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LABORATORY STUDY

## Beneficial Effects of Calcium Oral Coadministration in Gentamicin-Induced Nephrotoxicity in Rats

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### Abstract

Frequent therapeutical use of an aminoglycoside antibiotic gentamicin (GM) is limited by its nephrotoxic effects often characterized by both morphological and functional alterations of kidney leading to acute renal failure. The aim of this study was to examine the effect of dietary calcium supplementation on GM-induced nephrotoxicity in rats. Experiments were performed on 30 adult male Wistar rats divided into three groups of 10 animals each. G-group received GM intraperitoneally at a dose of 100 mg/kg; GCa-group received the same dose of GM concomitantly with 1 g/kg calcium carbonate given orally; and C-group, serving as control, received 1 mL/day of normal saline. All groups were treated during 8 consecutive days. Quantitative evaluation of GM-induced structural and functional changes of kidney was performed by histopathological, morphometrical, and biochemical analyses. Compared with control, G-group of rats were found to have diffusely and unequally thickened glomerular basement membrane with neutrophil cells infiltration. In addition, vacuolization of cytoplasm of proximal tubule cells with coagulation-type necrosis was observed. These GM-induced pathological lesions were significantly reduced in the rats of GCa-group. Morphometric analysis revealed statistically significant differences in the size of glomeruli (area, major and minor axes, perimeter), optical density, and roundness of glomeruli ( $p < 0.05$ ) between G and GCa groups. Biochemical analysis showed significant elevation in blood urea and serum creatinine concentrations, whereas potassium concentration was lowered in G-group compared with the other groups ( $p < 0.01$ ). It is concluded that oral supplementation of calcium during treatment with GM resulted in significant reduction of morphological and functional kidney alterations.

**Keywords:** gentamicin, nephrotoxicity, morphometry, calcium, rats

### INTRODUCTION

Gentamicin (GM) is an aminoglycoside antibiotic, which has long been and still is commonly used in the treatment of infections caused by Gram-negative bacilli, *Enterococcus* and *Staphylococcus*. The major adverse effects of GM are nephrotoxicity and ototoxicity. Although many hypotheses have been proposed and tested, the exact mechanisms of GM-induced nephrotoxicity still remain unclear. It has been shown that, during early time points in GM treatment (1–3 days), the antibiotic in low doses inhibits renal protein and phospholipid metabolism in rats.<sup>1</sup> Later alterations include degenerative changes (e.g., focal necrosis and apoptosis) and regenerative lesions (e.g., tubular cell proliferation

and differentiation). At higher doses, GM causes alterations in the balance of certain electrolytes, impairments of mitochondrial respiration, and inhibition of protein synthesis.<sup>2,3</sup>

Many studies have shown that reactive oxygen species (ROS) are involved in GM-induced renal damage. ROS directly act on cell components, including lipids, proteins, and DNA, destroying their structure. Peroxidation of membrane lipids during oxidative stress induces the fragmentation of polyunsaturated fatty acids and the release of various aldehydes and alkenes.<sup>4,5</sup> In addition, GM as a cationic amphiphilic drug may cause lysosomal phospholipidosis. Inhibition of lysosomal phospholipases, subsequent accumulation

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of phospholipids, and the formation of lysosomal myeloid bodies have been implicated as direct mechanisms of nephrotoxicity.<sup>1</sup> Moreover, correlative confocal microscopic and electron microscopic studies suggested a role of intracellular trafficking in GM-induced nephrotoxicity.<sup>6</sup> In addition, apoptosis is an important factor of GM toxicity, which could also contribute to clear off damaged cells and control compensatory proliferative response.<sup>7,8</sup>

In humans, nephrotoxicity is manifested clinically as proteinuria, enzymuria, fall in glomerular filtration rate, increase in levels of serum urea along with a slow rise in serum creatinine and hyposmolar urinary output, all leading to acute renal failure (ARF). In animals, tubular alterations have clearly been associated with the development of focal necrosis and apoptosis in the tubular epithelium, together with an extensive tubular and peritubular cell proliferation without an apparent change in kidney function.<sup>2</sup> There is a possibility that certain agents can protect the kidney from side effects of GM. Since GM-induced plasma and subcellular membrane damage appear to be critical pathogenetic events in nephrotoxicity, Ca<sup>2+</sup> may play a protective role in this serious adverse event.<sup>9</sup> This might involve competitive displacement of Ca<sup>2+</sup> from anionic phospholipids at the plasma and organelle membrane level, resulting in a decrease in Na-K-ATPase, adenylyl cyclase, mitochondrial function and ATP production, protein synthesis, solute reabsorption, and overall cellular function. The other possibility is increase the Ca<sup>2+</sup> solute flux, thereby competitively inhibiting the primary lesion: anionic phospholipid binding.<sup>3,10</sup> Accordingly, the main objective of the study was to examine the effect of dietary calcium loading on GM nephrotoxicity.

## MATERIALS AND METHODS

In this study, 30 healthy adult male Wistar albino rats, weighing 250–300 g, were used. The animals were kept under controlled temperature (20 ± 2°C), humidity (60%), and regular light cycle (12 h light/12 h dark) conditions. They were fed with standard rat chow and allowed free access to food and water during the experiments. All experimental procedures were conducted in accordance with the principles for the care and use of laboratory animals for scientific purposes contained in the European Union regulations (Directive 2010/63/EU) and USA Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

### Experimental Protocol

The animals were randomly divided into groups as follows: (1) Control or C-group (10 rats) received 1 mL/day 0.9% NaCl (normal saline) intraperitoneally; (2) G-group (10 rats) received GM (Galenika AD, Belgrade, Serbia) intraperitoneally at a daily dose of 100 mg/kg; (3) 10 rats in the GCa-group were treated

orally with calcium carbonate (CaCO<sub>3</sub>) (Alkaloid, Skopje, Former Yugoslav Republic of Macedonia) using gastric sonde at a dose of 1.0 g/kg concomitantly with the same dose of GM applied as in the G-group. The treatment protocols were repeated during 8 consecutive days. The animals were anesthetized 24 h after the last application (9 days after the beginning of the experiment) using 80 mg/kg ketamine (Ketamidol 10%, Richter Pharma AG, Wels, Austria) and sacrificed. Blood samples for biochemical analysis were taken from the aorta (2 mL), and the kidney was subsequently removed.

### Histological Analysis

After the kidneys were dissected out, tissues were fixed in 10% paraformaldehyde (in 0.1 M phosphate buffer saline) at room temperature for 48 h, then dehydrated through an ascending graded series of alcohol and embedded in paraffin wax. Tissue samples were cut at 5 μm thickness using a Histo Range Microtome (model: LKB 2218, LKB-Produkter AB, Bromma, Sweden) and stained with hematoxylin and eosin (HE) and Periodic Acid Schiff, according to conventional staining protocols. The histological sections were examined under the light microscope (Olympus BX50, Tokyo, Japan).

### Morphometric Analysis

For the morphometric analysis, light microscope (Olympus BX50, Tokyo, Japan) and Micro Image 3.0 (Olympus, Tokyo, Japan) image analysis and processing software were used. Spatial calibration by object micrometer (1:100), as well as optical density calibration were performed before each analysis. The following morphometric parameters were analyzed: glomerular area, major and minor axes, perimeter, diameter, roundness, and mean optical density. Detailed description of each measured parameter was given in our previous work.<sup>6</sup> In each animal, at least 20 glomeruli were examined, excluding columns of Bertin.

### Biochemical Analysis

Blood samples taken from the aorta were analyzed for markers of renal impairment. In plasma, creatinine, urea, sodium, and potassium concentrations were measured using an automatic biochemical analyzer (A25 Biosystems, Barcelona, Spain).

### Statistical Analysis

The results were expressed as mean values and SD for parameters obtained during the morphometric and biochemical analyses. Statistical significance for the differences between values of morphometric and functional parameters obtained from each group was tested by the multivariate analysis of variance (MANOVA) and Student's *t*-test using NCSS statistical software (NCSS Kaysville, UT, USA). In all cases, statistical significance was defined as *p* < 0.05.

## RESULTS

### Histological Analysis

Rats in the G-group had enlarged and paler glomeruli than those in the C-group. Glomerular basement membrane was irregularly thickened with neutrophil cell infiltration. In this group of animals, areas of proximal tubule epithelial cells undergoing necrosis and apoptosis, vacuolization of cytoplasm, and epithelial desquamation were found. Distal tubules had a normal histological appearance (Figures 1 and 2).

In the GCa experimental group of rats treated with GM and CaCO<sub>3</sub>, necrosis in proximal tubules was much less prevalent than in the G-group (Figure 3). In the GCa-group of rats, glomeruli were larger than those in the C-group, but were smaller than the glomeruli in the G-group of rats. Glomerular basement membrane in these animals was thinner than that in the G-group of rats. In the control group, glomerular basement membrane was thin and slender.

### Morphometric Analysis

Using MANOVA test, statistically significant differences between the control and the experimental G and GCa groups were found for all glomerular morphometric parameters (Table 1). Statistically significant differences were found between the G-group of rats and the control group in the size of glomeruli (area, major axis, minor axis, average diameter, and perimeter), optical density of glomeruli ( $p < 0.01$ ), and roundness of glomeruli ( $p < 0.05$ ). Analysis of glomerular morphometric parameters in the GCa-group of rats did not show statistically significant differences in relation to the control group. On the other hand, significant differences were found between the experimental G and GCa groups in the size of glomeruli (area, major and minor axes, perimeter), optical density, and roundness of glomeruli ( $p < 0.05$ ).

### Biochemical Analysis

Analysis of biochemical parameters using MANOVA test showed significant statistical differences between the control and G and GCa groups of animals (Table 2). The multivariate statistical analysis showed significant increase in blood urea and serum creatinine concentrations in the G-group when compared with the GCa-group ( $p < 0.01$ ). The concentration of potassium in the blood was significantly decreased ( $p < 0.01$ ) in the G-group of animals, whereas blood concentration of sodium was decreased, but without statistical significance in comparison with the GCa-group. Blood urea and serum creatinine concentrations in the GCa-group were significantly increased compared with the control group of animals ( $p < 0.01$ ).

## DISCUSSION

The results of this study indicated that Ca<sup>2+</sup> had ameliorative effect on GM nephrotoxicity as the oral

loading of rats with CaCO<sub>3</sub> exerted protective potential against renal dysfunction and structural damage caused by GM. The nephrotoxicity of GM occurs by selective accumulation of the drug in renal proximal convoluted tubules that leads to loss of its brush border integrity.<sup>11</sup> The GM nephrotoxicity involves renal free radical generation, reduction in antioxidant defense mechanisms, acute tubular necrosis, and glomerular congestion,<sup>2,12-15</sup> resulting in diminished glomerular filtration rate and renal dysfunction.<sup>16</sup> GM binds to membrane phospholipids, alters their turnover and metabolism, and, as a consequence, causes a condition known as phospholipidosis, which has been observed in humans<sup>17</sup> and experimental animals treated with the drug.<sup>18,19</sup> Lysosomal phospholipidosis results from the reduction in the available negative charge necessary for the correct function of phospholipases<sup>20</sup> and inhibition of their A1, A2, and C1 classes.<sup>21-23</sup> Phospholipidosis correlates tightly with the level of toxicity of aminoglycosides.<sup>19,24-26</sup> Some investigators demonstrated that GM acts as an iron chelator, and that the iron-GM complex is a potent catalyst of oxygen-derived radical formation.<sup>27-29</sup> The typical clinical manifestation of aminoglycoside toxicity is nonoliguric or even polyuric renal excretion dysfunction<sup>2,30-32</sup> accompanied by an increase in plasma creatinine, urea and other metabolic products of the organism, proteinuria, enzymuria, aminoaciduria, glycosuria, and electrolyte alterations (hypercalciuria, hypomagnesuria, hypocalcemia, and hypomagnesemia).<sup>26,33,34</sup> GM-induced nephrotoxicity is structurally associated with the occurrences of cellular desquamation, glomerular atrophy, tubular necrosis, tubular fibrosis, epithelial edema of proximal tubules, glomerular hypertrophy, perivascular edema inflammation, and glomerular congestion.<sup>16,35-42</sup> In this study, ARF was induced by a suprathreshold dose (100 mg/kg) administration of GM. Similarly to other studies, extensive segmental necrosis, vacuolization of the tubular epithelial cells' cytoplasm, and epithelial desquamation of proximal tubular cells were found. It is well known that GM accumulates in the lysosomes of kidney proximal tubular cells and causes apoptosis at clinically relevant doses.<sup>7,43</sup> The histopathological analysis showed that rats in the G-group had enlarged and paler glomeruli than the rats in the C-group, and the glomerular basement membrane was irregularly thickened with neutrophil cell infiltration. We also found areas of proximal tubule epithelial cells undergoing necrosis and apoptosis, vacuolization of cytoplasm, and epithelial desquamation. Histological analysis of glomeruli and proximal tubules in the experimental group of rats treated with GM and Ca<sup>2+</sup> showed that glomeruli were larger than in the C-group, but were smaller than the glomeruli in the G-group of rats. Glomerular basement membrane in these animals was thinner than that in the G-group of rats, and proximal tubular injuries were much less prevalent than in the G-group.

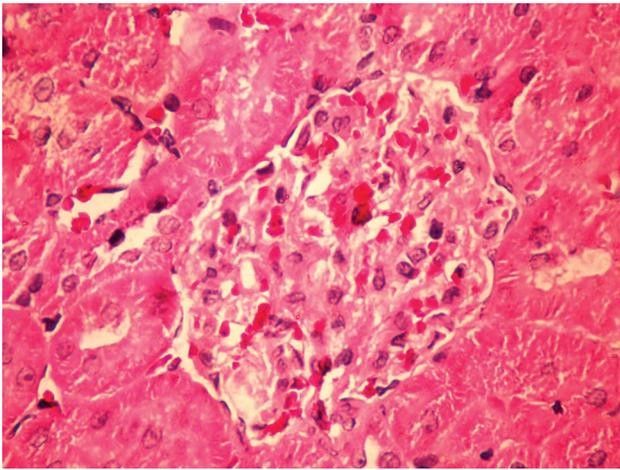


Figure 1. Kidney tissue of control (C) group of animals (HE  $\times 400$ ).

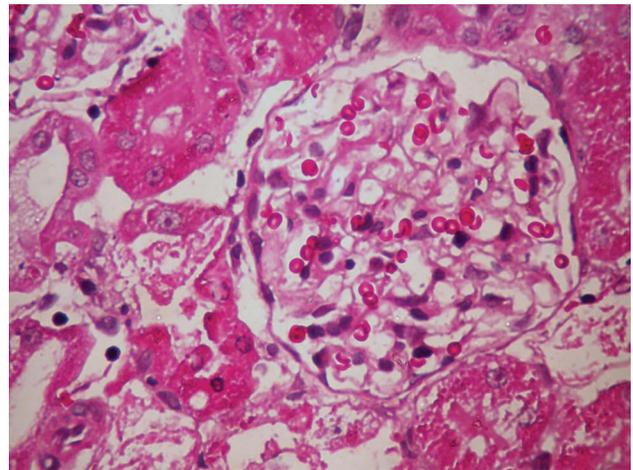


Figure 2. Histopathological view of renal sections in G-group (HE  $\times 400$ ).

Levels of blood urea and serum creatinine in the G-group were statistically significantly increased, whereas the values of serum sodium and potassium were decreased in comparison with the control. The values of blood urea and serum creatinine concentrations in the GCa-group were statistically significantly lower compared with the G-group. The concentration of serum potassium in the GCa-group was significantly higher compared with the G-group, whereas sodium values were increased, but without statistical significance. Remarkable elevation of urea and creatinine in the group of rats treated only with GM is an indicator of severe tubular necrosis. Morphometric analysis showed statistically significant differences between the

control and the experimental G and GCa groups. Statistically significant differences were found between the G-group of rats and the control group in the size of glomeruli (area, major axis, minor axis, average diameter, and perimeter), optical density of glomeruli ( $p < 0.01$ ), and roundness of glomeruli ( $p < 0.05$ ). Differences were also found between the experimental G and GCa groups in the size of glomeruli (area, major and minor axes, perimeter), optical density, and roundness of glomeruli ( $p < 0.05$ ). On the contrary, the glomerular morphometric parameters in the GCa-group of rats did not show statistically significant differences in relation to the control group. Oxidative stress causes  $\text{Ca}^{2+}$  influx into the cytoplasm from the

Table 1. Glomerular morphometric parameters in the control group (C-group), animals treated with GM only (G-group), and animals treated with GM and  $\text{CaCO}_3$  (GCa-group).

Variable	C-group	G-group	GCa-group
Area ( $\text{mm}^2$ )	9491.15 $\pm$ 1321.39*	12,799.86 $\pm$ 2098.87	10,222.84 $\pm$ 2177.32**
Optical density	0.33 $\pm$ 0.06*	0.28 $\pm$ 0.02	0.32 $\pm$ 0.08**
Major axis (mm)	129.72 $\pm$ 9.88*	142.51 $\pm$ 12.39	131.49 $\pm$ 18.10**
Minor axis (mm)	92.98 $\pm$ 8.26*	113.91 $\pm$ 12.98	93.17 $\pm$ 22.221**
Diameter (mm)	107.01 $\pm$ 7.89*	124.94 $\pm$ 10.94	109.83 $\pm$ 23.52
Perimeter (mm)	387.41 $\pm$ 26.49*	437.35 $\pm$ 35.86	392.60 $\pm$ 45.17**
Roundness	1.21 $\pm$ 0.06*	1.23 $\pm$ 0.02	1.22 $\pm$ 0.05**

Notes: Data are presented as the mean  $\pm$  SD.

\* $p < 0.01$  versus G-group.

\*\* $p < 0.05$  versus G-group.

Table 2. Biochemical analysis of serum levels of electrolytes, blood urea, and creatinine in the control or C-group, G-group, and GCa-group of rats.

Serum concentration	C-group	G-group	GCa-group
Sodium (mmol/L)	148.52 $\pm$ 4.64	143.84 $\pm$ 5.59	148.24 $\pm$ 5.80
Potassium (mmol/L)	5.84 $\pm$ 0.39*	4.38 $\pm$ 0.83	5.56 $\pm$ 0.60*
Urea (mmol/L)	6.83 $\pm$ 0.56*,**	47.68 $\pm$ 7.71	7.86 $\pm$ 0.54*
Creatinine ( $\mu\text{mol/L}$ )	63.55 $\pm$ 7.68*,**	474.76 $\pm$ 57.97	66.72 $\pm$ 8.08*

Notes: Data are presented as the mean  $\pm$  SD.

\* $p < 0.01$  versus G-group.

\*\* $p < 0.01$  versus G-group.

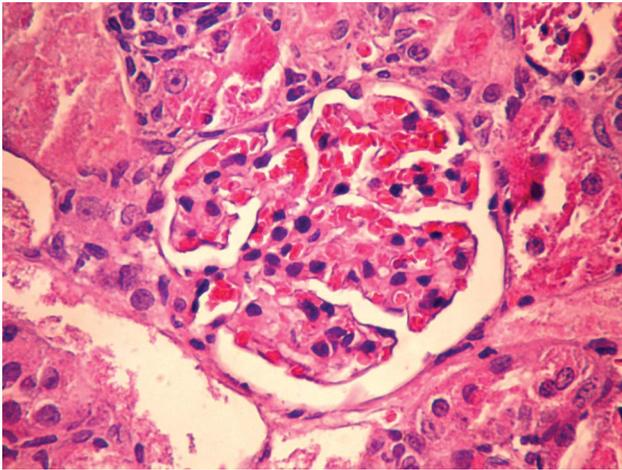


Figure 3. Histopathological view of renal sections in GCa-group (HE ×400).

extracellular environment and from the endoplasmic reticulum or sarcoplasmic reticulum (ER/SR) through the cell membrane and the ER/SR channels, respectively. Rising  $\text{Ca}^{2+}$  concentration in the cytoplasm causes  $\text{Ca}^{2+}$  influx into mitochondria and nuclei. In mitochondria,  $\text{Ca}^{2+}$  accelerates and disrupts the normal metabolism leading to cell death. In nuclei,  $\text{Ca}^{2+}$  modulates gene transcription and nucleases that control cell apoptosis. Both in nuclei and cytoplasm  $\text{Ca}^{2+}$  can regulate phosphorylation/dephosphorylation of proteins, and as a result can modulate signal transduction pathways.<sup>44</sup> Several investigators have demonstrated that calcium supplementation reduces the nephrotoxic effect likely through competitive inhibition of calcium channels in the proximal tubule.<sup>9,45–47</sup> It is suggested that high doses of GM cause wasting, and that  $\text{Ca}^{2+}$  loading increases the delivery of the ion to the kidney and prevents the binding of GM to the brush border membranes. It is also suggested that the mechanism of the protective action of  $\text{Ca}^{2+}$  may involve competitive displacement of  $\text{Ca}^{2+}$  from anionic phospholipids at the plasma and organelle membrane level, resulting in a decrease in Na–K–ATPase, adenylyl cyclase, mitochondrial function and ATP production, protein synthesis, solute reabsorption, and overall cellular function. The other possibility is increase the  $\text{Ca}^{2+}$  solute flux, thereby competitively inhibiting the primary lesion: anionic phospholipid binding.<sup>3,10</sup> Another possibility may be that  $\text{Ca}^{2+}$  prevents critical cellular derangements induced by GM within the renal tubular cell rather than on its cell surface. Since GM has been demonstrated to induce alterations in the structure and function of a variety of subcellular membranes produced by the antibiotic,<sup>48,49</sup> prevention of subcellular membrane dysfunction by calcium may be an additional mechanism for its protective effect. The influence of dietary sodium manipulations on the protective effect of oral calcium loading suggested that  $\text{Ca}^{2+}$  could have a major subcellular effect in preventing GM-induced

renal cell injury. Since sodium and calcium transport is directly linked to one another along the proximal tubule, volume expansion leads to the inhibition of  $\text{Ca}^{2+}$  transport and sodium depletion to elevations in  $\text{Ca}^{2+}$  transport along the proximal tubule.<sup>9,50</sup> This study indicated that  $\text{Ca}^{2+}$  could provide a significant protective effect against GM-induced ARF. We conclude that  $\text{Ca}^{2+}$  is an effective, safe, and practical agent that can reduce GM nephrotoxicity.

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