

Attenuation of gentamicin-induced nephrotoxicity: trimetazidine versus *N*-acetyl cysteine[†]

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ABSTRACT: Gentamicin (G) is a highly nephrotoxic aminoglycoside. It was used to experimentally induce nephrotoxicity in male Wistar rats. To find a drug capable of protecting the nephron we assayed a cardioprotector (trimetazidine, TMZ) and a hepatoprotector (*N*-acetyl cysteine, NAC). The rats were divided into six groups ($n = 8$): (A) control without drugs; (B) treated with 50 mg kg⁻¹ per day (i.p.) of G for 7 days; (C) diet supplemented with 20 mg kg⁻¹ per day of TMZ for 7 days; (D) treated with 10 mg kg⁻¹ per day (i.p.) of NAC for 7 days; (E) pretreated for 7 days with 20 mg kg⁻¹ per day of TMZ and during the following 7 days with G + TMZ; (F) pretreated for 7 days with 10 mg kg⁻¹ per day (i.p.) of NAC and during the following 7 days with G + NAC. Urea and creatinine as well as the excretion of urinary γ -glutamyl transpeptidase (GGT_u) and urinary *N*-acetylglucosaminidase (NAG_u) were determined and structural and ultrastructural studies were carried out. Group B was used as a G-induced nephrotoxicity control. Pretreatment with TMZ (E) showed a protector effect against induced nephrotoxicity, with no biochemical or functional changes nor alterations in histoarchitecture or ultrastructure. Pretreatment with NAC (F) showed no protector effect against G-induced nephrotoxicity since no statistically significant differences were found with respect to the control group with G. We conclude that G-induced nephrotoxicity is attenuated by the cytoprotective effect of TMZ. We may infer that TMZ inhibits the reabsorption and consequently the accumulation of G in the proximal tubule cell. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: nephrotoxicity; *N*-acetyl cysteine; trimetazidine; gentamicin; aminoglycoside

INTRODUCTION

The role of the kidney in relation to plasma clearance and maintenance of metabolic homeostasis makes it vulnerable to the toxic effects of drugs. Concentrations of medications and their metabolites found in glomerular, tubular and interstitial renal cells may induce changes in renal function and structure. Renal toxicity can be the result of hemodynamic changes, direct cellular or tissular injury, inflammatory tissular injury and/or obstruction of renal excretion (Choudhury and Ahmed, 2006).

Renal dysfunction and injury secondary to medications is frequent and can appear as injury and/or renal failure. Certain drugs disturb renal perfusion and induce loss of the filtering capacity. Others directly cause vascular, tubular and glomerular injury and interstitial cell injury, which result in loss of the renal function with clinical findings that include microangiopathy, Fanconi syndrome, acute tubular necrosis, acute interstitial nephritis, nephrotic syndrome, obstruction, nephrogenic diabetes insipidus, electrolytic abnormalities and chronic renal failure. The understanding of the mechanisms involved and the finding of early biomarkers help in finding strategies to prevent or minimize renal injury (Kleinknecht *et al.*, 1987). The incidence of drug-induced nephrotoxicity is difficult to determine. However, aminoglycoside-induced nephrotoxicity is above 36%.

Gentamicin (G) is an aminoglycoside frequently used in the treatment of infections caused by Gram-negative bacteria (Appel and Neu, 1987; Sastrasinh *et al.*, 1982). The renal excretion of G exposes to high concentrations of the drug a large area of the

surface of the glomerular capillary endothelium and tubular epithelium, the consequence of this being nephrotoxicity.

Previous studies (Paller *et al.*, 1984) have demonstrated that the nephrotoxic effect of G can be mediated by reactive oxygen species (ROS). Other authors have suggested that hydroxyl and superoxide anions are mediators of ischemic tissue injury and of the physiopathology of kidney disease (Baud and Ardaillou, 1986; Walker and Shah, 1988; Guidet and Shah, 1989a). In particular, hydrogen peroxide generation has been demonstrated *in vivo* in two models of acute renal failure (Guidet and Shah, 1989b; Demling *et al.*, 1986).

Our previous papers demonstrated the renal injury caused by G, shown in a significant decrease in glomerular filtration, decrease in creatinine clearance, coincident with the increase in malondialdehyde and decrease in antioxidant enzymes such as glutathione peroxidase and glutathione reductase. This would cause structural changes in the renal cell that would culminate in renal tubular necrosis (De la Cruz Rodríguez *et al.*, 1997).

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[†]This article was published online on 08 January 2010. An error was subsequently identified in the title and running header. This notice is included in the online and print versions to indicate that both have been corrected on 12 May 2010.

In this work we tested two drugs in order to prevent G-induced nephrotoxicity: trimetazidine and *N*-acetyl cysteine. Trimetazidine (TMZ), 1–2,3,4 tri-metoxibencil piperazine dihydrochloride, is a drug used for its cardioprotector effects since it prevents cell death secondary to transient myocardial ischemia that occurs during reperfusion – ‘reperfusion injury’ (Schafer *et al.*, 2001; Piper *et al.*, 1998; Di Lisa *et al.*, 2001; Pornin *et al.*, 1990).

TMZ is a cytoprotector whose place of action, mechanism and chronological order of effect are not yet known in depth. Among the mechanisms described we can mention hemodynamic changes (Maridonneau-Parini and Harpey, 1985), reduction in the toxicity of oxygen-derived free radicals (Williams *et al.*, 1993), decrease in the inflammatory reaction (Guarnieri *et al.*, 1997), optimization of the energetic metabolism and decrease in the utilization of fatty acids in favor of carbohydrates (Marzilli, 2003).

Numerous researchers have reported the protector effect of TMZ on ROS-induced renal failures during ischemia/reperfusion in rats. Some of them attributed the renoprotective effect of TMZ to its antioxidant and oxygen free radical scavenger activity (Kaur *et al.*, 2003).

N-acetyl cysteine (NAC) is a reduced thiol. A precursor of L-cysteine, it is one of the amino acids necessary for glutathione synthesis. Some researchers have attributed to it a hepatoprotective effect and others have mentioned it as a crucial factor in renal tissue protection against contrast medium-induced nephropathy. Contrast media are vasoconstrictor drugs and the generation of extracellular ROS could mediate the action of vasoconstrictors such as angiotensin II, thromboxane A, endothelin, adenosine and norepinephrine. Several experimental models have demonstrated that ROS are responsible for glomerular injury. In some protocols, NAC was added to the contrast media in order to improve kidney function and prevent nephropathy in patients with mild to moderate renal insufficiency (Lauterburg *et al.*, 1983; Brigouri and Marenzi, 2006; Marenzi *et al.*, 2006; Persson *et al.*, 1960).

The aim of this work is to find a drug or medicament capable of exerting a protector effect against G-induced nephrotoxicity. For that purpose, we designed schemes in assay animals treated with G and with G + TMZ and G + NAC, under different conditions in order to observe the biochemical, structural and ultrastructural changes that took place at the kidney level.

MATERIAL AND METHODS

Animals and Drugs

The experiments were carried out in 10-week-old male Wistar rats weighing 180–200 g housed in metabolic cages with a 12 h light–dark cycle (light on at 7:00 a.m.) at 20°C and 60% humidity. The animals were fed a standard rodent diet and drinking water.

All experimental procedures complied with the regulations of the European Union (86/60/EEC) and the recommendation of the Federación de Sociedades Sudamericanas de la Ciencia de Animales de Laboratorio- FESSCAL (Federation of South American Societies of Laboratory Animal Science).

Animals were given the following drugs: G (Bagó, Glebomicina 400 mg); trimetazidine (Servier, Vastarel 20 mg); and *N*-acetylcysteine (Farmasierra-*N*-Acetilcisteína, 200 mg).

Experimental Design

The animals were divided into six groups ($n = 8$). The groups were treated as follows: group A (control) – animals fed with a standard rat/mouse diet for 7 days; group B (G control) – animals fed with

a standard diet, with G subcutaneously administered at a dose of 50 mg kg⁻¹ per day (i.p.) for 7 days; group C (TMZ control) – animals fed with a standard diet supplemented with 20 mg kg⁻¹ per day of TMZ, drug powder added to the feed for 7 days; group D (NAC control) – animals given a standard diet and treated with 10 mg kg⁻¹ per day of NAC i.p. for 7 days; group E (TMZ + G) – animals given a standard diet supplemented with 20 mg kg⁻¹ per day of TMZ for 7 days prior to the assay and during the 7 days of the assay with 20 mg kg⁻¹ per day of TMZ and G at a dose of 50 mg kg⁻¹ per day; and group F (NAC + G) – animals given a standard diet and treated with 10 mg kg⁻¹ per day of NAC i.p. for 7 days prior to the assay and during the 7 days of the assay with 10 mg kg⁻¹ per day of NAC i.p. and G at a dose of 50 mg kg⁻¹ per day.

Throughout the assay, the animals were monitored with daily evaluations of appearance, behavior, food intake and activity. Body weight was determined at the end of the experiment (data not shown).

Blood and Urine Samples

At the beginning and end of the different treatments, blood samples were collected by tail vein puncture and by intracardiac puncture respectively, with no anticoagulant for the biochemical studies.

In groups E and F, during the last 7 days of the assay, blood samples were extracted daily by tail vein puncture for urea and serum creatinine determination and urine samples were collected for the determination of diuresis and of urinary excretion of γ -glutamyl transpeptidase (GGT_u) and urinary *N*-acetylglucosaminidase (NAG_u), as biomarkers of tubular damage.

Methods

Nitrogen compounds in blood, urea and creatinine were determined using the urease method and Jaffe's colorimetric method, respectively, supplied by Wiener Laboratories (Fawcett and Scott, 1960; Biggs and Cooper, 1961). Urea concentration in blood was expressed in g l⁻¹ and creatinine concentration in mg l⁻¹.

Diuresis was determined and expressed as milliliters per 24 h. Urine samples were used to determine GGT_u activity with the modified Szasz kinetic method, supplied by Wiener Laboratories (Szasz, 1969), and for the determination of NAG_u activity the spectrophotometric method of Horak *et al.* (1981) was used.

Histological Study

On day 7 for groups A–D and day 14 for groups E and F, the animals were decapitated without previous sedation. Then they were bled and their kidneys were removed and prepared for structural and ultrastructural studies. Small portions of each kidney previously separated, washed with physiological solution and fixed with a formaldehyde solution 10% were embedded in paraffin. They were cut into 2–3 μ m sections using a slide microtome and treated with hematoxylin–eosin staining (Martoja and Martoja-Pierson, 1970; Spannhof, 1966). These histological slices were observed using an Axiostar plus Zeiss optical microscope.

The tissue slices fixed by immersion for 60 min at 4°C in a solution containing 1.5% glutaraldehyde and 1% formaldehyde in buffer pH 7.40 were treated for observation through an electron microscope. After successive washings followed by dehydration

and embedding, the slices were placed in resin capsules. Then they were cut again into adequate sections for ultrastructural studies. The Zeiss EM 109 transmission electron microscope of the Laboratorio de Microscopía Electrónica del Noroeste Argentino, LAMENOA (Electron Microscopy Laboratory of Northwestern Argentina) was used.

Statistical Analysis

We conducted a battery of statistical procedures to test for the equality of means across different groups (for diuresis, urea and creatinine). We performed the ANOVA *F*-test for the equality of means and rejected (at a significance level of 5%) the null hypothesis that the means of the different groups are the same. After this, we conducted Fisher's least significant difference (LSD) test to test the equality of means in all the possible group pairings. Further details can be found in the Statistical Appendix.

RESULTS

Figure 1 shows diuresis expressed in milliliters per 24 h in the treated animal compared with the control group. TMZ did not alter diuresis (A vs C); G caused an 80% decrease in diuresis (A vs B), which was interpreted as a result of the G-induced nephrotoxicity; NAC did not alter diuresis (A vs D). The animals in group E, pretreated with TMZ and during the last 7 days with TMZ + G, showed no significant changes in diuresis with respect to the control (A vs E). The animals in group F, pretreated with NAC and with NAC + G for the last 7 days, showed a marked oliguria (A vs F).

Figures 2 and 3 show a statistically significant increase in urea and serum creatinine in the group treated with G with respect to the control group (A vs B). These biochemical findings show the experimental nephrotoxicity induced by the treatment with G at a dose of 50 mg kg⁻¹ per day.

Group E, treated previously with TMZ for 7 days to which G was added during the last 7 days, showed the protector effect of TMZ on the renal function. The nitrogen compounds urea and creatinine in group E were within the ranges of groups A and C. However, the therapeutic scheme applied to group F reflected a behavior similar to group B. NAC in these conditions did not exert a renoprotective effect.

Figure 4 shows the effect of pretreatment with TMZ and of pretreatment with NAC on the behavior of the nitrogen compounds urea and creatinine on the animals treated during the last 7 days with G. It can be seen that glomerular filtration is conserved with the previous treatment with TMZ 20 mg kg⁻¹ per day and that pretreatment with NAC did not modify G-induced nephrotoxicity.

We studied the urinary excretion of two enzymes: γ glutamyl-transpeptidase (GGT_u) and NAG_u in order to assess the effect of the different treatments at the level of the tubular function.

It was interesting to compare the urinary excretion of GGT_u and NAG_u with serum creatinine in the treated groups E and F. Figures 5 and 6 show the correlation between serum creatinine and the excretion of enzymes: GGT_u and NAG_u.

In this way, the protector effect of TMZ on the renal function of the animals treated previously with TMZ and for the last 7 days with TMZ + G has been demonstrated. No significant changes were found in group F (pretreated with NAC and for the last 7 days with NAC + G) with respect to B.

In order to relate the biochemical changes observed to the histoarchitecture of the renal parenchyma, we studied histological slices of rat kidney from the different groups, which were observed through a light microscope with hematoxylin–eosine staining.

Figure 7 shows the renal cortex with its corpuscles and Bowman's space conserved in the animals in the control group (A). The tubules, most of them proximal, show their characteristic shape and arrangement. Uniform interstitial tissue can be observed.

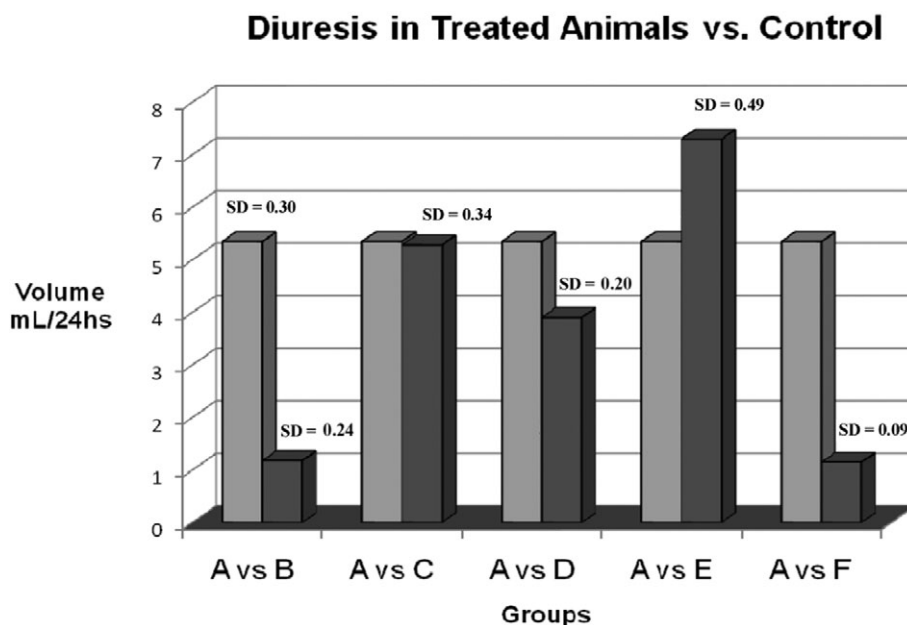


Figure 1. Diuresis in the treated animals compared with the controls. G caused an 80% decrease in diuresis (A vs B). TMZ did not alter diuresis (A vs C). The animals pretreated with TMZ and TMZ + G showed no significant changes (A vs E). (Standard deviations on top of columns.)

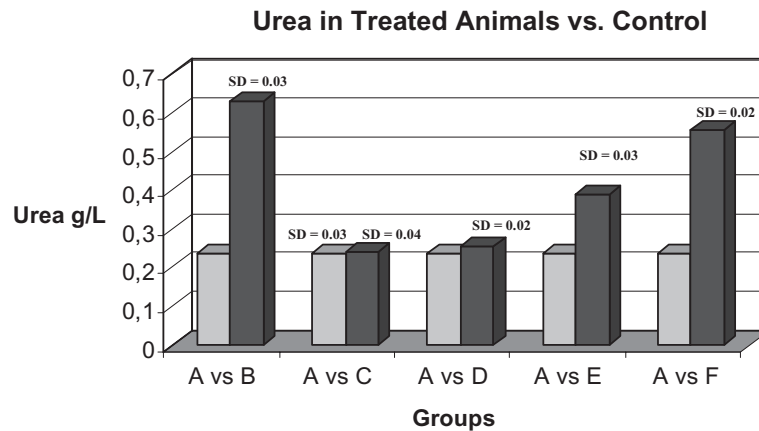


Figure 2. Serum urea in treated animals compared with the controls. The rats treated with G show increase in urea respect to the control group (A vs B). The animals pretreated with TMZ and TMZ + G showed the protector effect of TMZ on the renal function. (A vs E). NAC did not exert a renoprotective effect (A vs F). (Standard deviations on top of columns.)

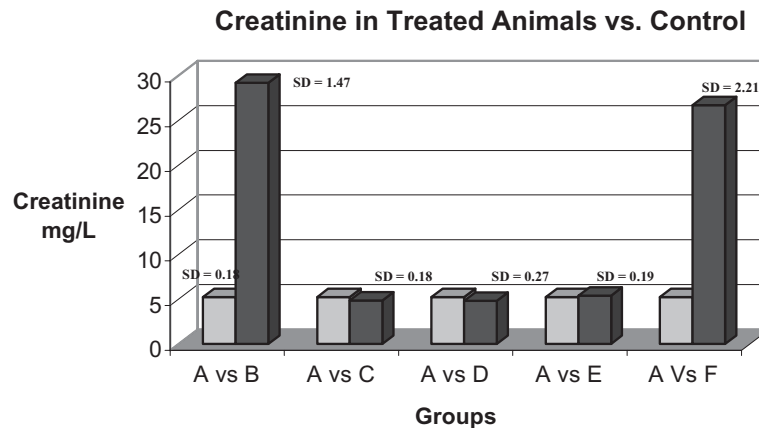


Figure 3. Serum creatinine in the treated animals compared with the controls. The rats treated with G show increase in creatinine with respect to the control group (A vs B). The animals pretreated with TMZ and TMZ + G show the protector effect of TMZ on the renal function (A vs E). NAC did not exert a renoprotective effect (A vs F). (Standard deviations on top of columns.)

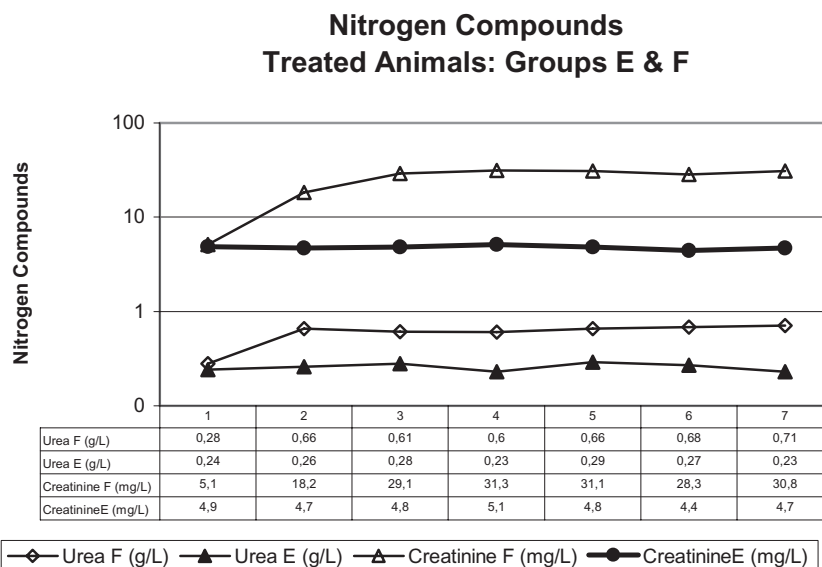


Figure 4. Nitrogen compounds in the treated groups E and F. Values found during the last 7 days of the experiment. The glomerular filtration was conserved with the previous treatment with TMZ 20 mg kg⁻¹ per day. Pretreatment with NAC did not modify G-induced nephrotoxicity.

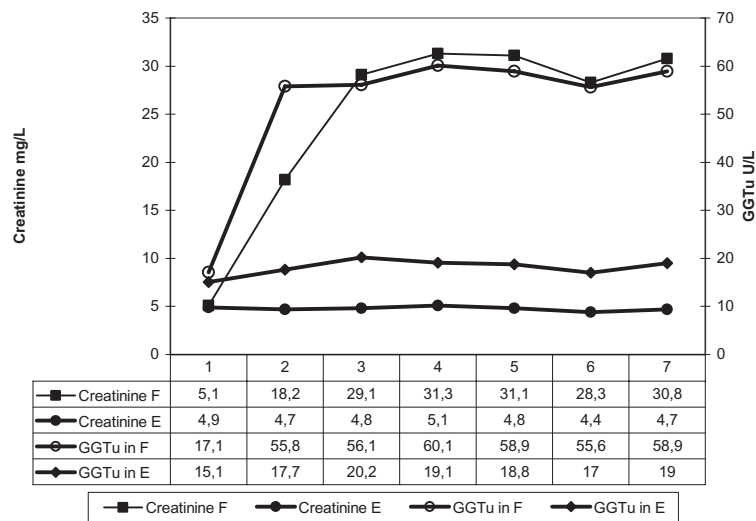
Urinary Gamma Glutamyl Transpeptidase vs Creatinine
Treated Animals in Groups E & F

Figure 5. Relationship between serum creatinine and GGT_u in treated animals. Values found in groups E and F. The figure shows the correlation between serum creatinine and the excretion of GGT_u .

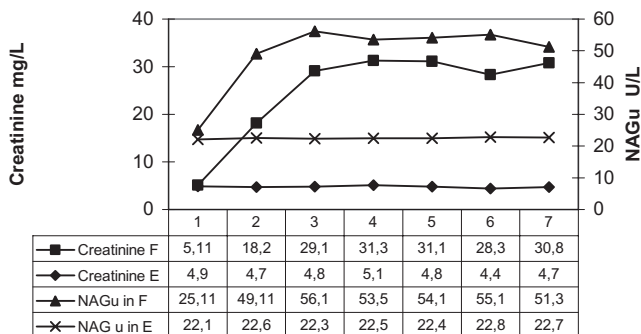
Urinary N-Acetyl Glucosaminidase vs Creatinine
Treated animals in Groups E & F

Figure 6. Relationship between serum creatinine and NAG_u in treated animals. Values found in groups E and F. The figure shows the correlation between serum creatinine and the excretion of NAG_u .

In order to determine the experimental induction of nephrotoxicity with a dose of 50 mg kg^{-1} per day of G, histological slices from the kidney of rats in group B were analyzed. In the center of Figure 8 we can see a glomerulus with conserved structure. Changes can be seen at the level of the proximal convoluted tubules: the tubular epithelium shows vacuolization and edematization of the cytoplasm. Cytoplasmic vacuolization would correspond to the edematization of the mitochondria and other organelles. The nuclei are displaced and in some cases are found in the tubular lumen, which suggests that they have undergone hydropic degeneration. We can also see desquamated cells toward the tubular lumen and loss of the brush border of the apical pole of the renal tubular cell.

Figure 9 shows the effects of TMZ in previous treatment for 7 days, followed by 7 days of simultaneous treatment with TMZ + G (group E), where the preserved renal histoarchitecture can be observed, similarly to the control group (A). On the upper right

hand margin of the microphotograph, we can see the renal capsule membrane, showing the integrity of the renal parenchyma.

Figure 10 shows the effects of NAC in previous treatment for 7 days, followed by 7 days of simultaneous treatment with NAC + G (group F). We can see the tubular epithelium with modifications similar to the ones described in group B treated only with G at a dose of 50 m kg^{-1} per day. We can see the conserved renal glomeruli. The structural findings described above and the biochemical changes shown justify the study of the ultrastructure.

Figure 11 shows the normal ultrastructure of the renal tubular epithelium of male Wistar rats (group A). We can see two cells of the tubular epithelium with their nuclei located in the basal pole. In this pole we can see the mitochondria with an oval shape arranged perpendicularly to the basal membrane.

Figure 12 shows the effect of the previous treatment for 7 days with doses of 20 mg kg^{-1} per day with TMZ and for the last 7 days with TMZ + G (group E). We can see the epithelium of the proximal convoluted tubule with its apical pole showing the brush border conserved in its structural characteristics.

Figure 13 shows two cells belonging to the renal tubular epithelium of rats treated with G and NAC (group F). In one of them, the one on the right, we can see clearly the presence of the nucleus. We can see mitochondria with altered shapes, sizes and arrangement in relation to the control group. In the basal pole of the cells we can see the mitochondria uniformly arranged perpendicularly to the basal membrane of the epithelium.

DISCUSSION AND CONCLUSIONS

Our results in animals treated with G showed alterations in the histoarchitecture such as necrosis and changes in the renal tubular cells, which agree with those obtained by other authors (Cuppige *et al.*, 1977; Mouedden *et al.*, 2000). In humans, the first sign of renal injury after the administration of aminoglycosides is an increase in the urinary excretion of several tubular

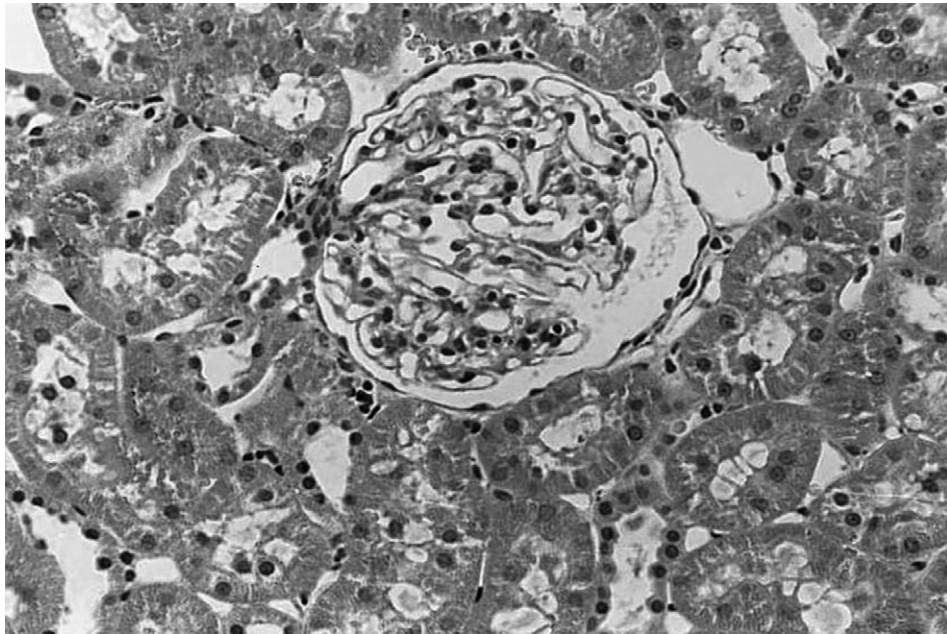


Figure 7. Renal cortex with its corpuscles and Bowman's space conserved in the animals in control Group (A). Histological slices with hematoxylin-eosin staining (20 \times).

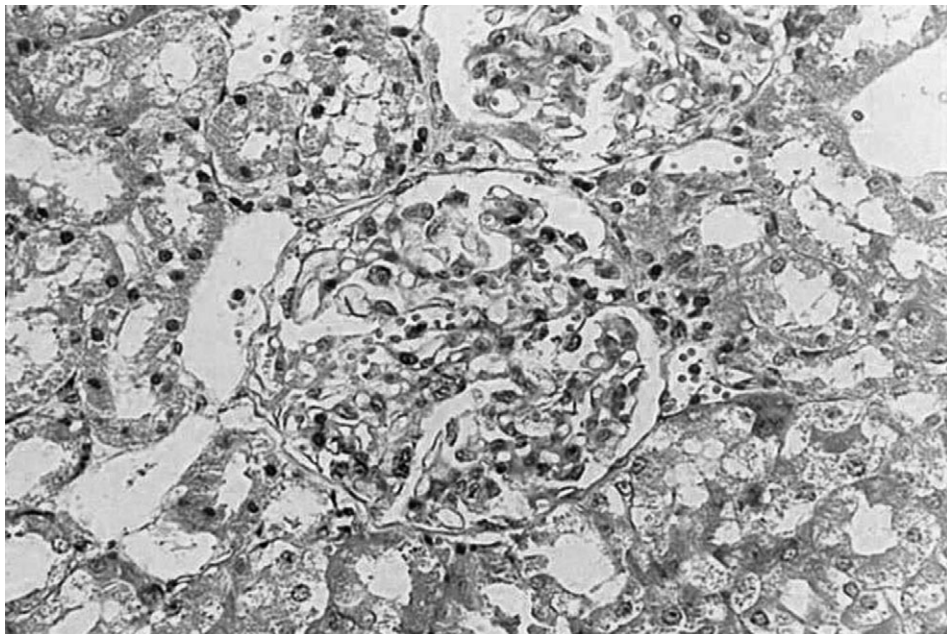


Figure 8. Renal cortex with hematoxylin-eosin staining (20 \times). Animals treated with 50 mg/Kg/day of Gentamicina. A central glomerulus with conserved structure and changes in the proximal convoluted tubules.

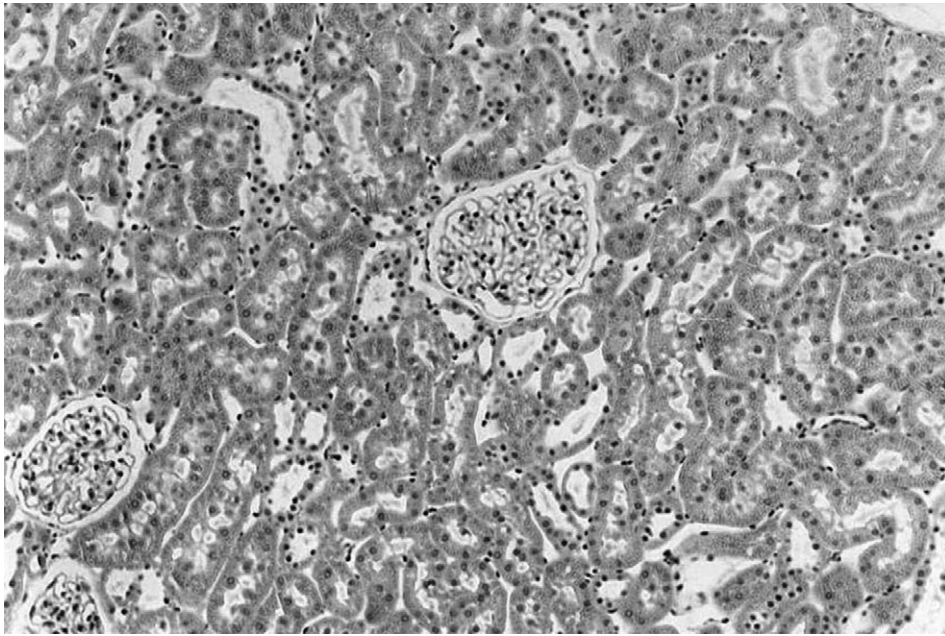


Figure 9. Renal cortex with hematoxylin-eosin staining (10 \times). Animals in Group E previously treated with 20 mg/Kg/day of TMZ and during the last 7 days with TMZ+G. The preserved renal histoarchitecture can be observed.

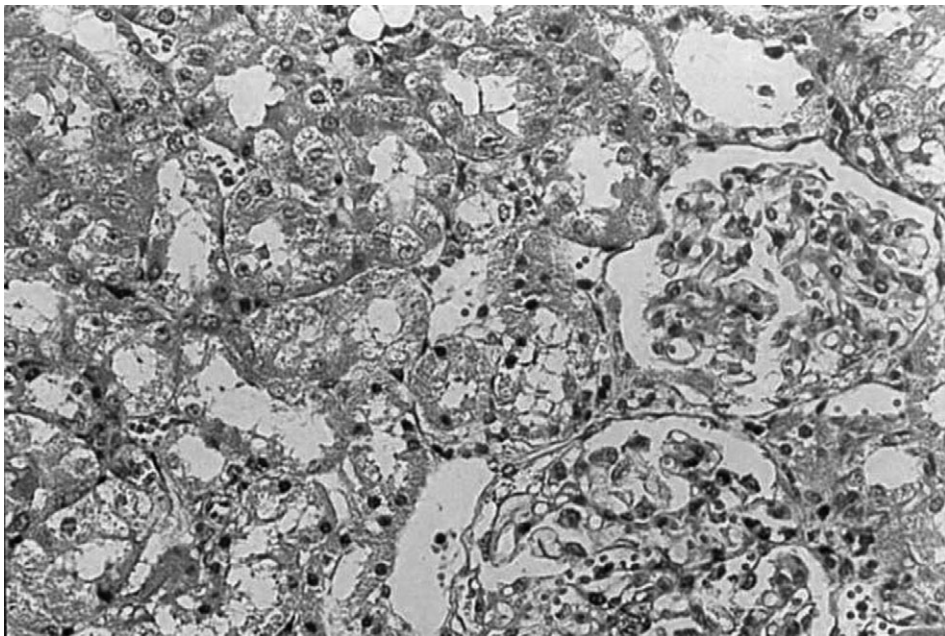


Figure 10. Renal cortex with hematoxylin-eosin staining (20 \times). Animals in Group F previously treated with 10 mg/Kg/day of NAC and during the last 7 days with NAC+G. The tubular epithelium with modifications similar to the ones described in Group B treated only with G at dose of 50 mg/Kg/day.

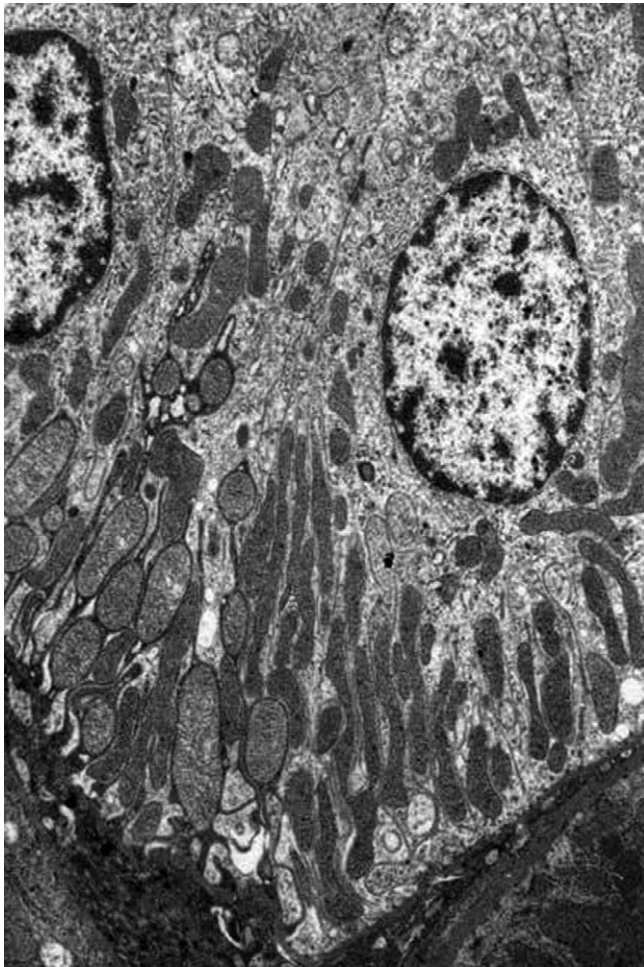


Figure 11. Epithelium of the proximal convoluted tubule of control animals (A). Two cells of the renal tubular epithelium with their nuclei located in the basal pole. The mitochondria are arranged perpendicularly to the basal membrane. Electron Microphotograph (4.960 \times).

enzymes – γ -glutamyltranspeptidase, alanine aminopeptidase, β -D-glucosaminidase and alkaline phosphatase – proteinuria and increase in the excretion of β -2 microglobulin. There are also alterations in the urinary sediment such as leukocyturia and cylindruria, and finally a decrease in glomerular filtration, with an increase in ureic nitrogen and plasma creatinine. Critical conditions such as dehydration and septicemia potentiate the nephrotoxic effect of the aminoglycoside and can result in permanent renal damage (Mouedden *et al.*, 2000; Tulkens, 1989).

The nephrotoxic effect of G was assayed in group B. The results, shown in Figs 2 and 3, increase in the nitrogen compounds urea and creatinine, are related to a decrease in glomerular filtration, with a diminution in diuresis. These results agree with previous works (Ahijado and Garcia, 2000; Appel and Neu, 1987).

In our work, we found that GGT_u is an early marker of proximal tubular cell injury in cyclosporin A-induced nephrotoxicity (De la Cruz Rodríguez *et al.*, 1996, 2007). That is why we considered enzymuria, GGT_u and NAG_u , as early and sensitive markers of nephrotoxicity.

One of the mechanisms proposed to explain aminoglycoside-induced nephrotoxicity indicates that these polycationic molecules are filtered in the glomerulus and reabsorbed at the level

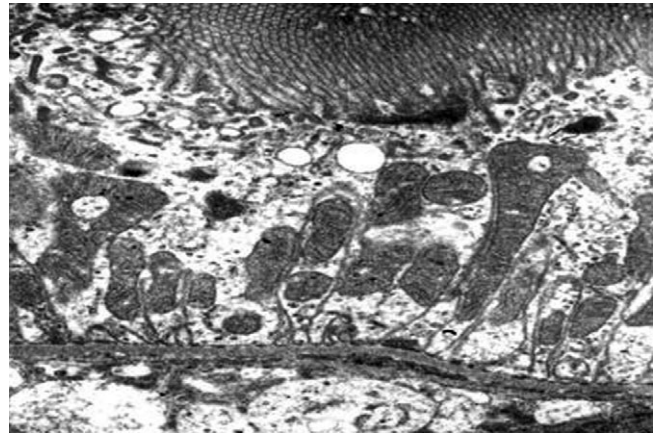


Figure 12. Epithelium of the proximal convoluted tubule of animals in Group E. Protector effect of pretreatment with TMZ and for the last 7 days with TMZ+G. The epithelium with its apical pole shows the brush border conserved in its structural characteristics. Electron Microphotograph (4.960 \times).

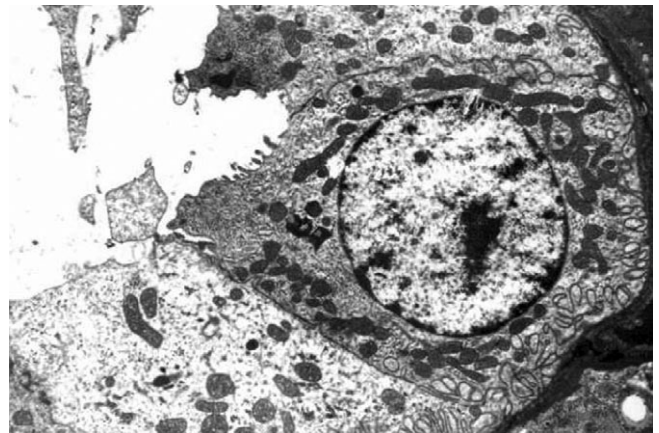


Figure 13. Epithelium of the proximal convoluted tubule of animals in Group F. Effect of pretreatment with NAC and for the last 7 days with NAC+G. Two cells belonging to the renal tubular epithelium: the one on the right shows the nucleus and the mitochondria with altered shapes, sizes and arrangement in relation to the control Group (A). Electron Microphotograph (4.960 \times).

of the proximal tubules, reaching in the tubular renal cell a concentration 5–50 times higher than plasma (Sastrasinh *et al.*, 1982; Kanuss *et al.*, 1983; Moestrup *et al.*, 1995). This reabsorption implies the union of the aminoglycosides to negatively charged phospholipids located on the brush border of the membranes of the renal tubular cells, with later internalization by pinocytosis. No conclusive evidence exists of tubular secretion of these agents; quantitatively, most aminoglycosides excreted in the urine correspond to filtrates (Ahijado and Garcia, 2000).

Vaamonde *et al.* (1996) demonstrated the absence of a glycoprotein, megaline, on the brush border of the proximal tubule in a rat model with streptozotocin induced diabetes mellitus concomitantly with a decrease in the intracellular transport of gentamicin and absence of renal damage secondary to G. An improvement in the diabetic condition with insulin in this model resulted in megaline expression, G accumulation in the renal cortex and reappearance of aminoglycoside-induced nephrotoxicity (Vaamonde *et al.*, 1997).

Previous studies (Baud and Ardaillou, 1986; Walker and Shah, 1988) have shown that the nephrotoxic effect of G can be mediated by ROS. Recent studies suggest that free radicals such as the superoxide and hydroxyl anions are mediators of ischemic tissue injury and of the physiopathology of renal diseases (Guidet and Shah, 1989a, b). In particular, the generation of hydrogen peroxide has been demonstrated *in vivo* in two models of acute renal failure.

On the basis of the above, we used G as a model of nephrotoxicity experimental induction. For the purpose of finding a drug that would exert a protective effect against G-induced nephrotoxicity, we assayed two drugs: TMZ and NAC.

The animals in group E inoculated with G from day 8 of the previous treatment with TMZ showed conserved renal function with values within the normal ranges for urea and creatinine. We also found normal excretion of GGT_u and NAG_u . The protector effect of TMZ was studied in its micro- and ultrastructure. In Fig. 9, using a light microscope, the preserved histoarchitecture can be seen, as well as the integrity of the capsule membrane and of the renal parenchyma. In Fig. 12, using an electron microscope, we can see the epithelium of the proximal tubule with its brush border conserved in its structural characteristics and also the mitochondria uniformly arranged perpendicularly to the basal membrane.

However, the animals in group F pretreated with NAC and simultaneously inoculated with G + NAC did not reflect differences with respect to group B. We conclude that NAC does not exert a protector effect against G-induced nephrotoxicity.

In Fig. 13, using an electron microscope, two cells belonging to the renal tubular epithelium of rats treated with G and NAC (group F) with alterations in shape, size and arrangement of mitochondrias can be observed.

With these results we conclude that previous treatment with TMZ exerts a protector effect on G-induced nephrotoxicity. Among the different mechanisms proposed to explain the cytoprotective effect of TMZ, we think that this drug would act at the level of the brush border, preventing reabsorption and accumulation of G in the renal tubular cell. This could be due to the inhibition of the membrane receptor, megaline, reported by Vaamonde *et al.* (1996, 1997) in a diabetic rats model with absence of expression of this glycoprotein and inhibition of the nephrotoxic effect of G.

On the other hand, Breton and Brown (1998) found that the cold preservation of renal tissues for a transplant affected the cytoarchitecture and function of the renal tubular cells, showing alteration in membrane proteins such as megaline, a glycoprotein located in the proximal convoluted tubules.

Other authors (Onbasile *et al.*, 2007) used TMZ to prevent renal injury caused by cold ischemia-reperfusion in an autotransplant model of pig kidney.

Unlike numerous authors (Breton and Brown, 1998; Hauet *et al.*, 2000; Onbasile *et al.*, 2007; Walker and Shah, 1988; Guidet and Shah, 1989a), we consider that TMZ would not play the role of scavenger, since in previous assays in animals treated with G + TMZ for 7 days we were unable to show its cytoprotector effect. Our results suggest a new mechanism of action of TMZ, which would require further studies.

Acknowledgments

We wish to thank the following institutions: Secretaría de Ciencia y Técnica (Science and Technique Department) of the Universidad

Nacional de Tucumán for grant number CIUNT 26/D 422 and Wiener Laboratory for providing the necessary reagents. We also want to acknowledge outstanding statistical and editorial support from Cesar Sosa Padilla, Ph.D.(c).

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STATISTICAL APPENDIX

In this Appendix we report some summary statistics as well as the results on the mean-comparison test (for diuresis, urea and creatinine) that we performed on the six groups. The summary statistics for groups A–F ($n = 8$) are:

Groups	Diuresis		Urea		Creatinine	
	Mean	SD	Mean	SD	Mean	SD
A	5.35	0.30	0.23	0.03	5.26	0.18
B	1.18	0.24	0.63	0.04	29.19	1.47
C	5.28	0.34	0.24	0.03	4.89	0.18
D	3.91	0.20	0.24	0.02	4.82	0.27
E	7.30	0.49	0.33	0.03	5.14	0.19
F	1.15	0.09	0.52	0.02	23.72	2.21

Testing for equality of six group means – diuresis

Assuming homogeneity (i.e. holding the assumption that the covariance matrices are the same across the groups):

	Statistic	F (d.f.1, d.f.2)	F	Prob > F
Wilks's λ	0.02	5 42	1176.16	0.00
Pillai's trace	0.98	5 42	1176.16	0.00
Lawley–Hotelling trace	58.81	5 42	1176.16	0.00
Roy's largest root	58.81	5 42	1176.16	0.00

If we remove the homogeneity assumption and test for the equality of means, we obtain:

Wald's χ^2 (5)	9042.40
Prob > χ^2	0.00 (chi-squared approximation)
Prob > χ^2	0.00 (James's approximation)

We can see that, either allowing for homogeneity or not, we can reject the hypothesis of equality of means (i.e. $H_0: \mu_{\text{Diuresis}_A} = \mu_{\text{Diuresis}_B} = \mu_{\text{Diuresis}_C} = \mu_{\text{Diuresis}_D} = \mu_{\text{Diuresis}_E} = \mu_{\text{Diuresis}_F}$ is rejected).

Given this rejection of the null hypothesis, we proceed to implement the Fisher's LSD method for comparing the means of the different groups. The table below presents both the LSD value (lower triangular partition) and the $|\mu_{\text{group}_i} - \mu_{\text{group}_j}|$ (upper triangular partition).

	A	B	C	D	E	F
A	█					
B	0.30	█				
C	0.30	0.30	█			
D	0.30	0.30	0.30	█		
E	0.30	0.30	0.30	0.30	█	
F	0.30	0.30	0.30	0.30	0.30	█

If $|\mu_{\text{group}_i} - \mu_{\text{group}_j}| \geq \text{LSD}_{\text{group}_i, \text{group}_j}$ then we reject the null hypothesis $H_0: \mu_{\text{group}_i} - \mu_{\text{group}_j}$. Hence, we are able to reject all of the pairwise mean comparisons except for $H_0: \mu_B = \mu_F$ and $H_0: \mu_A = \mu_C$.

Testing for equality of six group means – urea

Assuming homogeneity (i.e. holding the assumption that the covariance matrices are the same across the groups):

	Statistic	F (df1, df2)		F	Prob > F
Wilks's λ	0.03	5	42	577.76	0.00
Pillai's trace	0.97	5	42	577.76	0.00
Lawley–Hotelling trace	28.32	5	42	577.76	0.00
Roy's largest root	28.32	5	42	577.76	0.00

If we remove the homogeneity assumption and test for the equality of means, we obtain:

Wald χ^2 (5)	3180.24
Prob > χ^2	0.00 (chi-squared approximation)
Prob > χ^2	0.00 (James's approximation)

We can see that, either allowing for homogeneity or not, we can reject the hypothesis of equality of means (i.e. $H_0: \mu^{Urea}_A = \mu^{Urea}_B = \mu^{Urea}_C = \mu^{Urea}_D = \mu^{Urea}_E = \mu^{Urea}_F$ is rejected).

Given this rejection of the null hypothesis, we proceed to implement the Fisher's LSD method for comparing the means of the different groups.

The table below presents both the LSD value (lower triangular partition) and the $|\mu_{group,i} - \mu_{group,j}|$ (upper triangular partition).

	A	B	C	D	E	F
A	█	0.40	0.01	0.01	0.10	0.29
B	0.03	█	0.39	0.39	0.30	0.11
C	0.03	0.03	█	0.00	0.09	0.28
D	0.03	0.03	0.03	█	0.09	0.28
E	0.03	0.03	0.03	0.03	█	0.19
F	0.03	0.03	0.03	0.03	0.03	█

If $|\mu_{group,i} - \mu_{group,j}| \geq LSD_{group,i,group,j}$ then we reject the null hypothesis $H_0: \mu_{group,i} = \mu_{group,j}$. Hence, we are able to reject $H_0: \mu_A = \mu_B, H_0: \mu_A = \mu_E, H_0: \mu_A = \mu_F, H_0: \mu_B = \mu_C, H_0: \mu_B = \mu_D, H_0: \mu_B = \mu_E, H_0: \mu_B = \mu_F, H_0: \mu_C = \mu_E, H_0: \mu_C = \mu_F, H_0: \mu_D = \mu_E, H_0: \mu_D = \mu_F$ and $H_0: \mu_E = \mu_F$.

Clearly, we are not able to reject the equality of the remaining pairwise comparison of means.

TESTING FOR EQUALITY OF SIX GROUP MEANS – CREATININE

Assuming homogeneity (i.e. holding the assumption that the covariance matrices are the same across the groups):

	Statistic	F (df1, df2)		F	Prob > F
Wilks's λ	0.01	5	42	1902.79	0.00
Pillai's trace	0.99	5	42	1902.79	0.00
Lawley–Hotelling trace	93.37	5	42	1902.79	0.00
Roy's largest root	93.37	5	42	1902.79	0.00

If we remove the homogeneity assumption and test for the equality of means, we obtain:

Wald χ^2 (5)	6166.72
Prob > χ^2	0.00 (chi-squared approximation)
Prob > χ^2	0.00 (James' approximation)

We can see that either allowing for homogeneity or not, we can reject the hypothesis of equality of means (i.e. $H_0: \mu^{Creatinine}_A = \mu^{Creatinine}_B = \mu^{Creatinine}_C = \mu^{Creatinine}_D = \mu^{Creatinine}_E = \mu^{Creatinine}_F$ is rejected).

Given this rejection of the null hypothesis, we proceed to implement the Fisher's LSD method for comparing the means of the different groups. The table below presents both the LSD value (lower triangular partition) and the $|\mu_{group,i} - \mu_{group,j}|$ (upper triangular partition).

	A	B	C	D	E	F
A	█	23.93	0.37	0.44	0.12	18.46
B	1.08	█	24.30	24.37	24.04	5.47
C	1.08	1.08	█	0.07	0.26	18.83
D	1.08	1.08	1.08	█	0.33	18.90
E	1.08	1.08	1.08	1.08	█	18.57
F	1.08	1.08	1.08	1.08	1.08	█

If $|\mu_{group,i} - \mu_{group,j}| \geq LSD_{group,i,group,j}$ then we reject the null hypothesis $H_0: \mu_{group,i} = \mu_{group,j}$. Hence, we are able to reject $H_0: \mu_A = \mu_B, H_0: \mu_A = \mu_F, H_0: \mu_B = \mu_C, H_0: \mu_B = \mu_D, H_0: \mu_B = \mu_E, H_0: \mu_B = \mu_F, H_0: \mu_C = \mu_E, H_0: \mu_D = \mu_E$ and $H_0: \mu_E = \mu_F$.

Clearly, we are not able to reject the equality of the remaining pairwise comparison of means.