THE EFFECT OF CALCIUM CHANNEL BLOCKER VERAPAMIL ON GENTAMICIN NEPHROTOXICITY IN RATS

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Abstract

Aminoglycoside antibiotics are obligated nephrotoxins and inevitably cause renal failure during prolonged use. Experimental models of gentamicin-induced nephrotoxicity have shown histopathological, ultrastructural and functional alteration with blood urea nitrogen and serum creatinine increase leading to acute renal insufficiency (ARI). The aim of our study was to emphasize effects of verapamil, a calcium channel blocker, on gentamicin-induced ARI in rats. Experiments were done on 50 male Wistar rats (250-300 g) divided in three experimental groups. G-group animals (20 rats) were treated daily with gentamicin in dose of 100 mg/kg during 8 days. GV-group animals (20 rats) were treated daily with verapamil in dose of 3 mg/kg and the same dose of gentamicin as in G-group during 8 days. The control group (10 rats) received 1 ml/day saline intraperitoneally. Histological examinations were done using hematoxylin and eosin, periodic acid Schiff and methenamine silver staining methods. Morphometric parameters included measurement of glomerular area, major and minor axis, perimeter, diameter, roundness, and mean optical density. Biochemical analyses were used to determine concentrations of blood urea, serum creatinine, sodium and potassium. In G-group rats' glomerular basement membrane was diffusely and unequally thickened with polymorphonuclear neutrophils infiltration, while coagulation-type necrosis and vacuolization of cytoplasm of proximal tubules epithelial cells were observed. In GV-group rats' glomeruli were slightly enlarged with thickened basement membrane in some segments but without coagulation-type necrosis. Morphometric analyses showed statistically significant differences between the G-group and control group of animals in glomerular size, mean optical density and average roundness ($p<0,05$). On the other hand, morphometric analyses between GV-group and control group animals did not show statistically significant differences in any of parameters measured. Blood urea and serum creatinine concentration in G-group were significantly elevated in comparison with GV-group ($p<0,05$) but sodium and potassium levels in G-group were decreased compared to GV-group without statistical significance. Our results show that verapamil modify some of morphological and functional kidney alterations induced by gentamicin.

KEY WORDS: MTHFR, gentamicin, verapamil, nephrotoxicity, morphometry, rats

Introduction

A rather frequent administration of the aminoglycoside antibiotic gentamicin in the clinical practice has shown its unquestionable nephrotoxic effect (1). Even in low concentrations, gentamicin is bactericidal, but apart from vestibulotoxicity, it also shows a particularly high nephrotoxicity which can lead to acute renal insufficiency (ARI). Because of the potential nephrotoxicity of gentamicin, the administration of substances which would prevent or reduce the alteration of kidneys, and thus development of renal insufficiency, has been investigated in recent years. More recent study (2) has showed that the administration of gentamicin in a single daily dose significantly reduce a risk of gentamicin nephrotoxicity, although there is still a high incidence of gentamicin-induced ARI. Nephrotoxicity induced in experimental models (3,4,5) showed histopathological, ultrastructural and functional renal impairments in the form of tubular desquamation and necrosis as well as the enhanced blood urea nitrogen and creatinine. The predilection sites of damage are the renal cortex, i.e. glomeruli and proximal tubules. The exact mechanism of the cell impairment and the way in which gentamicin enters the cell are unclear. The stimulation of nephrotoxic effect of gentamicin is availed by the morphological and functional characteristics of the kidneys such as a large renal blood flow and therefore by a great proportional exposure of the renal parenchyma to toxic effects, high oxygen consumption, susceptibility of the renal tissue to hypoxia and the degree of the permeability of proximal tubules. There are numerous publications pointing out to the protective effect of verapamil possibly associated with blocking the calcium influx to the impaired cells or to the vasodilatory effects of verapamil which improve the renal blood flow and thereby moderate the development of acute renal insufficiency (6, 7, 8, 9). The aim of our experimental investigation was to determine the eventual preventive effect of the calcium channel blocker verapamil on gentamicin induced acute renal insufficiency in rats.

Material and Methods

All studies were performed on adult male Wistar rats, weighing 250 – 300 g. Animals were housed in a central facility under controlled conditions (12 h light/dark cycle and room temperature of 20°C ± 2°C) and with free access to food and water. All experimental procedures were conducted in accordance with the principles for the care and use of laboratory animals in research. The investigation conforms to the regulations of the European Union and USA Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (National Academy of Science Press, Washington, DC, 1996). The total number of 50 animals was divided in 3 groups, one of which was used as a sham control. The experimental group of animals or G-group (20 rats) received gentamicin (Galenika AD, Belgrade, Serbia) intraperitoneally in a daily dose of 100 mg/kg. GV-group animals (20 rats) were treated daily with verapamil (Galenika AD, Belgrade, Serbia) in dose of 3 mg/kg and the same dose of gentamicin as in G-group during 8 days. The control group of animals or C-group (10 rats) received 1 ml/day saline intraperitoneally. Both experimental and control group were treated over the period of 8 consecutive days. Following the last application, nine days after the beginning of the experiment, all animals were anaesthetized using 80mg/kg ketamine (Ketamidor 10%, Richter Pharma AG, Wels, Austria) and then sacrificed. Immediately after vivisection 2ml blood from aorta was taken for biochemical analysis. The kidneys were sectioned and fixed in 10% paraformaldehyde (in 0,1 mol/dm^3 phosphate buffer saline), dehydrated in graded alcohols and processed for paraffin wax embedding. Then, kidney slices were cut on 5 μm thick sections using HistoRange microtome (model: LKB 2218, LKB-Produkter AB, Bromma, Sweden) and stained with hematoxylin-eosin (HE), PAS (Periodic Acid Schiff) and Jones methenamine silver according to conventional staining protocols as described by Bancroft and Stevens (10). Histological slides were analyzed using light microscope (Olympus BX50, Tokyo, Japan) and Micro Image 3.0 (Olympus, Tokyo, Japan) image analysis and processing software was used for the morphometric analysis. Spatial calibration, by object micrometer (1:100), as well as optical density calibration was performed before each analysis. Morphometric parameters measured during the analysis of glomeruli were area, major and minor axis, perimeter, diameter, roundness and mean optical density. As previously mentioned, after finishing the experiment, blood samples were taken from aorta and analyzed for markers of renal impairment. Plasma creatinine, blood urea, sodium and potassium concentrations were measured using an automatic biochemical analyzer (A25 Biosystems, Barcelona, Spain). The data obtained from morphometric and functional measurements of each experimental group were expressed as mean value and standard deviation and

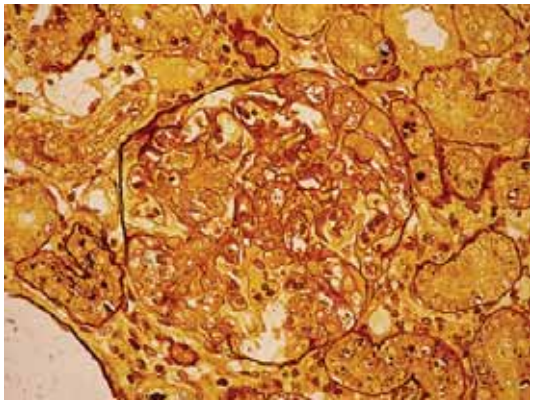

FIGURE 1. The glomerular basement membrane thickened diffusely in the G-group of animals. Jones staining method (magnification x 40).

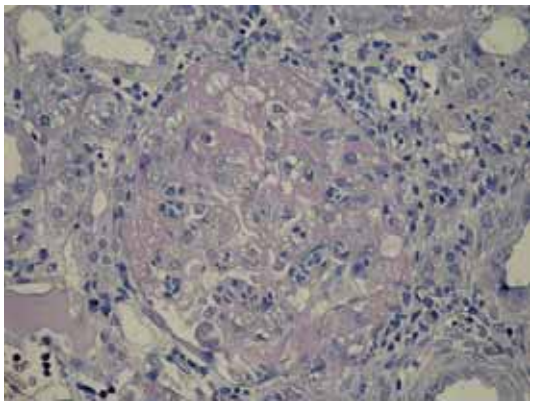

FIGURE 2. The presence of neutrophilic leucocytes in certain glomerular capillaries in the G-group of animals. PAS staining method (magnification x 40).

were analyzed by multivariate analysis of variance (MANOVA) using NCSS statistical software (NCSS Kaysville, Utah, USA). Paired comparisons between animal groups were performed using Student's t–test. In all cases, statistical significance was inferred for p<0,05.

Results

The kidney sections taken from the experimental G-group of animals showed that the glomerular basement membrane was thickened diffusely, and it was of unequal thickness (Figure 1). The presence of polymorphonuclear neutrophils was noticed in certain glomerular capillaries as well (Figure 2). In this group of experimental animals, the fields of necrosis of coagulation type were found in a large number of proximal tubules as well as the vacuolization of the cytoplasm of cells still possessing nucleus. The distal tubules were of normal appearance. The presence of dark inclusions in the epithelial cell cytoplasm of proximal tubules was observed in the sections stained with silver methenamine (Figure 3).

In GV-group of animals glomeruli were somewhat enlarged, and the glomerular basement membrane was thickened only in some segments of the glomeruli (Figure 4). In this group of animals, the fields of coagulation-type necrosis were not found. In some epithelial cells of proximal tubules dark inclusions and cytoplasm vacuolization were observed (Figure 5). The application of the MANOVA test for all glomerular morphometric parameters showed statistically significant differences between the control and the experimental G and GV groups: (*Wilks Lambda* = 0,25; *Rao's R* = 4,76; p< 0,001). The application of the t-test showed the following statistically significant differences: area, optical density, minor axis, perimeter and roundness (p<0,01 for each parameter). Statistically significant differences were found

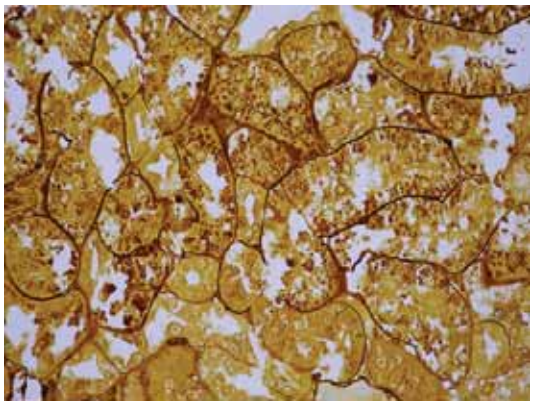

FIGURE 3. The fields of necrosis of coagulation type in a large number of proximal tubules as well as the vacuolization of the cytoplasm of cells still possessing nucleus in the G-group of animals. The distal tubules were of normal appearance. The presence of dark inclusions in the epithelial cell cytoplasm of proximal tubules was observed in the sections stained with silver methenamine. Jones staining method (magnification x 40).

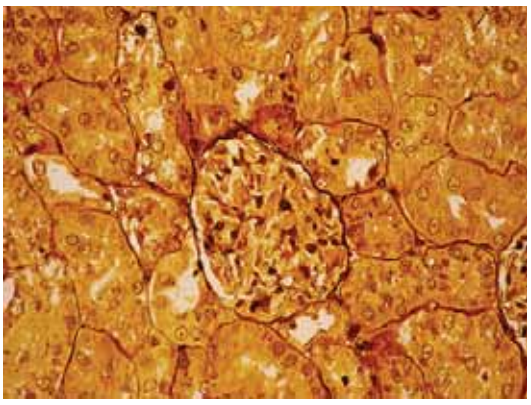

FIGURE 4. Somewhat enlarged glomeruli, and the glomerular basement membrane thickened only in some segments of the glomeruli in the GV-group of animals. Jones staining method (magnification x 40).

Variable	G-group	GV-group	Control
Area (μm²)	12800,489±2163,198*	10222,84±2177,326#	9491,350±1325,407
Optical density	0,28 4±0,012*	0,33±0,071#	0,363±0,0400
Major Axis (μm)	142,466±12,405*	137,45±17,897	129,428±10,188
Minor Axis (μm)	113,811±12,353*	99,14±22,221#	93,060±8,4268
Average diameter (μm)	124,793±11,044*	115,48±25,335	107,063±7,9038
Perimeter (μm)	437,529±36,362*	392,6±45,174#	381,805±27,501
Roundness	1,206±0,021**	1,27±0,051#	1,237±0,0469

Data are presented as mean ± SD. * p<0,01 vs. control **p<0,05 vs. control #p<0,05 vs. G-group

TABLE 1. Morphometric parameters of glomeruli in G-group, GV-group and control group of rats

Parameter	G-group	GV-group	Control
Sodium	144,46±6,07	146,10±5,71	149,33±4,49
Potassium	4,36±0,88*	4,57±0,54*	5,9±0,39
Urea	49,24±8,79*#	42,24±6,27*	6,86±0,58
Creatinine	488,20±62,25*#	424,50±76,10*	64,2±7,96

Data are presented as the mean ± SD. * p<0,001 vs. control #p<0,05 vs. GV-group

TABLE 2. Biochemical analysis of serum levels of electrolytes, blood urea, and creatinine in G- group, GV-group and control group of rats.

between the G-group of rats and the control group as follows: in the size of glomeruli (area, major axis, minor axis, average diameter and perimeter) (p<0,01); optical density of glomeruli (p<0,01) and roundness of glomeruli (p<0,05). Statistical analysis of glomerular morphometric parameters in the GV-group of rats did not show statistically significant differences in relation to the control group. Statistically significant differences found between the experimental G and GV groups were as follows: in the size of glomeruli area, minor axis, perimeter, optical density and roundness of glomeruli (p<0,05) (Table 1). The application the MANOVA test for all laboratory parameters showed high statistically significant differences between the experimental G and GV groups and the control groups of rats: (Wilks Lambda = 0,017; Rao's R = 40,18; p< 0,001) The application of the t-test showed a statistically significant difference in the values of urea and creatinine (p<0,001). The mean values of urea and creatinine serum concentrations found in the G-group of rats were statistically significantly enhanced, while the mean values of potassium serum concentrations in the G group were statistically significantly reduced, in relation to the same values in the control group (p<0,001). There were no statistically significant differences between the values of sodium serum concentrations in the G group and the control group. The values of urea and creatinine serum concentrations in the GV-group of rats were statistically significantly enhanced, while the values of potassium serum concentrations were statistically significantly reduced, in relation to the same values in the control group of rats (p<0,001). The values of urea and creatinine serum concentrations found in the G-group of rats were statistically significantly enhanced in relation to the same values found in the GV-group (p<0,05). There were no statistically significant differences between the values of sodium and potassium serum concentrations found in the G group in relation to the same values found in the GV group (Table 2).

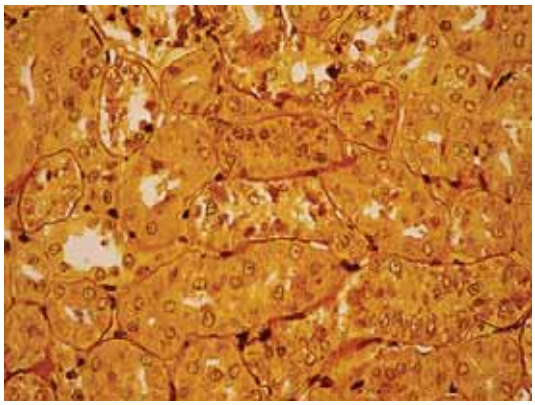

FIGURE 5. Dark inclusions and cytoplasm vacuolization in some epithelial cells of proximal tubules in the GV-group of animals. HE staining method (magnification x 40).

Discussion

Because of its strong bactericidal effect, gentamicin is widely used antibiotic in the treatment of infections caused by gram-negative microorganisms. However, the data found in the literature also speak of its nephrotoxic effect demonstrated in a number of experimental studies in which gentamicin acute renal insufficiency was induced (3, 4, 5). In our study, gentamicin given in a supratherapeutic dose (100mg/kg) induced acute renal insufficiency (ARI) in rats. The histopathological changes observed in these animals consisted of enlargement of glomeruli and glomerular basement membrane alterations with unequal thickness in some of its segments. The neutrophilic leucocytes were present in some glomeruli capillaries. The changes in the proximal

tubules were dominant and manifested in the form of segmented necrosis of the coagulation type, cytoplasm vacuolization of tubular epithelial cells with preserved nuclei and multitude of dark inclusions ("myeloid body"). The structural changes in the distal tubules are not found. These changes mostly coincide with the changes already described by other authors (11, 12, 13). In relation to the control group, morphometric analysis of the glomeruli of G-group of rats showed signs of glomerular impairment such as the enlargement of glomeruli with reduction in optical density of glomeruli and roundness ($p<0,05$). The above mentioned results point out that there are morphological and functional changes in the glomeruli caused by the effect of gentamicin. In the mentioned group of animals, the biochemical analysis showed the most significant increase in serum urea and creatinine as a sign of the functional alterations of kidney, whereas the values of serum sodium and potassium were reduced in relation to the GV-group. However, the obtained values were not statistically significant. This is rather customary having in mind that the above laboratory parameters and electrolytes are secreted predominantly by glomerular filtration. The morphological changes in the proximal tubules reduced sodium and potassium reabsorption, and consequently increased the urinary excretion of these electrolytes. In his study Matsuda (14), showed that the electrolyte composition of the renal tubular cells in the gentamicin nephrotoxicity was different in relation to the necrotic and non-necrotic tubular cells of the proximal tubules. It was showed that the histological impairment was present only in the proximal tubules with sodium and potassium concentration in the necrotic tubular cells which was lower than in control, whereas the concentrations of sodium in the non-necrotic cells were somewhat higher than those observed in proximal tubules of control group. This showed that the sodium and potassium serum levels correlate with the histopathological findings in the cells of proximal tubules where gentamicin expressed its main nephrotoxic effect, which coincides with our findings. The presence of neutrophils in the glomerular capillaries confirms that the renal microcirculation and glomerular hemodynamics were impaired by the administration of gentamicin. If the changes in the kidneys and glomeruli are primarily due to changed microcirculation and hemodynamics in the capillaries, the removal of those would abolish gentamicin effects in renal nephrotoxicity. As a consequence of gentamicin toxic effect, there comes to the impairment and decay of different cell organelles (lysosomes, mitochondria). The impairment of organelles leads to necrosis and desquamation of the cells. Our results showed that the most severe necrosis and desquamation was registered in the proximal tubules of animals treated with gentamicin only. Gentamicin increases the entry of Ca^{++} in the mesangial cells and the intracellular concentration of cytosol Ca^{++}. The increased concentrations of Ca^{++} cause the contraction of the mesangial cells along with consequent glomerular contraction. The contraction of glomeruli and the mesangial cells leads to the reduction in ultrafiltration coefficient, and the reduction in glomerular filtration. This would be a logical explanation of the given hemodynamic impairments as a main characteristic of toxic renal failure. The main components of disturbed renal hemodynamics are the following: reduction in renal blood flow in accordance with the increase in renal vascular resistance, reduction in ultrafiltration surface and ultrafiltration coefficient, tubular necrosis with consequent tubular obstruction, and impairment of water and electrolytes reabsorption (15). Further, our experiments showed a minor impairment of glomeruli and tubules in animals treated with both gentamicin and verapamil (3mg/kg b.w./24h). Glomeruli were insignificantly enlarged, and the basement membrane of glomerular capillaries was thickened only in some segments of glomeruli. There were no signs of necrosis in the proximal tubules, but only vacuolization and the presence of dark inclusions were noted in the cytoplasm of some cells. The results of morphometric investigations of glomeruli of GV-group did not show statistically significant difference in relation to the control group of rats. In relation to the G-group the differences were found in the size of glomeruli, glomerular optical density and roundness as well ($p<0,05$). The values of the area were lower in relation to the G-group pointing out to certain protective properties of verapamil in gentamicin induced ARI. The values of serum creatinine and urea in the GV-group of rats were enhanced in relation to the control group, but reduced in relation to G-group. The sodium and potassium serum concentrations were enhanced in relation to the group treated with gentamicin alone, but significantly reduced ($p<0,001$) in relation to the control group. With its vasodilatory effects, verapamil increased the kidney blood flow. The increase in blood flow (microcirculation correction) also corrected the morphological changes and reduced the urea and creatinine retention. The experiments showed that verapamil did not fully abolish the nephrotoxic effect of gentamicin. Further, this suggests that the change in the microcirculation was not the only reason of the impairment of the glomeruli and capillaries. On the other hand, a better hemodynamics at the level of glomerular capillaries (re-

duced contractions of the mesangial cells) also means a higher exposure of the glomeruli and their structures to gentamicin. Partly, it could lead to a certain vicious circle at the level of the kidneys. The protective role of verapamil was proved in some experimental models of ARI. This effect can be associated with the blockade of Ca^{++} influx in the impaired cells or with vasodilatory effects (6). The protective function of verapamil in gentamicin nephrotoxicity can also be emphasized by proving the existence of competitive relationship between verapamil and gentamicin to the common transport system of the cell membrane, monitoring their effects on the transport system in brush border membrane vesicles. The results of this investigation demonstrated that both gentamicin and verapamil are substrates for the transport system of renal organic cations. The authors conclude that a high similarity of substrates should have a protective function in the gentamicin-induced nephrotoxicity (7). In the conditions of gentamicin-induced impairment i.e. the cell apoptosis and necrosis, the increased concentration of intracellular Ca^{++} activates phospholipases, nucleases and proteases which lead to the impairment of the plasma membrane and further entry of Ca^{++} ions leading to irreversible impairment of the cells. In these conditions, verapamil as a Ca^{++} channel blocker ceases further entry of Ca^{++} from the extracellular space into the cell by blocking the entry of Ca^{++} through the slow channels which are opened by activating the corresponding receptors "receptor calcium channels". It is considered that verapamil deforms the slow channels and interferes with Ca^{++} liberation from the sarcoplasmatic reticulum. In this way, verapamil interrupts the mechanisms of further impairment of the cell in which the main part plays the redistribution of intracellular Ca^{++}. The intrarenal effects of Ca^{++} blockers verapamil on Ca^{++} influx are expressed by the reduction in cellular and functional changes and impairments. Structural alterations in the proximal tubules are significantly reduced after the treatment with verapamil and range within normalization, absence of necrosis, but also visibly damaged cells in the form of vacuolization and reduction in the brush border. The vascular effects of verapamil on the epithelium are of protective character considering that verapamil slows down the increase of intracellular Ca^{++} in the renal microcirculation epithelium thus improving the endothelial dysfunction (16). The recovery of renal perfusion is evident in the reduction of the overall renal vascular resistance resulting from the vasodilatory effects of verapamil on glomerular structures that are afferent arterioles and contractile elements of the mesangial cells (17).

Conclusion

The results of our investigation showed the unquestionable nephrotoxic effect of gentamicin with pronounced changes in glomeruli and proximal tubules. The concomitant administration of verapamil with gentamicin led to the reduction in the morphological and functional changes. Our experimental study showed that administration of verapamil ameliorates gentamicin-induced acute renal insufficiency.

List of Abbreviations

ARI	-	acute renal insufficiency
G-group	-	experimental group of animals treated with gentamicin
GV-group	-	experimental group of animals treated with gentamicin and verapamil
C-group	-	control group of animals

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