells of distal tubules. Data are presented as the mean ± SD. *p < 0.05 versus C group, **p < 0.001 versus C group, #p < 0.05 versus GM group, ##p < 0.001 versus GM group.**

Table 4. **Interstitial morphometric parameters of the experimental and control group of rats**

Morphometric parameter	C	GM	GMCE
AREA (μm^2)	32.76 ± 2.18	17.75 ± 1.23*	21.96 ± 2.54*#
PERIMETER (μm)	24.22 ± 0.97	15.31 ± 0.57*	17.31 ± 1.17*##
FERETS DIAMETER	10.16 ± 0.54	5.36 ± 0.27*	6.23 ± 0.44*#
CIRCULARITY	0.7009 ± 0.0399	0.9381 ± 0.0087*	0.9151 ± 0.0099*
OPTICAL DENSITY	0.4589 ± 0.0226	0.6218 ± 0.0607*	0.5176 ± 0.0314**##

Data are presented as a mean ± SD.

*p<0.001 vs. C group; **p<0.05 vs. C group; #p<0.01 vs. GM group; ## p<0.001 vs. GM group

Morphometric parameters of interstitial cell nuclei from the experimental and control groups are presented in Table 4. The tested parameters (area, perimeter, Feret´s diameter, circularity, optical density) in the GM group of animals were significantly different compared to the control group of rats, whereby area, perimeter and Feret's diameter were significantly lower ($p < 0.001$), while circularity and optical density were significantly higher ($p < 0.001$). Area and Feret´s diameter in the GMCE group were significantly increased compared to the GM group ($p < 0.01$). The values of perimeter in GMCE group were significantly increased ($p < 0.001$), while the values of optical density were significantly decreased compared to the GM group ($p < 0.001$).The values of area, perimeter and Feret´s diameter in the GMCE group were significantly lower ($p < 0.001$), while circularity was significantly higher ($p < 0.001$) compared to the control group. Optical density in the GMCE group was also higher compared to the control group ($p < 0.05$).

DISCUSSION

Aminoglycoside antibiotics, including gentamicin, can cause nephrotoxicity in humans. Proximal tubular cells appear to be the most severely affected in patients treated with gentamicin or amikacin (26). Gentamicin binds the cell wall phospholipids, thus blocking the chain reactions of the phosphatidyl inositol pathway leading to the impairment of cell integrity (27).

In our experimental study, acute renal failure (ARF) was induced by intraperitoneal injection of gentamicin. Histological and morphometric analysis showed direct cellular toxic injury primarily on the proximal but also partly on the distal tubules, which indicates gentamicin nephrotoxicity.

Rats from the GM group were found to have proximal tubular impairment in the form of coagulation necrosis, epithelial desquamation and cytoplasmic vacuolization. Other tubules showed prenecrotic changes with condensed cytoplasm and hyperchromatic nuclei which caused tubular lumen to be dilated. The obtained results are in accordance with other studies (16). In Jones-stained microscopic slides, GBM of GM treated rats was unequally thickened. Marked coagulation necrosis was noticed with the presence of dark inclusions and cytoplasmic vacuolization of cells that posses nuclei, which is in accordance with our previous results (28).

GEC group of rats showed clearly alleviated glomerular changes when compared to the GM group where glomeruli were mildly enlarged (Table 3). Some of the proximal tubules contained hyaline cylinders, but no overt coagulation necrosis was present. Some of the proximal tubulocytes had cytoplasmic vacuoles. In Jones-stained microscopic slides, it was recorded that the GBM thickness of rats from the GEC and control group was significantly smaller than the one in the GM group. This result was confirmed through morphometric analysis which assessed the GBM thickness of all experimental groups.

Serum urea and creatinine concentrations in the GM group of rats were significantly increased, while sodium and potassium levels were significantly decreased in comparison with the GMCE and control group. These findings indicate renal damage, precisely proximal tubular impairment with the retention of uremic toxins and electrolyte imbalance, in the form of hyponatremia and hypokalemia.

To the best of authors' knowledge, there are no available data concerning the morphometric analysis of glomerular and tubular parameters in this experimental model, except those that are part of our previous research that confirmed correlation of histological changes of previously mentioned kidney structures with the analysis of some morphometric parameters (29).

Our results implicate that in gentamicin-induced ARF, histological findings are compatible with the morphometric ones. Morphometric analysis of the GM group showed a significant decrease in optical densities of proximal and distal tubules, which clearly indicates nuclear damage of tubular cells caused by gentamicin.

Morphometric analysis of proximal and distal tubules of the GEC group showed significantly higher values of nuclear optical densities when compared to the GM group, which demonstrates considerable protective effects of vitamin C and E co-supplementation on nuclear chromatin, which is in accordance with the results of other authors who investigated the preventive effects of vitamin C against a double-strand DNA damage induced by UV light. Their research found that vitamin C showed dose-dependent protection against DNA deterioration. It is assumed that the protection is managed through the vitamin C effect on highly compact polynucleosomic structures (30).

Morphometric analysis of proximal tubules recorded a significantly smaller epithelial area and a higher nucleo-cytolasmic ratio. The reason might lie in the fact that the measurements could be done only on tubules that underwent the initial phase of necrosis, but were not completely necrotic. That pathology development caused a decrease in the amount of cytoplasm in the proximal tubules. It is in accordance with previous studies that demonstrated proximal tubules (S1 and S2 segments) to

be the primary location of gentamicin toxicity (31). The same study of glomerules recorded results that implicate that GEN group glomerules were enlarged which correlates our histological findings. This indicates that gentamicin apart from affecting the proximal tubules, also exerts its toxic effects on glomerular capillaries. It is well known that gentamicin causes proliferation and apoptosis of glomerular mesangial cells (32). In our study, due to cell counting, we established that in this model of gentamicin nephrotoxicity, apoptosis is the dominant process. Interstitium study showed that the cells with smaller nuclei with increased circularity prevail, possibly because of massive leukocyte peritubular infiltration that is common in gentamicin damage. This peritubular infiltration can be brought in connection with increased amounts of AOPP in GM group, which is known to be the true proinflammatory mediator in uremia (33). Vitamin C and E injection alleviated histological and morphometric changes caused by gentamicin, which indicates their protective effect, probably through antioxidative activity.

Gentamicin-induced morphological changes cause functional impairment such as: glomerular chemo-dynamic disruption with the increse of capillary resistance, reduction of renal perfusion, reduction of ultra-filtration area, ultrafiltration coefficient (Kf) and glomerular filtration, retention of uremic toxins and electrolite disturbance.

In the complex mechanism of gentamicin nephrotoxicity, oxidative stress is probably the main pathological cause of cell damage. In our study, we confirmed the development of oxidative stress through investigating the levels of biochemical parameters MDA, CAT and AOPP. The amount of MDA is an indicator of lipid peroxidation and our study confirmed other data which indicated that gentamicin caused oxidative changes of cellular lipids (16). This cellular lipid damage, especially cell membrane, can be directly connected with the pathohistological findings which include tubule cell lyisis and consequenitional desquamation (34). Such changes are in good correlation with the decrease in proximal tubule cell area and nucleo/cytoplasimic ratio found in GM group (Figure 3).

Catalase is one of the most important endogenous antioxidative enzymes. Our research documented that gentamicin decreased its activity and thus disturbed the balance between production and elimination of ROS. This probably further disrupted cellular function, signaling pathways and caused cell death.

AOPP is a parameter that indicates a degree of oxidatively modified proteins. Up to date, in this experimental model its levels were not estimated. Increased AOPP in the GM group indicates that GM caused considerable ROS-mediated protein damage. Our results are in accordance with other studies which documented that gentamicin nephrotoxicity is caused by: production of ROS, increased intracellular Ca^{2+} concentration, activetion of lipid peroxidation and synthesis of various cytokines, PG, TX A2 and TNF-alpha (35). All together, they cause cellular damage through an increase in NO and the occurrence of endothelial dysfunction, apoptosis and necrosis. These changes in cell function possibly resulted in alteration of proximal tubule cell appearance, i.e. their size (area) and nucleo/cytoplasmic ratio (Figure 1 and 3). The role of superoxide anion in gentamicin toxicity is noted when different amount of superoxide dismutase was found to be effective in its reduction (36). Co-treatment of vitamin C and E together with gentamicin considerably improved oxidative status and decreased oxidative parameters changes induced by it.

Some studies showed that the use of antioxidants reduces GM-induced nephropathy (37, 38). Vitamin C is a non-enzymatic antioxidant and active reducing agent in many biological processes (19). It attenuates gentamicin caused lipid peroxidation and glutathione depletion. Many studies investigated dose-dependent protective effects of vitamin C on gentamicin-induced ARF (39). Its use would prevent free radical activation and consecutive progression of oxidative stress-mediated kidney damage. Some researchers studied cooperability between vitamin C and glutathione (40) and concluded that vitamin C treatment prevents gentamicin-induced reduction of renal glutathione. Vitamin C considerably decreases the level of mutations caused by H_2O_2. Glutathione depletion causes cytotoxicity and increases H_2O_2-induced mutation rate, but their frequency is considerably reduced in impaired cells that were previously treated with vitamin C. These results clearly indicate that high intracellular calcium concentrations can protect human cells against mutations induced by oxidative stress (41).

Investigations showed that gentamicin-induced lipid peroxidation could be inhibited by injecting medium and high doses of vitamin C. It is assumed that low concentration of vitamin C can cause increase in lipid peroxidation, while high concentration decreases it (42). Vitamin C capability to significantly decrease *in vivo* lipid peroxidation enables antioxidants from food to maintain membrane integrity in renal tissue in oxidative stress (43, 44). Also, vitamin C pretreatment prevents serum creatinine increase and reduction of its clearance in gentamicin-induced nephrotoxicity (45). Vitamin C, as an antioxidant, either inhibits chain reaction in reactive oxygen

species production or mediates their elimination before they manage to cause disruption of renal function. Vitamin C is able to regenerate other antioxidants by acting as co-antioxidant (46). It is most often applied as co-supplementation with vitamin E which ensures better antioxidative activity and protective effect in gentamicin-induced nephrotoxicity (47). Vitamin C decreases gentamicin-induced enzyme increase (40). Previous vitamin E injection, apart from inhibition of GM-induced activity of urinary enzymes, also prevents a reduction of GSH levels without considerable improvement of GFR. Co-supplementation of vitamin C and E significantly reduces GM-induced nephrotoxicity, due to maintenance of GFR and GSH levels and prevention of increased activity of urinary enzymes (47). In increased concentrations, antioxidative vitamins inhibit pathology development. Vitamin E is the main endogenous antioxidant which through reacting with oxygen species prevents their chain reaction and protects plasma membrane (18, 48). However, antioxidant depot, such as vitamin E, gradually decreases due to interaction with free radicals. Vitamin C use helps restoring them, whereas high doses of vitamin C act as an oxidative agent (49). Thus, in this study, in order to amplify their effects, dosage of vitamins C and E were modified in their co-supplementation. Other study also documented protective effects of their co-supplementation in GM-induced nephrotoxicity. Similarly to our results, co-treatment with vitamin C and probucol improves biochemical parameters of renal function and antioxidant enzymes level in GM-induced toxicity (13). Other studies found synergism between vitamin C and selenium in the prevention of renal damage (20, 50), documented attenuation of lipid peroxidation and increase in the levels of antioxidative enzymes and thiole components when applying alpha tocopherol and ascorbic acid. Besides the biochemical level (increase in antioxidative renal defenses), our study detected a significant reduction in kidney structure changes, such as glomerules and tubules (Table 2 and Figure 3), that are the direct consequence of gentamicin damaging effects.

CONCLUSION

Our results indicate that co-treatment with vitamin C and E considerably reduces histological and functional glomerular and tubular impairment in gentamicin nephrotoxicity, which is in correlation with our previous study when we separately investigated the protective effects of these two vitamins against gentamicin nephrotoxicity (29, 51). Since literature data implicate that gentamicin causes oxidative stress that initiates the whole specter of complex biochemical reactions with the consequence of cellular damage, in our experiment we demonstrated that vitamin C and E co-supplementation, possibly through enhancing of endogenous antioxidative renal defense, decreases or inhibits free radical activity. Through this action, their co-treatment has protecttive effect, alleviates morphological and functional kidney damage, decreases urea and creatinine concentrations, which was documented in our experimental model.

Acknowledgment

This work was supported by the Ministry of Science and Technological Development, Republic of Serbia (grant 175092).

References

1. Mwengee W, Butler T, Mgema S, et al. Treatment of plague with gentamicin or doxycycline in a randomized clinical trial in Tanzania. Clin Infect Dis 2006; 42: 614-21. https://doi.org/10.1086/500137

2. Martínez-Salgado C, Eleno N, Morales AI, et al. Gentamicin treatment induces simultaneous mesangial proliferation and apoptosis in rats. Kidney Int 2004; 65: 2161-71. https://doi.org/10.1111/j.1523-1755.2004.00642.x

3. Balakumar P, Rohilla A, Thangathirupathi A. Gentamicin-induced nephrotoxicity: do we have a promising therapeutic approach to blunt it? Pharmacol Res 2010; 62: 179-86. https://doi.org/10.1016/j.phrs.2010.04.004

4. Stojiljkovic N, Stoiljkovic M, Mihailovic D, et al. Beneficial effects of calcium oral coadministration in gentamicin-induced nephrotoxicity in rats. Ren Fail 2012; 34: 622-7. https://doi.org/10.3109/0886022X.2012.664809

5. Bae EH, Kim IJ, Joo SY, Kim EY, Choi JS, Kim CS, et al. Renoprotective effects of the direct renin inhibitor aliskiren on gentamicin-induced nephrotoxicity in rats. J Renin Angiotensin Aldosterone Syst 2013; 15: 348-61. https://doi.org/10.1177/1470320312474853

6. Kalayarasan S, Prabhu PN, Sriram N, et al. Diallyl sulfide enhances antioxidants and inhibits inflammation through the activation of Nrf2 against gentamicin-induced nephrotoxicity in Wistar rats. Eur J Pharma-col 2009; 606: 162-71. https://doi.org/10.1016/j.ejphar.2008.12.055

7. Lee IC, Kim SH, Lee SM, et al. Melatonin attenuates gentamicin-induced nephrotoxicity and oxidative stress in rats. Arch Toxicol 2012; 86:1527-36. https://doi.org/10.1007/s00204-012-0849-8

8. Sue YM, Cheng CF, Chang CC, et al. Antioxidation and anti-inflammation by haem oxygenase-1 contribute to protection by tetra-methylpyrazine against gentamicin-induced apoptosis in murine renal tubular cells. Nephrol Dial Transplant 2009; 24: 769-77. https://doi.org/10.1093/ndt/gfn545

9. Juan SH, Chen CH, Hsu YH, et al. Tetramethylpyrazine protects rat renal tubular cell apoptosis induced by gentamicin. Nephrol Dial Transplant 2007; 22: 732-9. https://doi.org/10.1093/ndt/gfl699

10. Selby NM, Shaw SM, Woodier N, et al. Gentamicin-associated acute kidney injury. Q J Med 2009; 102: 873-80. https://doi.org/10.1093/qjmed/hcp143

11. Begg EJ, Barclay ML, Kirkpatrick CM. The therapeutic monitoring of antimicrobial agents. Br J Clin Pharmacol 2001; 52: 35S-43S. https://doi.org/10.1046/j.1365-2125.2001.00377.x

12. Pedraza-Chaverrí J, Maldonado PD, Medina-Campos ON, et al. Garlic ameliorates gentamicin nephrotoxicity: relation to antioxidant enzymes. Free Radic Biol Med 2000; 29: 602-11. https://doi.org/10.1016/S0891-5849(00)00354-3

13. Abdel-Naim AB, Abdel-Wahab MH, Attia FF. Protective effects of vitamin E and probucol against gentamicin-induced nephrotoxicity in rats. Pharmacol Res 1999; 40: 183-7. https://doi.org/10.1006/phrs.1999.0494

14. Morales AI, Detaille D, Prieto M, et al. Metformin prevents experimental gentamicin-induced nephropathy by a mitochondria-dependent pathway. Kidney Int 2010; 77: 861-9. https://doi.org/10.1038/ki.2010.11

15. Li YC, Shih YM, Lee JA. Gentamicin caused renal injury deeply related to methylglyoxal and -(carboxyethyl) lysine (CEL). Toxicol Lett 2013; 219: 85-92. https://doi.org/10.1016/j.toxlet.2013.01.024

16. Veljković M, Pavlović DR, Stojiljković N, et al. Morphological and morphometric study of protective

effect of green teain gentamicin-induced nephrotoxicity in rats. Life Sci 2016; 147: 85-91. https://doi.org/10.1016/j.lfs.2016.01.035

17. Randjelovic P, Veljkovic S, Stojiljkovic N, et al. Protective effect of selenium on gentamicin-induced oxidative stress and nephrotoxicity in rats. Drug Chem Toxicol 2012; 35: 141-8. https://doi.org/10.3109/01480545.2011.589446

18. Manali BP, Deshpande S, Shah G. Evaluation of efficacy of vitamin e and N-Acetyl cysteine in gentamicin-induced nephrotoxicity in rats. Ren. Fail. 2011; 33: 341-7 https://doi.org/10.3109/0886022X.2011.560987

19. Parveen N, Ahmad S, Shadab GG. Iron induced genotoxicity: attenuation by vitamin C and its optimization. Interdiscip. Toxicol. 2014; 7: 154-8. https://doi.org/10.2478/intox-2014-002

20. Djeffal A, Messarah M, Boumendjel A, et al. Protective effects of vitamin C and selenium supplementation on methomyl-induced tissue oxidative stress in adult rats. Toxicol Ind Health 2015; 31: 31-43. https://doi.org/10.1177/0748233712468020

21. Kedziora-Kornatowska K, Szram S, Kornatowski T, et al. Effect of vitamin E and vitamin C supplementation on antioxidative state and renal glomerular basement membrane thickness in diabetic kidney. Nephron Exp Nephrol 2003; 95: 134-43. https://doi.org/10.1159/000074840

22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem 1951; 193: 265-75.

23. Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Int 1996; 49: 1304-13. https://doi.org/10.1038/ki.1996.186

24. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-8. https://doi.org/10.1016/0003-2697(79)90738-3

25. Goth L. A simple method for determination of serum catalase activity and revision of reference range. Clin Chim Acta 1991; 196: 143-51. https://doi.org/10.1016/0009-8981(91)90067-M

26. Prayle A, Watson A, Fortnum H, Smyth A. Side effects of aminoglycosides on the kidney, ear and balance in cystic fibrosis. Thorax 2010; 65: 654-8. https://doi.org/10.1136/thx.2009.131532

27. Poole, K. Bacterial stress responses as determinants of antimicrobial resistance. J Antimicrob Chemother 2012; 67: 2069-89. https://doi.org/10.1093/jac/dks196

28. Stojiljkovic N, Mihailovic D, Veljkovic S, et al. Glomerular basement membrane alterations induced by gentamicin administration in rats. Exp Toxicol Pathol 2008; 60: 69-75. https://doi.org/10.1016/j.etp.2008.02.007

29. Stojiljkovic N, Stojiljkovic M, Randjelovic P, et al. Cytoprotective effect of vitamin C against gentamicin-induced acute kidney injury in rats. Exp Toxicol Pathol 2012; 64: 69-74. https://doi.org/10.1016/j.etp.2010.06.008

30. Yoshikawa Y, Hizume K, Oda Y, et al. Protective effect of vitamin C against double-strand breaks in reconstituted chromatin visualized by single-molecule observation. Biophys J 2006; 90: 993-9. https://doi.org/10.1529/biophysj.105.069963

31. Zhou Y, Vaidya VS, Brown RP, et al. Comparison of kidney injury molecule-1 and other nephrotoxicity biomarkers in urine and kidney following acute exposure to gentamicin, mercury, and chromium. Toxicol Sci 2008; 101: 159-70. https://doi.org/10.1093/toxsci/kfm260

32. Martinez-Salgado C, Lopez-Hernandez FJ, Lopez-Novoa JM. Glomerular nephrotoxicity of aminoglycosides. Toxicol Appl Pharmacol 2007; 223: 86-98. https://doi.org/10.1016/j.taap.2007.05.004

33. Wang JC, Zhao Y, Chen SJ, et al. AOPPs induce MCP-1 expression by increasing ROS-mediated activation of the NF-κB pathway in rat mesangial cells: inhibition by sesquiterpene lactones. Cell Physiol Biochem 2012; 32: 1867-77. https://doi.org/10.1159/000356619

34. Nath KA, Norby SM. Reactive oxygen species and acute renal failure. Am. J. Med. 2000; 109: 665-78. https://doi.org/10.1016/S0002-9343(00)00612-4

35. Abdelsameea AA, Mohamed AM, Amer MG, Attia SM. Cilostazol attenuates gentamicin-induced nephrotoxicity in rats. Exp Toxicol Pathol 2016; 68: 247-53. https://doi.org/10.1016/j.etp.2016.01.002

36. Cuzzocrea S, Mazzon E, Dugo L, et al. A role for superoxide in gentamicin-mediated nephropathy in rats. Eur J Pharmacol 2002; 450: 67-76. https://doi.org/10.1016/S0014-2999(02)01749-1

37. Ateşşahin A, Karahan I, Yilmaz S, et al. The effect of manganese chloride on gentamicin-induced nephrotoxicity in rats. Pharmacol Res 2003; 48: 637-42. https://doi.org/10.1016/S1043-6618(03)00227-5

38. Pedraza-Chaverrí J, Gonzalez-Orozcoa AE, Maldonado PD, et al. Diallyl disulfide ameliorates gentamicin induced oxidative stress and nephropathy in rats. Eur J Pharmacol 2003; 473: 71-8. https://doi.org/10.1016/S0014-2999(03)01948-4

39. Lusania M, Antunes G, Darin J, Bianchi M. Protective effects of vitamin C against cisplatin induced nephrotoxicity and lipid peroxidation in adult rats: a dose dependent study. Pharmacol Res 2000; 41: 405-11. https://doi.org/10.1006/phrs.1999.0600

40. Meister A. Glutathione, ascorbate, and cellular protection. Cancer Res 1994; 54: 1969-75.

41. Lutsenko E, Carcamo J, Golde D. Vitamin C prevents DNA mutation induced by oxidative stress. J Biol Chem 2002; 277: 16895-9. https://doi.org/10.1074/jbc.M201151200

42. Halliwell B, Chirico S. Lipid peroxidation: its mechanism, measurement and significance. Am J Clin Nutr 1993; 57: 715-25. https://doi.org/10.1093/ajcn/57.5.715S

43. Ali BH. Agents ameliorating or augmenting experimental gentamicin nephrotoxicity: some recent research. Food Chem Toxicol 2003; 41: 1447-52. https://doi.org/10.1016/S0278-6915(03)00186-8

44. Lloberas N, Torras J, Herrero-Fresneda I, et al. Postischemic renal oxidative stress induces inflammatory response through PAF and oxidized phospholipids. Prevention by antioxidant treatment. FASEB J. 2002; 16: 908-10. https://doi.org/10.1096/fj.01-0880fje

45. Spargias K, Alexopoulos E, Kyrzopoulos S, et al. Ascorbic acid prevents contrast-mediated nephropathy in patients with renal dysfunction undergoing coronary angiography or intervention. Circulation 2004; 110: 2837-42. https://doi.org/10.1161/01.CIR.0000146396.19081.73

46. Naidu KA. Vitamin C in human health and disease is still a mistery? An Overview Nutr J 2003; 2: 7-16.

47. Kadkhodaee M, Khastar H, Faghihi M, et al. Effects of cosupplementation of vitamins E and C on gentamicin-induced nephro-toxicity in rat. Exp Physiol 2005; 90: 571-6. https://doi.org/10.1113/expphysiol.2004.029728

48. Varzi HN, Esmailzadeh S, Morovvati H, et al. Effect of silymarin and vita-min E on gentamicin-induced nephrotoxicity in dogs. J Vet Pharmacol Ther 2007; 30: 477-81. https://doi.org/10.1111/j.1365-2885.2007.00901.x

49. Paolini M, Pozzetti L, Pedulli GF, et al. The nature of prooxidant activity of vitamin C. Life Sci 1999; 64: 273-8. https://doi.org/10.1016/S0024-3205(99)00167-8

50. Patra RC, Swarup D, Dwivedi SK. Antioxidant effects of alpha tocopherol, ascorbic acid and L-methionine on lead induced oxidative stress to the liver, kidney and brain in rats. Toxicology 2001; 162: 81-8. https://doi.org/10.1016/S0300-483X(01)00345-6

51. Stojiljković N, Ilić S, Veljković, M, Todorović J. Effects of supplementation with vitamin E on gentamycin-induced acute renal failure in rats. FU Med Biol 2014; 16: 61-6.

Korelacija između biohemijskih i morfometrijskih parametara kod nefrotoksičnosti izazvane gentamicinom: uloga suplementacije vitaminima C i E

Nenad Stojiljković[1], Sonja Ilić[1], Milica Veljković[1], Pavle Ranđelović[1], Dragan Radovanović[2], Jovan Todorović[3], Nikola Stojanović[3]

[1]*Univerzitet u Nišu, Medicinski fakultet, Departman za fiziologiju, Niš, Srbija*
[2]*Univerzitet u Nišu, Fakultet za sport i fizičko obrazovanje, Departman za fiziologiju, Niš, Srbija*
[3]*Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija*

SAŽETAK

Cilj ovog istraživanja bio je ispitivanje efekata istovremene aplikacije vitamina C i E kod nefrotoksičnosti izazvane gentamicinom, kao i kvantifikacija oštećenja bubrežnih struktura morfometrijskom analizom. Eksperimenti su izvedeni na 24 Wistar pacova koji su bili podeljeni u tri eksperimentalne grupe od po osam životinja. Životinje u GM grupi bile su tretirane gentamicinom u dozi od 100 mg/kg, dok su životinje iz GMEC grupe dobijale vitamin E u dozi od 100 mg/kg i vitamin C u dozi od 200 mg/kg zajedno sa gentamicinom. Životinje iz kontrolne grupe primale su fiziološki rastvor NaCl. U GM grupi utvrđen je porast koncentracija uree i kreatinina u serumu, statistički značajne promene parametera oksidativnog stresa (CAT, MDA, AOPP), kao i značajne strukturne promene na histopatološkom nalazu isečaka bubrega. Morfometrijska analiza je pokazala povećanu debljinu glomerularne bazalne membrane i signifikantne promene glomerularnih i tubularnih parametara u GM grupi. Analiza parametara oksidativnog stresa pokazala je da aplikacija vitamina C i E (grupa GMCE) statistički značajno umanjuje nefrotoksične efekte gentamicina. Takođe, histološka i morfometrijska analiza pokazala je da je istovremena primena vitamina C i E dovela do značajnog smanjenja histoloških i morfometrijskih promena struktura bubrega izazvanih gentamicinom. Ovo istraživanje pokazalo je da vitamini C i E mogu značajno da ublaže gentamicinom indukovane morfološke i funkcionalne promene bubrega.

Ključne reči: **Gentamicin, vitamin C i E, nefrotoksičnost, morfometrija, oksidativni stres**