Nephroprotective effect of C-phycocyanin on renal damage induced by kanamycin in rats

Zulema Ramírez-Carmenate[1], Sandra Rodríguez-Salgueiro[2*], Mariuska Matos-Terrero[3], Amelia Capote-Crespo[4], Diana García del Barco-Herrera[3], Lucía González-Núñez[4]

[1]Biomarkers Group, Molecular Immunology Center, Havana, Cuba
[2]Microscopy Laboratory, Infectious Diseases Division, National Center for Scientific Research, Havana, Cuba
[3]Pharmaceutical Division, Center for Genetic Engineering and Biotechnology, Havana, Cuba
[4]Department of Human Morphophysiology II-III, Latin American School of Medicine, Havana, Cuba

*Correspondence to
Sandra Rodríguez
Salgueiro, PhD;
Email:
sandra.rodriguez@cnic.
edu.cu

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Abstract

Introduction: Pharmacological properties of C-phycocyanin are related to its antioxidant activity. The use of aminoglycoside antibiotics is limited due to the risk to induce nephrotoxicity. Agents with antioxidant properties reduce renal damage caused by aminoglycosides.
Objectives: This study aimed to evaluate the effect of C-phycocyanin on glomerular and tubular morphologies during recovery phase after the damage induced by a chronic treatment with kanamycin in rats.
Materials and Methods: Twenty-nine adult male Wistar rats divided into five groups were treated for 20 days with;solution of sodium chloride 0.1 mol/L, pH 7.2-7.4 (PBS) (Control group), kanamycin (500 mg/kg) (Kanamycin group) and the other three groups received C-phycocyanin (5 or 10 mg/kg) plus kanamycin (500 mg/kg) (Phycocyanin Concomitant 5, Phycocyanin Concomitant 10 and Phycocyanin Pretreated 10). The group Phycocyanin Pretreated 10 received a previous treatment with C-phycocyanin during 4 days. Eight weeks after the last treatment, kidneys were removed, embedded in paraffin and stained with hematoxylin and eosin and periodic acid–Schiff (PAS). Glomeruli and proximal tubules were evaluated by light microscopy in 10 histological fields of cortical area, using qualitative, semi-quantitative and quantitative morphological studies.
Results: The group Phycocyanin Pretreated 10 showed the best effects minimizing glomerular and tubular damages and increasing the density of preserved tubules. The group Phycocyanin Concomitant 10 had effect on proximal tubules only. These two groups decreased the global renal damage caused by kanamycin.
Conclusion: C-phycocyanin accelerates the recovery of glomeruli and proximal tubules damaged by kanamycin in rats, possibly due to its antioxidant properties.

Introduction

The biliprotein C-phycocyanin is a photosynthetic pigment extracted from blue-green algae such as *Spirulina* sp. (1). It has various pharmacological properties related to its antioxidant activity (2-4). C-phycocyanin is a major component of the dietary supplement spirulina, which is frequently used in many countries due to its nutritional value (5,6). Nephroprotective effects of C-phycocyanin have been previously proven in models of nephrotoxicity induced by oxalate in rats (7,8) and by cisplatin in mice (9-11). This has also been confirmed in models of inflammation and fibrosis in mice (12) and diabetic nephropathy in rats (13).
The aminoglycoside antibiotics are often used in clinical practice, mainly in the treat-

Core tip
C-phycocyanin accelerates the recovery of glomeruli and proximal tubules damaged by kanamycin in rats, possibly due to its antioxidant properties.

ment of serious infections caused by gram negative bacteria (14). However, the clinical benefits of these agents are affected by its most persistent and serious side effects: ototoxicity and nephrotoxicity (15-18).
The production of reactive oxygen species (ROS) is the central pathophysiological mechanism of renal damage induced by aminoglycosides (19). The ROS initially leads to death of proximal tubule epithelial cells (acute tubular necrosis phase) (20,21) and subsequently, to vascular and mesangial

contraction in glomeruli; while tubular epithelium regenerates (recovery phase) (22). In addition, an inflammatory process is favored (23).

The use of nephroprotective substances could neutralize aminoglycoside toxicity and enhance their clinical efficacy (24). In relation to experimental results, antioxidant agents have decreased nephrotoxicity caused by these drugs (25-29). Protective effect of C-phycocyanin and *Spirulina* sp. on renal tubular morphology has been demonstrated in models of acute injury induced by gentamicin in mice (30-32).

Objectives

In a preceding study of our group, the protective effect of C-phycocyanin on proximal tubules was achieved. This effect was assessed during the recovery phase of kanamycin injury in rats and mice (33).

This study aimed to evaluate the effect of C-phycocyanin on glomerular and tubular morphologies during recovery phase of damage induced by a chronic treatment with kanamycin in rats. It would be valuable to gain an antioxidant therapy concomitant to aminoglycoside treatment, designed to accelerate the regeneration of renal tubules and prevent late effects of these drugs in the renal glomeruli.

Materials and Methods
Animals

Twenty nine Wistar rats, weighing 320-350 g, from the National Center for Laboratory Animal Production of Havana were used. All procedures were performed as approved by the International Committee for Animal Care in accordance with the Cuban regulations for animal experimentation and ethical research guidelines. The animals were provided with standard diet and water ad libitum before and during the experiment.

Study design

Kanamycin-100 (ampoules of 100 mg/mL, AICA Laboratories, Havana, Cuba); C-phycocyanin (30% purity from *Spirulina platensis,* Biodelta PTY Ltd, South Africa) and a solution of sodium chloride 0.1 mol/L, pH 7.2-7.4 (PBS) were used. C-phycocyanin at a dose of 2.5 mg/mL in sterile PBS was prepared.

Rats were divided into five groups; Control, Kanamycin, Phycocyanin Concomitant 5, Phycocyanin Concomitant 10, and Phycocyanin Pretreated 10. C-phycocyanin

and kanamycin were administered once daily for 20 days (Table 1). Control animals received PBS intraperitoneally. Euthanasia was performed 8 weeks after the period of treatments and kidneys were removed for histological study.

Histological preparation

Kidneys were sectioned by a sagittal section through the hilum, fixed in 10% formalin in PBS and embedded in paraffin. Sections of 3 μm thick were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) (34).

Morphological analysis

A PAS-stained kidney histological section per animal was used. Ten cortical fields randomly chosen from the upper to the lower pole were evaluated using an OLYMPUS BX 53 light microscope at ×400 magnification. The images were obtained with a CDP 73 digital camera attached to the microscope.

Semi-quantitative analysis
Glomerular damage

Complete glomeruli in each histological field were evaluated. The following variables were assessed: mesangial hypercellularity (presence of more than three nuclei in mesangium); mesangial expansion (wide mesangial PAS-positive areas) and presence of glomerular synechiae (adhesion to Bowman's capsule through either a fibrillary or cellular junction) (35).

Tubular damage

For tubular damage evaluation, only the transversely-sectioned proximal tubules were considered in each histological field. The tubular damage was classified as reversible or irreversible (36). The reversible damage was identified by the brush border absence. The irreversible damage was recognized by the variables: presence of necrotic cells, presence of casts or detached cells in the lumen, and alterations of the tubular basal membrane (thickening, denudation or discontinuity).

To analyze variables of glomerular and tubular damage, 0 was assigned to the normal histological structure and 1 to the pathological condition. Quantification of the variables was performed by a previously described method (37) using a software developed for ".Net platform, by means of the integrated development environment Visual Studio 2008 and C# language." The damaged glomeruli

Table 1. Experimental schedule

Groups	Treatment	Doses (mg/kg)	Duration (days)	Administration route
Kanamycin	kanamycin-100	500	20	Subcutaneous
Phycocyanin Concomitant 5	C-phycocyanin	5	20	Intraperitoneal
	Kanamycin-100	500	20	Subcutaneous
Phycocyanin Concomitant 10	C-phycocyanin	10	20	Intraperitoneal
	Kanamycin-100	500	20	Subcutaneous
Phycocyanin Pretreated 10	C-phycocyanin	10	24	Intraperitoneal
	Kanamycin-100	500	20	Subcutaneous

and damaged tubules per histological field were averaged, obtaining 10 values per animal for each variable.

Quantitative analysis

For each animal the density of proximal tubules with preserved brush border per cortical area was calculated. The images were taken at ×400 magnification and the following equation was used (38).

$$\text{Density of preserved tubules} = \frac{PPT}{A \times B}$$

Where:

PPT- quantity of preserved proximal tubules, showing the brush border

A- Image area (0.24 μm²)

B- Histological fields evaluated (10 fields)

The Image J (v. 1.39 u) software was used to measure the area of each image (39).

Ethical issues

The research was approved by ethical committee of National Center for Scientific Research, Havana, Cuba. Prior to the experiment, the protocols were confirmed to be in accordance with the guidelines of Animal Ethics Committee of National Center for Scientific Research, Havana, Cuba.

Statistical analysis

GraphPad Prism 5.00 software for Windows was used. The percentage of damaged glomeruli and damaged tubules by histological field was compared between groups. Statistical differences between treatment groups were determined by non-parametric Kruskal-Wallis and Dunn's multiple comparison tests. The comparison between reversible and irreversible tubular changes was done using the Wilcoxon test. Density of preserved tubules was compared between groups using chi-square and Fisher's exact tests. Differences were considered statistically significant when $P \leq 0.05$.

Results

Qualitative analysis

Glomeruli

Glomerular morphology in the Control group was characterized by the presence of normal renal corpuscles (Figure 1 A, B).

In the Kanamycin group, glomeruli with different degrees of injury – from partially damaged to completely degenerated – were observed (Figure 1 C-E). Areas of preserved glomeruli were also observed.

Partially damaged glomeruli showed synechiae, obliteration of capillary lumens in some lobules, hypercellularity, and increased mesangial matrix. The latter conferred them a widened appearance. In neighboring areas, extraglomerular interstitial inflammatory infiltrate was identified (Figure 1 C). Irregular Bowman's space dilation and glomerular tuft retraction were also observed (Figure 1 D). In the degenerated glomeruli, basement membrane thickening, marked retraction of glomerular tuft with obliter-

ation of capillary lumens, and marked widening of Bowman's space were observed (Figure 1 E).

In the groups Phycocyanin Concomitant 5 and Phycocyanin Concomitant 10, synechiae, obliteration of capillary lumens, hypercellularity and increased mesangial matrix were observed. In these groups, the basement membrane of Bowman's capsule was thickened in some glomeruli. In some cases, irregular Bowman's space dilation and glomerular tuft retraction were both noticed. However, these damages were less perceptible in the group Phycocyanin Concomitant 10 (Figure 1 F, G). The best results were obtained in the group Phycocyanin Pretreated 10, with large areas of preserved glomeruli (Figure 1 H).

Proximal tubules

In the Control group, the normal structure of the proximal tubules in the cortex was observed (Figure 2 A).

Large areas of damaged proximal tubules alternating with scarce areas of preserved tubules were observed in Kana-
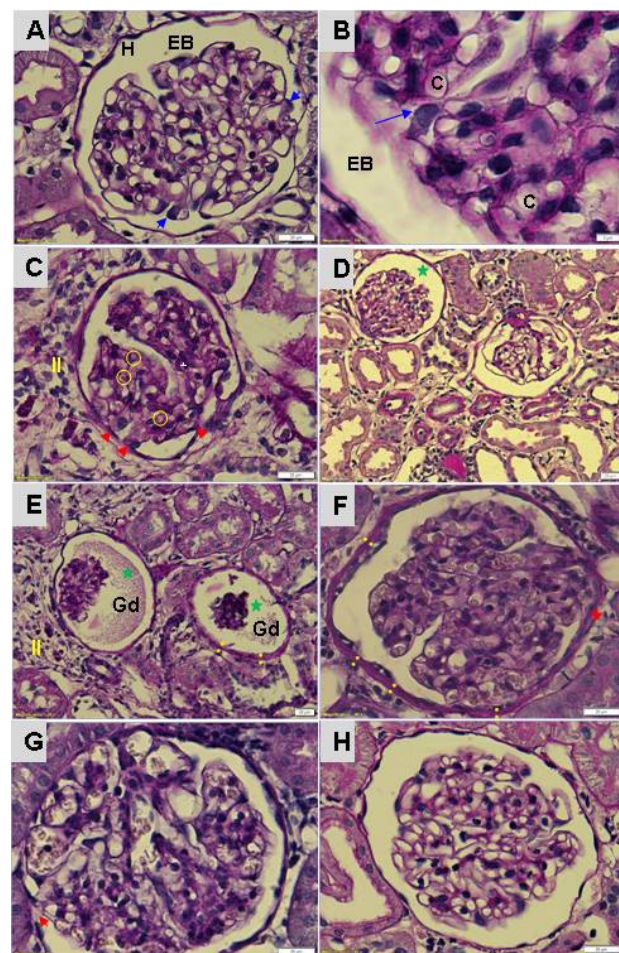
Figure 1. Glomerular morphology. PAS staining. (A, B) *Control*; (C, D, E) *Kanamycin*; (F) *Phycocyanin Concomitant 5*; (G) *Phycocyanin Concomitant 10*; (H) *Phycocyanin Pretreated 10*. H: Bowman's capsule parietal ephitelium, EB: Bowman's space, blue arrow: podocyte, red arrowhead: synechiae. Circle: obliterated blood vessel, green star: Bowman's space dilation, Gd: degenerated glomerulus, yellow points: basement membrane, II: inflammatory infiltrate. Bars 20 μm, except in (B) Bar 5 μm.
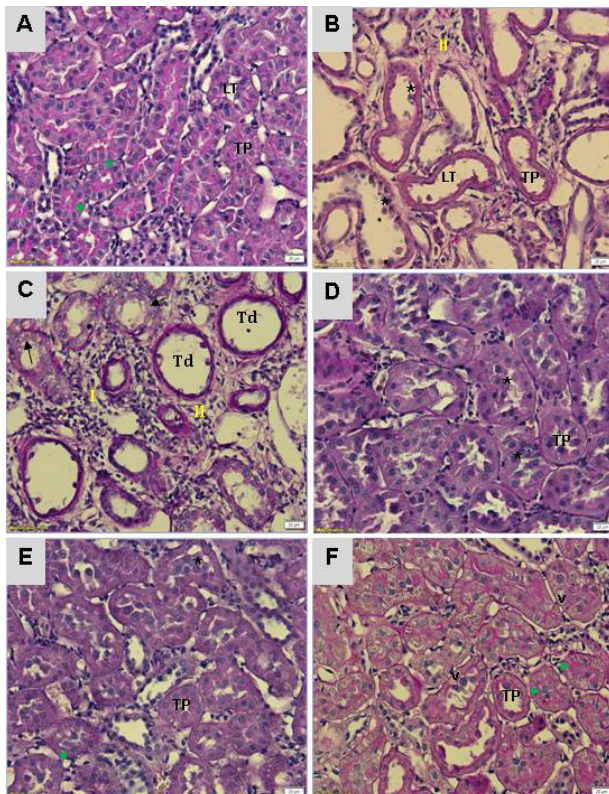
mycin group. In areas of injury, proximal tubular epithelial cells showed signs of necrosis, decreased height, apical localization of nuclei and partial or total loss of the brush border. Tubular lumens were widely dilated with detached cells and acidophilic substances (Figure 2 B).

Tubules with denudation of the basement membrane were observed. The histological damage was severe in some areas showing complete degeneration of the proximal tubules, thickened basement membranes and a notable dilation of tubular lumens. Furthermore, a large peritubular inflammatory infiltrate was identified in damaged regions (Figure 2 C).

In the three groups treated with C-phycocyanin there was a recovery of the proximal tubular morphology compared with Kanamycin group. Most of the tubules displayed their epithelial integrity and different degrees of brush border preservation. In these groups, neither denuded nor degenerated tubules were found (Figure 2 D-F).

Tubules with partially preserved brush borders were observed in Phycocyanin Concomitant 5 and Phycocyanin Concomitant 10 groups. However, in the latter, tubular areas with intact brush border were more extensive (Figure 2D, E).

The best condition of the proximal tubular morphology of the three groups was noticed in Phycocyanin Pretreated 10. Most tubules showed positive PAS staining in tubular basement membranes and brush borders (Figure 2 F). However, in these groups, the recovery of tubular morphology was not complete, since we have found different degrees of cytoplasmic vacuolization in the epithelium and detached cells in the lumen (Figure 2 D-F).

Semi-quantitative analysis
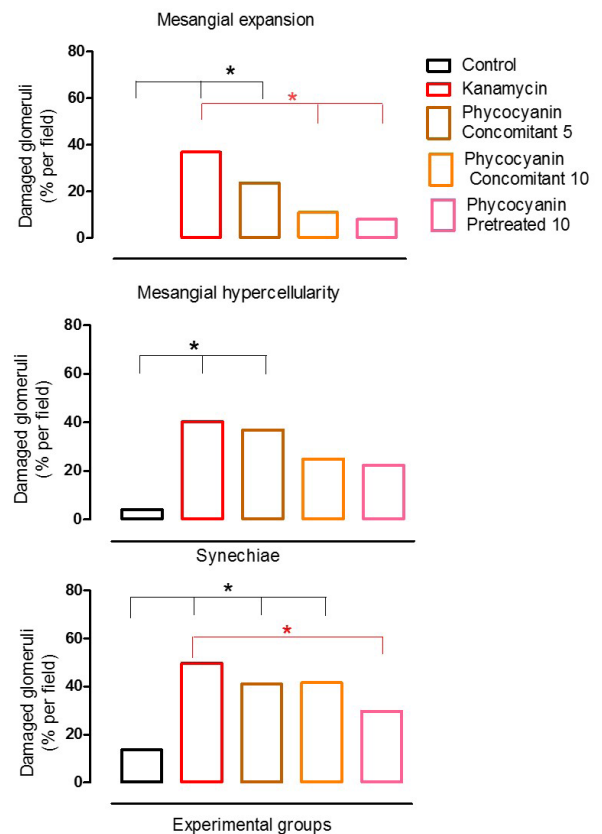#### Glomerular damage
Kanamycin caused an increase in mesangium at the expense of mesangial matrix and mesangial cells. The groups treated with C-phycocyanin 10 mg/kg showed a decrease in the mesangial matrix, with respect to Kanamycin group. However, mesangial hypercellularity in these groups treated with C-phycocyanin 10 mg/kg was similar to the Control group (Figure 3).

Kanamycin group had the highest percentage of glomeruli with synechiae. Only in Phycocyanin Pretreated 10 group there was a decrease of synechiae with respect to Kanamycin group (Figure 3).

In the assessment of glomerular damage (average of three variables), only in Phycocyanin Pretreated 10 group there was a decrease in glomerular injury with respect to *Kanamycin* group (Figure 4).
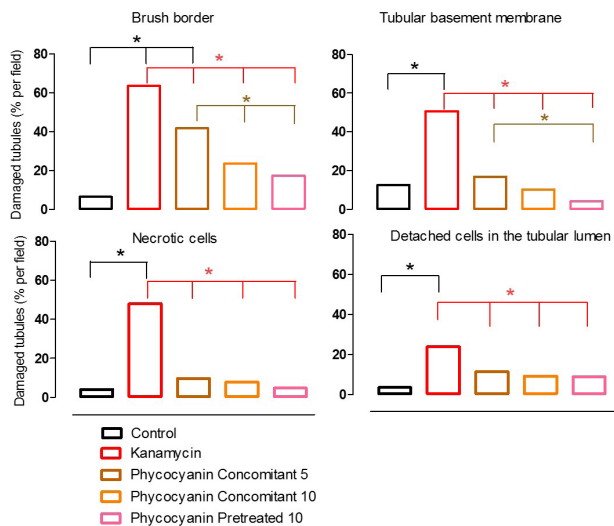
Tubular damage
All groups treated with C-phycocyanin had lower damage of brush border than Kanamycin group. The groups that received C-phycocyanin at dose of 10 mg/kg showed better brush border recovery, similar to Control group.

Figure 2. Tubular morphology. PAS staining. (A) Control; (B, C) Kanamycin; (D) Phycocyanin Concomitant 5; (E) Phycocyanin Concomitant 10; (F) Phycocyanin Pretreated 10. LT: tubular lumen, TP: proximal tubule, green arrowhead: brush border, II: inflammatory infiltrate, asterisk: detached cells, arrow: necrotic cell, Td: degenerated tubule, v: vacuolated cell. Bars 20 μm.

Figure 3. Variables of glomerular damage. Kruskal-Wallis test and Dunn's multiple comparison test. * $P \leq 0.05$
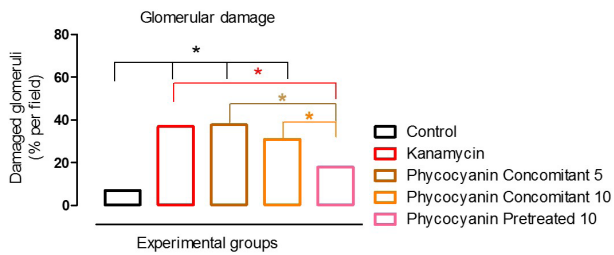
Figure 4. Glomerular damage (Kruskal-Wallis test and Dunn's multiple comparison test, *$P \leq 0.05$).

Figure 5. Variables of tubular damage (Kruskal-Wallis test and Dunn's multiple comparison test, * $P \leq 0.05$).

Figure 6. Reversible and irreversible tubular damages. Kruskal-Wallis test and Dunn's multiple comparison test. *$P \leq 0.05$. Reversible changes vs. Irreversible changes. Wilcoxon test. Different letters indicate statistical significant differences, $P \leq 00.05$.
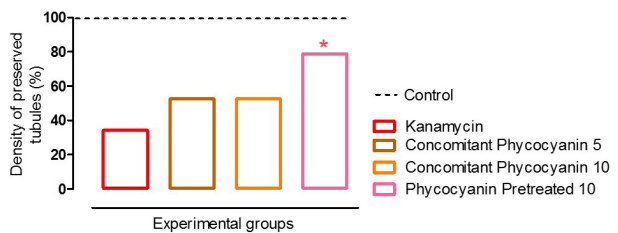
Figure 7. Density of preserved tubules ($\chi 2$ and Fisher tests, * $P < 0.0001$).

Figure 8. Global renal damage (Kruskal-Wallis test and Dunn's multiple comparison test * $P \leq 0.05$).

The best results were found in Phycocyanin Pretreated 10 group (Figure 5).

The treatments with C-phycocyanin induced a decrease in tubular basement membrane alterations in relation to Kanamycin group. Additionally, basement membrane alterations of the groups treated with C-phycocyanin did not differ from the Control group. The lowest values of basement membrane alterations were obtained in Phycocyanin Pretreated 10 group (Figure 5).
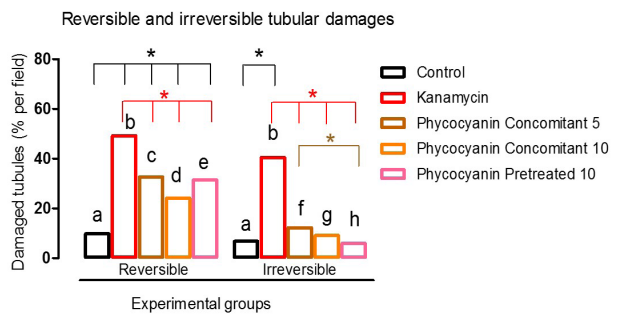
The presence of necrotic cells decreased in all groups treated with C-phycocyanin in relation to Kanamycin group, showing similar records to the Control group. C-phycocyanin also reduced the presence of detached cells in tubular lumens compared to Kanamycin group (Figure 5).

Reversible and irreversible tubular changes also decreased in groups treated with C-phycocyanin. Regarding to irreversible changes, Phycocyanin Pretreated 10 group showed the best recovery (Figure 6).

A comparison between reversible and irreversible changes within each treatment group was done. In the groups treated with C-phycocyanin there was a predominance of reversible changes versus irreversible changes (Figure 6).
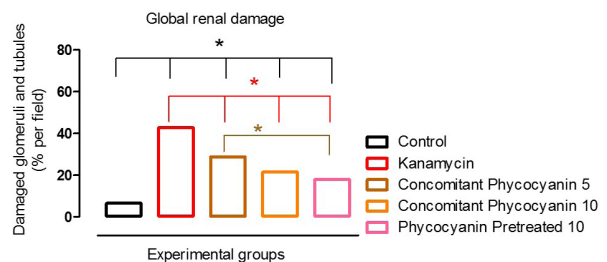
Quantitative analysis
Phycocyanin Pretreated 10 group showed the highest density of preserved tubules compared to Kanamycin group (Figure 7).

Global renal damage
C-phycocyanin treated groups displayed a decrease of Global renal damage induced by kanamycin. The lowest damage was observed in Phycocyanin Pretreated 10 group (Figure 8).

Discussion
Aminoglycosides induce significant morphological changes in the kidney leading to acute renal damage in many cases (40). This occurs because epithelial cells of the renal proximal tubules have a particular transport mechanism which increases the concentration of aminoglycosides (41). The kidney can react to the insult by starting a process of repair, whenever there is enough nutrients and oxygen and the tubular basement membrane's integrity is not irreparably damaged (20,42).

The pursuit of nephroprotective substances to reduce aminoglycosides toxicity, or accelerate kidney recovery is a priority (25,26,28,43). In the present research, the effect of C-phycocyanin on renal morphology was confirmed during the recovery phase after kanamycin-induced injury in rats, by means of qualitative, semi-quantitative and quantitative studies.

The experimental design used to replicate the renal damage induced by aminoglycosides led to changes in glomeruli and tubules and is consistent with previous studies of our group using kanamycin (33,35).

In the groups treated with C-phycocyanin, we observed wide areas of preserved glomeruli and tubules, which could be attributed to predominance of tissue repair in the kidneys of these animals.

In the glomeruli of the groups treated with C-phycocyanin at the dose of 10 mg/kg, no mesangial expansion was found and a tendency to reduce the number of mesangial cells was observed. Similarly, in a model of diabetic nephropathy in mice, protective effect of C-phycocyanin on mesangial expansion was found, linked to its antioxidant action (13). It has been suggested that the effect of antioxidants in glomeruli could be related to the inhibition of vasoconstriction and mesangial contraction (44). This line of thought allowed us to propose that C-phycocyanin contributed to mitigate the adverse effects of kanamycin on the intraglomerular mesangium due to its well documented antioxidant properties (2).

The lower values of stynechiae in the group Phycocyanin Pretreated 10, similar to Control group, could be related to beneficial effects of C-phycocyanin on renal function. This is the first report of the effect of a pretreatment with C-phycocyanin on glomerular morphology.

Concerning the effect of C-phycocyanin on proximal tubules, all treatments decreased both irreversible and reversible changes, with better recovery in the group Phycocyanin Pretreated 10. This is also the first report of the benefits of a pretreatment with C-phycocyanin on tubular morphology during the recovery phase after aminoglycosides-induced damage. A similar result was obtained in rats pretreated with *Spirulina platensis* using an acute model of gentamicin nephrotoxicity (32).

The protective effect of C-phycocyanin against kanamycin-induced damage in glomeruli and proximal tubules described in this work, could be related to its antioxidant activity (2,11). One of the most accepted mechanisms to explain acute renal damage due to aminoglycosides, is the generation of ROS in proximal tubule epithelial cells, caused by mitochondrial dysfunction (41,45). Antioxidant agents could act by weakening the aminoglycosides direct cytotoxicity, inhibiting vascular contraction and reducing the resulting inflammation (44).

Most studies concerning the nephroprotective effect of antioxidants against the damage induced by aminoglycosides have been performed during the acute phase (27,28). Previous studies of our group have shown that C-phycocyanin accelerates only tubular regeneration when administered concomitantly with kanamycin (33). The current results confirm what was obtained in the preceding study and demonstrate that protection is also expressed in the glomerular structures.

In addition, this work constitutes the first preclinical report of the positive effect of a pretreatment with C-phycocyanin in the recovery of glomerular and tubular damage in a model of kanamycin-induced nephrotoxicity in rats.

Conclusion

C-phycocyanin accelerates the recovery of glomeruli and proximal tubules damaged by kanamycin in rats, possibly due to its antioxidant properties.

Conflicts of interest

The authors declared no competing interests.

Authors' Contribution

ZRC performed the analysis of the histological samples, searched the related articles and prepared the draft. SRS, DGBH and LGN designed the experiments, supervised the study, review and edited the final manuscript equally. MMT and ACC provided technical assistance during the experiments. All authors read and signed the final paper.

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