Laboratory changes inherent to acute kidney injury induced by aminoglycosides in wistar rats

Alterações laboratoriais inerentes à lesão renal aguda induzida por aminoglicosídeos em ratos wistar

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Introduction

Kidney disease is a global public health problem, affecting over 750 million people worldwide3. The importance of the kidneys for survival is evident when observing the consequences of loss of kidney function. Individuals suffering from renal insufficiency progressively retain nitrogen metabolism products, lose the ability to dilute and concentrate urine and become unable to regulate their acid-base balance or maintain plasma electrolyte balance and levels4. Acute renal insufficiency is defined as the abrupt loss of glomerular kidney filtration, with consequent changes to hydroelectrolytic and acid-base balance4. This imbalance, in turn, leads to the accumulation of substances in the blood, such as urea and creatinine4.

Recently, the term “insufficiency” has been replaced by injury, since not all patients with acute kidney injury...
suffer from renal failure and not all display structural damage. Although the term has been replaced, the same acronym (AKI) has been maintained to refer to the syndrome\(^5\).

According to Kidney Disease Improving Global Outcomes (KDIGO) \(^6\), acute kidney injury (AKI) is defined in humans as an increase greater than 0.3 mg/dL of serum creatinine within 48 hours, a 1.5-fold increase of serum creatinine from baseline within 7 days, or urine output below 0.5 ml/kg/h for 6 hours. AKI\(^7\) represents a continuous series of mild, clinically unapparent renal lesions and nephron loss, causing severe acute renal insufficiency. This disease is considered an under-detected condition estimated to occur in 8-16% of hospitalized patients, and is now a well-established risk factor for chronic kidney disease\(^8\).

The International Renal Interest Society (IRIS) has developed a five-grade classification for the early recognition of AKI in dogs and cats. Grade 1 includes non-azotemic patients (creatinine less than 1.6 mg/dL) with proven AKI, presence of oliguria or anuria, or progressive increases in creatinine equal to or greater than 0.3 mg/dL at an interval of 48 hours. Grade 2 includes patients with static or progressive azotemia (creatinine between 1.7 and 2.5mg/dL), while Grades 3 comprises creatinine between 2.6 to 5.0mg/dL, Grade 4, between 5.1 to 10, 0mg/dL and Grade 5, above 10mg/dL.\(^7\)

The clinical presentation, diagnosis, therapy and prophylaxis for AKI are influenced by the pathophysiology of the process. In 1960, a three-form classification was proposed, namely pre-renal AKI, renal AKI and post-renal AKI\(^9\).

The AKI related to nephrotoxins, alternative drugs and infectious agents, as well as hospital admissions and associated procedures, are more pronounced in low- and medium-low-income countries, and contribute to the increased risk of mortality and chronic kidney disease (CKD) in these regions\(^10\). According to Carvalho\(^11\), impaired renal function in AKI results in hydroelectrolytic imbalance and accumulation of metabolism residues, causing azotemia, uremia, severe systemic impairment and high morbidity and mortality rates in dogs. Brown et al.\(^12\) reported two AKI outbreaks that occurred in dogs and cats in 2004 and 2007, associated with commercial animal feed.

According to KDIGO\(^6\) and Roy et al.\(^13\), sepsis is the most common trigger for AKI development in hospitalized patients, affecting about 36% of patients in ICUs, as this condition causes renal hypoperfusion and ischemia, followed by acute tubular necrosis, some of which comprise the main sepsis complications, leading to the mortality factor\(^14\). Nephrotoxic drugs are considered the main etiological AKI agents\(^15\). The frequency of nephrotoxicity due to aminoglycosides can reach 50% when therapy is performed for longer than two weeks.

The use of nephrotoxic substances has become more frequent and a series of drugs and nephrotoxic agents have been incorporated into the medical practice, such as new iodinated contrasts, immunosuppressants, non-hormonal anti-inflammatory drugs, antibiotics, antifungals and antiviral agents\(^16,6,13\). The evolution in the treatment of serious diseases and the emergence of increasingly effective drugs displaying different mechanisms of action is now a reality. However, these drugs result in several side effects, with nephrotoxicity as one of the most common and with the highest morbidity.\(^17\)

In human medicine, aminoglycosides are used for the treatment and prophylaxis of infections following cardiac surgery, sepsis and osteomyelitis\(^18\). In veterinary medicine, aminoglycosides are used for the regional perfusion of limbs in the treatment of lamiinitis\(^19\), osteomyelitis with intraossaoso antibiotic perfusion\(^20\), pleuropneumonia and septic arthritis\(^21\) in horses. In addition to the injectable form, formulations for otitis treatment and dermatological creams for domestic animals are also available. Aminoglycosides are also being used to treat infections caused by multi-resistant bacteria, such as MRSA (methylillin-resistant \emph{Staphylococcus aureus})\(^22\).

These drugs, however, exhibit characteristics that make them toxic as soon as they penetrate living tissue. For instance, binding to the tubular membrane occurs with megalin, an endocytotic receptor expressed on the apical membrane of the proximal tubular epithelium. Once bound to megalin, the aminoglycoside-megalin complex is transported into the cell and joins the lysosome, where it merges with pre-existing structures, causing progressive deposition of polar lipids, which adopt a concentric lamellar arrangement, forming so-called myeloid bodies. In addition, several other changes in organelles and enzymes, such as ribosomes, mitochondria and the Na/K-ATPase pump, also occur. Aminoglycosides gradually accumulate in lysosomes and induce morphological changes\(^15\). Acute toxic tubular necrosis is observed in histopathological findings, ranging from diffuse to multifocal, affecting the external cortical region to the corticomedullary region, characterized as proteinosis and multifocal mononuclear inflammatory infiltrates\(^23\). Since the nephrotoxicity mechanism is due to the accumulation of the drug in the contorted proximal tubule, the use of a single dose and a short treatment period are required to reduce risks.

Dehydration and other factors, such as association with nephrotoxic drugs, pre-existing kidney disease, advanced age, hypokalemia and metabolic acidosis, aggravate the injury\(^15,16\).

Since AKI is a rapidly evolving lesion, biomarkers must display early diagnosis characteristics and functions, such as free glomerular filtration and the absence of tubular...
secretions or reabsorption and extrarenal metabolism or excretion. Early biomarkers, such as low molecular weight proteins and tubular enzymes, and late ones, such as urea and creatinine, are noted\(^6,24\).

New biomarkers are emerging, with the most studied comprising NGAL, interleukin-18, KIM-1, cystatin-C, L-FABP, NAG, netrin-1, vanin-1 and MCP-1. Of these, NGAL is the most employed in clinical studies, while NGAL and L-FABP are the earliest, with KIM-1 and IL-18 are later detected with better specificity\(^25\).

With the purpose of reproducing an AKI model induced by aminoglycoside in normohydrated and dehydrated Wistar rats, this study investigated laboratory alterations inherent to acute renal injury caused by gentamicin and aggravated by dehydration.

**Material and methods**

This study was carried out at the Animal Anatomy Section of the Laboratory of Animal Morphology and Pathology (LMPA) belonging to the North Fluminense Darcy Ribeiro State University (UENF), following the experimental rules established by the Ethics Committee on the Use of Animals (CEUA), under protocol number 293. Twenty-four male Wistar rats (*Rattus norvegicus*) weighing between 300-450 g, were used, obtained from the Laboratory Animal Center (CECAL) at the Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, RJ). The animals were categorized into three groups of eight animals, Control, Genta, and Deh+Genta. The Genta group received 50 mg.kg\(^{-1}\) of gentamicin in two daily doses for eight days, the Deh+Genta group was subjected to water restriction during the experiment, receiving the same drug treatment as the Genta group, and the Control group received the same volume, in milliliters, of a 0.9% saline solution every 12 hours, for eight days (Table 1).

Euthanasia was performed at days 5 and 8, to assess histopathological liver conditions and, consequently, exclude the possibility of liver alterations causing increases in the serum gamma glutamyl transferase (GGT) enzyme. A 4 cm\(^3\) fragment of the liver of each animal was collected and placed in a previously identified container for routine histological processing. Hematoxylin and eosin (HE) staining was performed, and the samples were analyzed by light microscopy with an Olympus BX 41 microscope. Photomicrographs were acquired with a Nikon Eclipse 80i camera (Kurobane Nikon Co., Ltd, Otawara, Tochigi, Japan) using the NIS-Elements-BR software).

<table>
<thead>
<tr>
<th>Group/Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>NaCl 0.9% BID</td>
<td>NaCl 0.9% BID</td>
<td>NaCl 0.9% BID</td>
<td>NaCl 0.9% BID</td>
<td>NaCl 0.9% BID</td>
<td>NaCl 0.9% BID</td>
<td>NaCl 0.9% BID</td>
<td>Euthanasia 4 animals</td>
</tr>
<tr>
<td>Genta</td>
<td>Gentamicin BID</td>
<td>Gentamicin BID</td>
<td>Gentamicin BID</td>
<td>Gentamicin BID</td>
<td>Gentamicin BID</td>
<td>Gentamicin BID</td>
<td>Gentamicin BID</td>
<td>Euthanasia 4 animals</td>
</tr>
<tr>
<td>Deh + Genta</td>
<td>Water restriction + gentamicin BID</td>
<td>Water restriction + gentamicin BID</td>
<td>Water restriction + gentamicin BID</td>
<td>Water restriction + gentamicin BID</td>
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<td>Water restriction + gentamicin BID</td>
<td>Water restriction + gentamicin BID</td>
<td>Euthanasia 4 animals</td>
</tr>
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</table>

The animals received pelleted feed and water *ad libitum* throughout the experiment\(^26\), with the exception of the Deh+Genta group. The animals were kept in modified metabolic cages under a controlled photoperiod cycle (12/12 h) in a ventilated room at room temperature (20 °C).

Blood samples were obtained for complete blood counts and serum biochemical analyses of urea, creatinine and GGT. To this end, the animals were previously anesthetized (isoflurane, dose-effect), and cardiac puncture was performed with a 0.45 x 13 mm (26G) needle and a 1 mL syringe. Urine was also collected through metabolic cages, one animal in each box, to determine urinary GGT, creatinine and protein. These tests were carried out immediately prior to the beginning of the experiment to confirm the absence of systemic changes. To monitor AKI, blood urea and creatinine measurements were repeated every 48 hours and urinary
GGT, every 24 hours. To exclude the possibility of hepatic alteration leading to increased urinary GGT, this enzyme was also determined in the serum from each blood sample.

Hematological analyses

Hemograms were obtained with a Labtest SDH 3 Vet automatic analyzer, determining red, white and platelet cell counts. Hematocrit values were obtained by the microcentrifugation method (10,000 rpm, 5 min) followed by determination using a specific table. After obtaining the microhematocrit, the capillary was fragmented at the division between plasma and blood cells. The plasma was then placed in a previously calibrated refractometer to determine total proteins.

Biochemical analyses

Biochemical tests were performed using commercial Labtest kits employing a Bioplus 200 semiautomatic device. Creatinine determination was performed using the modified Jaffé method and serum urea levels were determined by a colorimetric enzymatic test. The values were calculated based on a calibration factor and urea concentrations were expressed in mg/dL. Serum GGT was also determined using the commercial Labtest kit. Absorbances (A1) were determined at 405 nm, simultaneously triggering a stopwatch. The readings were repeated after two minutes (A2).

Urinary analyses

Protein concentrations were determined in urine samples by colorimetry using the Sensiprot® kit (Labtest). Urinary GGT was determined by the same process previously described for serum GGT. The variables weight, feed and water consumption, urine output, serum urea and creatinine, serum GGT, urinary GGT, urinary protein, as well as hematological data, were evaluated by mixed models with the PROC MIXED procedure available in the Statistical Analysis System software (SAS System, Inc., Cary, NC, USA), and in case of significant differences, the Tukey test was applied.

Results and discussion

Weight, feed and water consumption

The Deh+Genta group animals presented the lowest mean weight and lowest feed consumption during the eight days of experiment. Accentuated weight loss can be attributed mainly to dehydration, probably associated with metabolic acidosis and anorexia caused by gentamicin intoxication(27). All three groups presented equal average weight values up to the fifth day of the experiment, while on the last days the Deh+Genta group presented lower average weight than the other two groups. The animals in the Control group consumed the most feed, while the animals submitted to dehydration consumed the least (Figures 1 and 2).

Anorexia and discomfort probably caused the lower water consumption observed in the Genta group in comparison with the Control up to the fifth day(27). However, in the last days to the experiment (up to the eighth day), Genta group animals exhibited the highest average water consumption compared to animals belonging to same group in the first euthanasia moment, but equal to the Control group for the same period (Figure 3). After the fifth day, the Genta group presented polydipsia, as also reported by Dantas et al.(23) and Melchert et al.(28).
Urinary debit (UD)

The average urinary debit values for the Genta and Control groups were equal at both euthanasia moments. These differs from the findings reported by Erdem et al.\(^{(27)}\) and Oliveira et al.\(^{(15)}\), who observed polyuria. Abdeen et al.\(^{(29)}\) also reported increased aquaporin-2 excretion in urine associated with polyuria and renal tubule damages. Because of the water restriction, the Deh+Genta group presented significantly lower average urinary debit values than the other two groups at both analyzed moments (Figure 4).

**Figure 4.** Mean variable urinary debit values (in mL). Number 1 is the interaction between the Control group and death on day 5; number 2 is the interaction between the Genta group and death on day 5; number 3 is the interaction between death on day 5 and the Deh+Genta group and numbers 4, 5 and 6 refer to the interaction between death on day 8 and the Control, Genta and Deh+Genta groups.

Serum urea and creatinine

The highest serum urea levels were observed in the Deh+Genta group, while the Control and Genta groups did not differ significantly from each other (Figure 5). Urea is a weak glomerular filtration predictor, as 40%-70% returns to the plasma by a passive tubular diffusion process which depends on the urinary flow.\(^{(30)}\) The increases in serum urea were only belatedly detected, on the eighth day of the experiment. Thus, we believe it is necessary to develop new biomarkers for early renal injury diagnosis, risk stratification and prognosis, as also indicated by Dusse et al.\(^{(11)}\). Other factors displaying no relation with renal function can significantly alter urea serum levels, such as diet, hepatic production rate, dehydration, trauma, congestive heart insufficiency, infection, sodium depletion and the use of corticosteroids, diuretics or tetracyclines.\(^{(10)}\)

**Figure 5.** Mean urea values (in mg/dL). Treatments (1 to 3, where 1 is the Control group, 2 is the Genta group and 3 is the Deh+Genta group) and death (4 and 5, where 4 is death on day 5 and 5 is death on day 8).

The average creatinine levels of the three groups were equal up to the fifth day of the experiment, while on the eighth day the animals that received gentamicin (Genta and Deh+Genta) exhibited equal average values, significantly different from the Control group. These results corroborate the findings reported by Oliveira et al.\(^{(35)}\), Cobrin et al.\(^{(24)}\) and Abdeen et al.\(^{(29)}\), who observed serum creatinine to be a belated renal marker, increasing only three days after renal injury. The water restriction of the Deh+Genta group was not significant in determining pre-renal azotemia and increased serum creatinine levels (Figure 6).

**Figure 6.** Mean creatinine values (in mg/dL). Number 1 is the interaction between the Control group and death on day 5; number 2 is the interaction between the Genta group and death on day 5; number 3 is the interaction between death on day 5 and the Deh+Genta group and numbers 4, 5 and 6 refer to the interaction between death on day 8 and the Control, Genta and Deh+Genta groups.

Various factors cause renal alterations, and the most commonly employed markers, urea and creatinine levels, only vary when approximately 66% to 75% of total nephron function is lost\(^{(33)}\). Therefore, the renal injury is present before the start of biochemical alterations in patients suffering from intrinsic renal insufficiency who demonstrate excess nitrogen in the blood.\(^{(34)}\)

Serum and urinary ggt

Serum GGT values were significantly higher in the Deh+Genta group compared to the other two groups (Figure 7). Dehydration reduces glomerular filtration rates and increases tubular reabsorption\(^{(35)}\). As urinary GGT is secreted by brush border cells of the proximal convoluted tubule, the tubular fluid is systemically absorbed, as indicated by the higher serum GGT levels observed in the Deh+Genta group until the eighth day of the experiment. Crivellenti et al.\(^{(36)}\) observed false positive urinary GGT values when administering a non-nephrotoxic drug in dogs. It is possible that the urinary GGT values observed herein originate from tubular lesions.

**Figure 7.** Mean serum GGT values (in UI/L). Number 1 is the interaction between the Control group and death on day 5; number 2 is the interaction between the Genta group and death on day 5; number 3 is the interaction between death on day 5 and the Deh+Genta group and numbers 4, 5 and 6 refer to the interaction between death on day 8 and the Control, Genta and Deh+Genta groups.
The urinary GGT levels in dogs reported by Dantas et al.\(^{23}\) and Melchert et al.\(^{28}\) indicated that GGT is a good early marker for acute tubular lesions, as this enzyme increased in urine three days after inducing AKI. Other more reliable biomarkers for the early detection of acute tubular lesions have been evaluated, based on increased urine levels within two days of AKI induction\(^{24}\). In the present study, urinary GGT values for the three groups did not show any interaction with euthanasia timing (Figure 8). The rats that received gentamicin presented equal and higher levels than the Control group, corroborating of Vicente-Vicente et al.\(^{37}\), who reported that gentamicin causes proteinuria and enzymuria. No histopathological alterations regarding hepatic tissue were noted, indicating that enzyme GGT alterations did not interfere in renal excretion, causing increased GGT urinary levels. Nale et al.\(^{38}\) observed hepatic alterations after intraperitoneal gentamicin administration at a daily dose of 40 mg/kg for 14 days. In turn, Noorei et al.\(^{39}\) observed a hepatotoxic gentamicin effect at a daily dose of 80 mg/kg for 7 days, with hepatocyte cord disarrangement with granular cytoplasm alterations, multifocal hepatocyte edema, congestion of the central, portal and sinusoidal blood vessels, focal degeneration and necrosis, alongside mononuclear cell infiltration. We used a daily dose of 100 mg/kg, divided into two equal applications, and still no hepatic impairment was observed, as reported by other authors.

**Urinary protein**

Urinary protein levels only increased after the fifth day of the experiment. The Genta group presented significantly higher levels than the Control and Deh+Genta groups. The animals submitted to dehydration presented lower urinary protein levels and urinary debit (Figure 9). By dividing the average values for urinary protein by the urinary debit, we obtained urinary protein/ml of urine, in mg. Employing this metric, the Deh+Genta and Genta groups were statistically equal (p = 0.46), while the Control group was significantly different than the Deh+Genta group (p = 0.01). No significant difference (p = 0.21) between the Control and Genta groups was observed. As mentioned previously, gentamicin causes proteinuria and enzymuria\(^{24, 28, 37}\), but severe dehydration masks these effects, resulting in false negative values.

**Hematology**

The hematology data were recorded before the start of the experiment to assure the animals were healthy, without concomitant affections. The average values of the hematological variables of the animals from all three groups were within the standards established for the species\(^{40}\).

The animals were submitted to another hemogram to compare the hematological findings 48 hours after the start of the experiment. The erythrocyte and hematocrit counts in the Deh+Genta group were significantly different (p < 0.05), i.e., the blood component values on the third day in this group were different from the other groups and higher than in the same group on the first day, due to higher plasma concentrations. The hematology of the Deh+Genta group indicated relative polycythemia, corroborating Lopes et al.\(^{41}\). Relative polycythemia is generally characterized by higher hematocrit counts due to dehydration, which, in turn reduces the plasma volume and increases plasma protein and red blood cell concentrations. Leucopenia is often associated with decreased neutrophils, and may be due to physiological causes or be drug-induced \(^{42}\) (Table 2).

**Table 2:** Means and standard errors of the hematological parameters between days 1 and 3 for the Deh+Genta group

<table>
<thead>
<tr>
<th>P-value</th>
<th>Day 3</th>
<th>Day 1</th>
<th>Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0005</td>
<td>8.76±0.13</td>
<td>7.93±0.13</td>
<td>Erythrocytes (x10⁸/µL)</td>
</tr>
<tr>
<td>0.318</td>
<td>13.14±0.27</td>
<td>12.24±0.27</td>
<td>Hemoglobin (g/dL)</td>
</tr>
<tr>
<td>0.0018</td>
<td>48.13±0.6</td>
<td>44.88±0.6</td>
<td>Hematocrit (%)</td>
</tr>
<tr>
<td>0.1912</td>
<td>4.96±0.65</td>
<td>6.22±0.65</td>
<td>Leucocytes (µL)</td>
</tr>
<tr>
<td>0.0971</td>
<td>8.15±0.15</td>
<td>7.76±0.15</td>
<td>Total proteins (g/dL)</td>
</tr>
</tbody>
</table>
Conclusions

The findings reported herein indicate that the administration of 100 mg/kg of gentamicin, BID, despite causing acute kidney injury, did not result in liver changes. The ARF model in Wistar rats induced by gentamicin and enhanced by dehydration was able to provoke polycythemia caused by dehydration after 48 hours of water restriction. It is evident that decreased glomerular filtration rates caused by dehydration increase the concentrations of blood components and mascara, urinary components and kidney tissue. Asear for efficient AKI biomarkers is, therefore, paramount, as the urinary enzyme GGT is not a reliable early biomarker to detect acute kidney injury.

Conflict of interests

The authors declare no conflict of interests.

Author Contributions


References


