

Histological and Biochemical Assessment of the Protective Influence of Rutoside Against Gentamicin-Related Nephrotoxicity in a Murine Model

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Abstract: This is a pilot investigation on the potential protective influence of rutoside (RUT), a plant-derived bioflavonoid, against gentamicin (GEN)-induced nephrotoxicity in an animal model. Thirty healthy adult male Sprague-Dawley rats were randomized to six treatment groups (n=5 each): sham control (plain normal saline solution [PNSS]); GEN control; RUT 75 and RUT 150 controls (RUT 75 and 150 mg/kg, respectively); and experimental groups RUT 75 + GEN and RUT 150 + GEN. Oral pretreatment with RUT was given for 14 days via gastric gavage. From days 15 to 21, single daily doses of GEN (80 mg/kg/dose) were given intraperitoneally. There was no mortality during and after GEN treatment. Twenty-four hours following the last dose of GEN, intracardiac blood was extracted for blood urea nitrogen (BUN) and serum creatinine determination. The kidneys were then immediately excised for histopathological evaluation. Results showed significant attenuation of renal tissue damage and lower BUN and serum creatinine levels in the RUT-treated experimental groups when compared with the GEN control. RUT effect on the extent of renal tissue injury, but not on the biochemical markers, appeared to be dose-dependent. These findings suggest the potential protective effects of RUT against the development of GEN-related nephrotoxicity in this murine model.

Key Words: bioflavonoid; gentamicin; nephroprotective; nephrotoxicity; rutoside

1. INTRODUCTION

Kidney disease is the 9th leading cause of death among Filipinos. One Filipino develops chronic renal failure every hour or about 120 Filipinos per million population per year. The most common causes of nephropathy in the Philippines are diabetes (44.6%), hypertension (23%), and nephritis (19.3%). Drugs have been shown to cause around 20% of community and hospital-acquired episodes of actual renal failure. Among older adults, the incidence of drug-

induced nephrotoxicity may be as high as 66% in the United States (Naughton, 2015).

Gentamicin is one very efficacious antibacterial drug used in the treatment of many severe infections (Selby, 2009). The most common adverse effects of gentamicin are nephrotoxicity and ototoxicity (Bello, 2009). Unfortunately, around 30% of patients treated with gentamicin for more than seven days showed signs of nephrotoxicity in the form of direct tubular

necrosis mainly in the proximal tubule because of the generation of reactive oxygen species (ROS) (Mazen, 2013). Gentamicin-related nephrotoxicity involves renal free radical generation, reduction in antioxidant defense mechanisms, acute tubular necrosis and glomerular congestion, resulting in diminished glomerular filtration rate and renal dysfunction (Balakumar, 2010). This results in electrolyte and acid-base abnormalities and retention of nitrogenous waste products such as urea and creatinine (Naughton, 2015).

Rutoside, or quercetin-3-rhamnosyl glucoside, is a ubiquitous plant-derived flavonoid widely found in Asia. It is a flavinoglycoside comprised of quercetin and the disaccharide rutinose (raminose and glucose) found in many plants, fruits, and vegetables (Mazen, 2013; Yang, 2008). It is also called vitamin P, with excellent therapeutic properties demonstrated in various animal models (Umar, 2012). It is a powerful antioxidant with various pharmacologic benefits particularly its excellent scavenging activity (Di Carlo, 2002; Duthie, 1999). Yang (2011) has suggested a mechanism that operates on the remarkable potency of rutoside to donate electrons to reactive free radicals thereby converting these radicals into more stable species and quenching the free radical chain reaction. Clinically, rutoside is used in the treatment of hemorrhoids, varicose veins, and leg ulcers, among others (Cesarone, et al., 2005).

This pilot study was conducted to explore the protective influence of rutoside against the development of gentamicin-related renal tissue injury in an experimental animal model.

The objective of this study was to determine effects of oral administration of rutoside on the development of renal tissue injury in adult male albino rats, specifically: (1) to describe histopathologic changes on the degree of tubular necrosis; (2) to measure the levels of BUN and creatinine; (3) to assess if the renal effect of rutoside is dose-dependent at 75 and 150 mg/kg body weight.

2. METHODOLOGY

2.1 Subjects

Thirty male Sprague Dawley rats around 6-8 weeks old, each weighing 150-200 grams, were procured from the University of the

Philippines (Manila, Philippines). These rats were housed and acclimatized for 14 days in the individual animal cages at San Beda College of Medicine Animal Science Laboratory (Manila, Philippines). The rats were kept at 22±5°C, with 12:12 hour light/day cycle, fed with standard chow pellet and was given free access to distilled drinking water by a personnel blinded to the study. Each rat was weighed before the start of the treatment, during treatment (on a weekly basis) and after the treatment.

The Institutional Animal Care and Use Committee (IACUUC) guidelines for proper animal handling were strictly observed throughout the experiment. The subjects kept were under the supervision of a licensed veterinarian who was blinded to the study.

2.2 Pre-treatment with Rutoside

Laboratory-grade rutoside powder was procured from ChangshaNulantChem Co., (Hunan, China). The dosages of rutoside utilized in the experimental treatment groups (Table 1) were adapted from Chua (2013). Rutoside powder was dissolved in PNSS and administered via gastric gavage once daily for 21 days. Plain NSS 1mg/kg body weight was given as placebo for the sham group.

Table 1. Control and Treatment Groups

Group (n=5)	Treatment
Sham	PNSS + PNSS
Rutoside 75	75mg/kg Rutoside + PNSS
Rutoside 150	150 mg/kg Rutoside + PNSS
GEN	PNSS + 80 mg/kg Gentamicin
Rutoside 75 + GEN	75mg/kg Rutoside + 80 mg/kg Gentamicin
Rutoside 150 +GEN	150 mg/kg Rutoside + 80 mg/kg Gentamicin

PNSS - plain normal saline solution 1mL/kg body weight; GEN – Gentamicin

2.3 Induction of Gentamicin

Gentamicin 80 mg/ kg was given via intraperitoneal injection to the GEN, Rutoside 75 + Gen, and Rutoside 150 + Gen groups from days 15-21. Gentamicin was administered 12 hours apart from rutoside.

2.4 Surgical Excision of Kidney and Blood Extraction

Twenty four hours after the last administration of gentamicin, the rats were anesthetized with tiletamine/zolazepam. Blood samples were collected via intracardiac puncture. Blood samples were then sent for measurement of BUN and creatinine levels.

Bilateral nephrectomy was performed immediately after the intracardiac puncture. The kidneys were excised, washed with NSS, incised sagittally into halves and individually contained in the properly labeled bottle with 10% buffered formalin solution.

2.5 Histopathologic Evaluation

Paraffin sections (4µm thickness) were prepared from each kidney, stained with hematoxylin and eosin (H & E). The tissue samples were examined under light microscope (Eclipse Ni-E, Nikon Instruments Inc., Japan), evaluated by a veterinary pathologist who was blinded to the study. Renal tubular necrosis was assessed using a standard scoring scheme adapted from Pai *et al.* (2012).

Table 2. Histological grading of degree of proximal tubule necrosis*

Grade	Description*
0	Normal
1	Mild usually single cell necrosis in sparse tubules
2	Moderate, More than one cell involved in sparse tubules
3	Marked tubules exhibiting total necrosis in almost every power field
4	Massive total necrosis

*Adapted from Pai *et al.* (2012).

2.6 Statistical Analysis

Two-way ANOVA was used to analyze the mean BUN and creatinine levels between groups with Tukey's HSD test to account for multiple comparisons. Shapiro-Wilk test and Bartlett's test were applied to check the assumptions of normality of distribution and

homogeneity of variance, respectively. Kruskal-Wallis test was utilized in the analysis of median histologic grades with Wilcoxon rank-sum test for pairwise comparisons. Stata 12 was the statistical software of choice for this study.

3. RESULTS AND DISCUSSION

3.1 Histopathologic Findings

There was no mortality among the subjects during and after treatment with gentamicin.

Histopathologic analysis of the kidney showed normal tissue (median grade 0) in the sham, Rutoside 75, and Rutoside 150 controls (Figure 1-A). There was massive total necrosis (median grade 4) in the GEN control (Figure 1-B), confirming the histopathologic diagnosis of gentamicin-related nephrotoxicity. The Rutoside groups showed significant attenuation of renal injury, with median grades 1 and 3 for Rutoside 75 + GEN and Rutoside 150 + GEN, respectively.

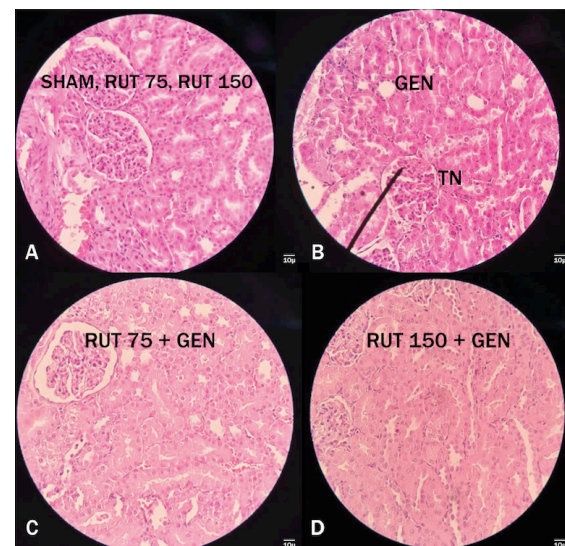


Figure 1. Representative tissue sections of the kidney

In this study, a standard grading scheme was adapted (Pai *et al.*, 2012) to quantify the histopathologic findings (Table 1). Individually comparing the sham, Rutoside 75, and Rutoside 150 against the GEN group showed no statistically significant difference in the histopathologic grade. The treatment groups

(Rutoside 75 + GEN and Rutoside 150 + GEN), when compared with the GEN group, showed a significant attenuation in the degree of renal tissue injury. *Post hoc* analysis showed the following trend from normal, uninjured kidney to increasing severity of renal tissue injury: Sham < Rutoside 150 + GEN < Rutoside 75 + GEN < GEN group.

As to dose-dependency, it appeared that treatment with Rutoside 150 + GEN showed a significantly lesser degree of renal tissue injury when compared to treatment Rutoside 75 + GEN ($p < 0.05$). These findings may suggest that the attenuation of renal tissue injury by the rutoside effect was dose-dependent.

3.2 BUN Levels

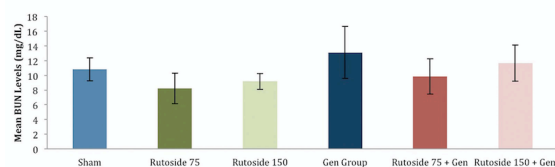


Figure 2. Mean blood urea level nitrogen levels (mg/dL).

Among all the treatment groups, mean blood urea nitrogen (BUN) level was highest in the GEN group. When compared to the GEN group, the mean BUN levels of the sham, Rutoside 75 + GEN, and Rutoside 150 + GEN were lower by 21.2% ($p=0.019$), 24.7% ($p>0.05$), and 11.1% ($p>0.05$), respectively.

Analysis of the treatment groups revealed that at least one of the treatment groups has a different BUN level. *Post hoc* analysis showed the following trend from lowest to highest BUN levels: Sham < Rutoside 75 + GEN < GEN = Rutoside 150 + GEN. These findings suggest that rutoside was able to attenuate increase in BUN levels only at a concentration of 75 mg/kg. The dose-dependent influence of rutoside was not demonstrated using this biochemical marker.

3.3 Creatinine Levels

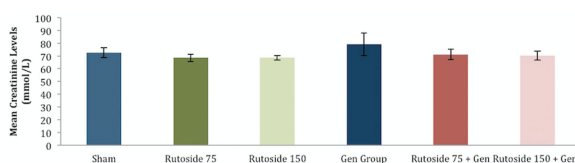


Figure 3. Mean serum creatinine levels (mmol/L).

Among all treatment groups, mean creatinine level was highest in the GEN group. When compared to the GEN group, the mean creatinine levels of the sham, Rutoside 75 + GEN and Rutoside 150 + GEN were lower by 9.1% ($p=0.0456$), 10.1% ($p>0.05$), and 11.1% ($p>0.05$), respectively. The mean creatinine level of Rutoside 150 + GEN when compared with Rutoside 75 + GEN was by 1.1%.

Post hoc analysis showed the following trend from the lowest to the highest creatinine level: Sham < Rutoside 150 + GEN < Rutoside 75 + GEN < GEN.

When compared with the GEN control, there were significantly lower BUN and serum creatinine levels in the Rutoside-treated groups ($p<0.05$). However, Rutoside effect on these biochemical markers did not appear to be dose-dependent ($p>0.05$).

3.4 Discussion

The histopathologic findings of the sham group showed no cell necrosis. Normal architecture of the renal capsule and parenchyma was observed. Renal corpuscles appeared morphologically normal with double walled Bowman's capsule surrounding the glomerulus. The proximal convoluted tubules (PCT) of normal kidneys showed narrow lumen, occupied by striated brush borders and a regular basal lamina lined by a single layer of pyramidal shaped cells. Distal convoluted tubules (DCT) attained a wide lumen surrounded by numerous smaller sized cuboidal cells.

The GEN group showed marked tubules exhibiting total necrosis in almost every power field and massive total necrosis on left and right, respectively. There were degenerative changes in most parenchymatous elements, as well as marked congestion in the cortical veins, multiple areas of necrosis of the tubules, thickening of the arterial walls, and renal tubules were partially filled with red, proteinaceous materials which were suggestive of early renal damage. Histopathologic examination revealed loss of PCT brush borders and tubular epithelial cell desquamation, hypertrophied podocytes pedicles, and thickened glomerular basement membrane as well as tubular cell vacuolization. The glomeruli appeared to have reduced cellularity and dilated urinary spaces. Severe PCT tubular degenerative features were observed, where

tubular lumen appeared wide and contained cellular debris and most of them showed swollen outlines. Mononuclear cellular infiltrations among renal tubules were also noted. Light microscopy revealed that most DCT showed few changes.

Gentamicin, an aminoglycoside, was used to induce renal tissue injury. It is believed that it has the capacity to generate reactive oxygen species (ROS) leading to relative deficiency in intrinsic antioxidant enzymes (Mazen, 2013) ultimately leading to tubular necrosis and renal failure.

Rutoside 75 + GEN group revealed moderate necrosis with more than one cell involved in sparse tubules and marked tubules exhibiting total necrosis in every power field on left and right, respectively. Only slight histopathologic changes were observed. It was demonstrated by moderate congestion in the glomerulus. Mild to moderate tubular necrosis were also detected in PCT of this group. The DCT did not show significant tubular damage.

Rutoside 150 + GEN group revealed mild injury, with usually single cell necrosis in sparse tubules on both the left and right kidneys. The tubules were more regular with moderate glomerular cellularity, minimal blood congestion and slightly narrow urinary space. Rare tubular necrosis and inflammatory cells among renal tubules were detected. PCT appeared slightly organized with regular lumen occupied by evident brush borders; large numbers of epithelial cells were less hypertrophied. No significant findings in DCT architecture.

Histopathologic findings in the GEN group revealed marked necrosis as compared to the group pretreated with rutoside 75mg/kg and 150mg/kg, respectively. Hence, both groups pretreated with rutoside showed attenuation of renal injury caused by gentamicin.

Rutoside probably attenuated renal effect by inhibiting damage through the conversion of the reactive oxygen species into a more stable species and quenching the free radical chain reaction (Yang, 2008) in the proximal convoluted tubules of the kidneys caused by gentamicin. It is also possibly due to the selective accumulation of the drug in the proximal convoluted tubule that leads to the damage of the brush border (Whiting, 1996).

Groups treated with rutoside had lower BUN values compared to those who were not, and thus exhibited potential protective rutoside effect. Increasing the rutoside dosage up to 150 mg/kg did not correlate with further decrease in BUN compared with 75 mg/kg, thus using rutoside for lowering BUN was not dose-dependent.

The highest mean serum creatinine level was found in the GEN group and was significantly higher when compared to the sham control, suggesting that there was damage in renal function by gentamicin. These changes reflected the severity of renal insufficiency which occurred in association with the sudden fall in glomerular filtration rate because of the majority of administered GM enters specifically the proximal tubular epithelial cells, binds to anionic phospholipids in the target cells inducing abnormalities in the function and metabolism of multiple intracellular membranes and organelles then developed injury in the proximal tubular epithelial cells of kidney that caused acute renal failure (Mazen, 2013).

Drug-induced nephrotoxicity is usually measured by BUN and creatinine levels as a marker of injury. In our study, the BUN and creatinine did not specifically show direct correlation with the histopathologic data. The creatinine levels were correlated to the histopathologic result compared to BUN levels. This may be explained by the plasma concentration and 24-hour urinary excretion of creatinine that are not affected by dietary changes, metabolic rate and urine excretion rate. As a biochemical marker, serum creatinine level is more significant than BUN level in the earlier phases of injury (Erdem, 2000).

4. CONCLUSIONS

In this model of gentamicin-related nephrotoxicity, pre-treatment with rutoside resulted in significant attenuation of renal tissue damage and significantly lower BUN a serum creatinine levels. Rutoside effect on the extent of renal tissue injury, but not on BUN and serum creatinine levels, was dose-dependent at 75 and 150 mg/kg body weight.

For future studies, the following are recommended:

1. To increase the duration of treatment of rutoside for further assessment of its therapeutic effect;

2. To determine other organ effects of rutoside used as a protective supplement;
3. To perform pre- induction and post- induction of creatinine and BUN levels;
4. To identify renal damage using other biochemical marker such as urine albumin;
5. To measure oxidative assays such as glutathione, glutathione peroxidase, glutathione S-transferase, superoxide dismutase, xanthine oxidase, malondialdehyde, nitric oxide levels that causing renal damage;
6. To perform histopathologic examination of organs other than kidneys that can be affected by rutoside.

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