Gentamicin-induced Acute Kidney Injury in Equines is associated with Marked Acute Phase Response: An Experimental Study on Donkey (Equus asinus)

Maged El-Ashker1*, Engy Risha2, Fatma Abdelhamid2, Mohamed Salama3, Mahmoud El-Sebaei3 and Walaa Awadin4

Abstract
Recently, there is growing evidence suggesting that acute kidney injury (AKI) in human and laboratory animals is associated with an inflammatory response that could play a role in tissue damage. However, such link has not previously been addressed in equines. The present study was designed to evaluate the effect of gentamicin (GEN) administration on the development of AKI and systemic inflammatory response in equines using donkey as a model. GEN (10%) was administered intravenously in six donkeys at a dose of 20 mg kg⁻¹ BW thrice daily for 14 consecutive days. Three other donkeys were randomly assigned to receive saline solution and served as controls. The donkeys were clinically and sonographically examined throughout the experimental period. Blood and urine (U) samples were simultaneously collected at day (D) 7, and D 14 of GEN administration. Renal specimens from all donkeys were collected at D 14 and processed for routine histopathological examination. AKI was confirmed by sonography, laboratory measurements, histopathology and immunohistochemistry. Our findings showed that serum amyloid A, haptoglobin, fibrinogen, interleukin (IL)-6, interferon gamma, IL-10, sialic acid, serum creatinine, U- gamma-glutamyl transpeptidase, and fractional excretion of sodium were significantly higher at D 7 and D 14 compared with those of controls (P<0.05). In addition, values of IL-1β, sialic acid, serum urea, and U-sodium were higher at D 14 than those at D 7; however, they did not reach statistical significance. From the obtained results, it is clear that GEN-induced AKI in donkeys is associated with marked acute phase inflammatory response. This response could play a role in the development of AKI in donkeys. Targeting these inflammatory alterations could serve as a new therapeutic strategy to alleviate AKI in equines.

Keywords: Acute kidney injury; Equines; Inflammatory response; Immunohistochemistry

Introduction
The kidney is particularly susceptible to ischemia and toxins with a resultant vasoconstriction, endothelial damage, and activation of inflammatory processes. Indeed, there are many potential causes of acute kidney injury (AKI), and each has its own characteristics. In equines, AKI is usually pre-renal or renal in origin and is usually associated with hemodynamic or nephrotoxic insults. The main causes of AKI are nephrotoxins, aminoglycosides, oxytetracycline, and some steroid anti-inflammatory drugs (NSAIDs) [1]. Gentamicin (GEN) is a potent aminoglycoside antibiotic that is widely used in equines due to its bactericidal effect on gram-negative and some gram-positive micro-organisms [2]. Despite the frequent use of GEN in equine practices, its potential nephrotoxicity has been a real concern for veterinarians. GEN is often given two or thrice a day. However, in recent years, several experimental and clinical studies have demonstrated that a single daily dose could be as effective as, and possibly less toxic than, multiple dosing [3].

Both innate and adaptive immune responses are important contributors to the development of ischemic injury. The innate component is responsible for the early response to infection or injury and is foreign-antigen independent. The acute phase response (APR) also provides an early non-specific defense mechanism opposing tissue injury or inflammation before specific immunity is achieved [4]. APR is induced by cytokines that act as messengers between the site of injury and the liver cells that synthesize acute phase proteins (APPs). These cytokines are soluble messenger proteins produced by a wide range of different cell types, which exhibit their actions within a local environment or in a systemic manner to modify and regulate immunological and inflammatory reactions as a part of their effects [5]. Under normal circumstances, cytokines are present at low concentrations and may not even be detectable in body fluids or tissues [6]. The pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, are secreted primarily by monocytes in response to bacterial toxins or local tissue injury and diffuse into blood where they could be detected in waves or pulses [7]. They induce local effects on the cells adjacent to the site of injury, as well as their systemic effects since they can travel to different target organs via blood stream [4,8]. In addition, APPs can serve as a prognostic tool, as their magnitude and duration may reflect the severity of infection [9]. They can also provide information about the incidence of both clinical and sub-clinical infections in the herd [10]. Sialic acid (SA), on the other hand, can mediate a variety of cell-cell interaction during inflammation and immune response. Its levels have been shown to be elevated in animals with various disorders [11,12]. It could be used to increase SA-containing erythropoietin that has organ protective effects, and can be useful in the prevention of AKI [13].

Recently, there is a growing body of evidence suggests that the inflammatory response associated with AKI can play a role in kidney damage in laboratory animals [14]. However, such link has not previously been addressed in equines. Therefore, the present study was designed to evaluate the effect of GEN administration on the development of AKI and systemic inflammatory response in equines using donkey as a model.

Materials and Methods
Animals and clinical examination

The present study was conducted in accordance with principles...
of good clinical practice, and all the procedures were performed in accordance with the Ethics Committee of the National Research Centre, Egypt; registration number (09/189). For the current study, nine apparently healthy adult (1.40 ± 0.37 years, body weight: 180 ± 25 kg) male donkeys (Equus asinus) were randomly selected and subjected to thorough clinical examination. Two weeks prior to the experiment, the selected animals were de-wormed by using Ivermectin (Equiwell, ADWIA, Egypt) with an oral dose of 0.2 mg kg⁻¹ BW. The donkeys were fed on corn silage, bran, and tibn. Food and water were offered ad-libitum. The animals were clinically monitored throughout the experimental period. A special concern was given to the heart and respiratory rates, rectal temperature, mucous membrane color, appetite, water intake, general demeanor, posture, urination and defecation patterns, hair coat, cecal and colon motility. Complete blood picture and renal function tests were performed to all donkeys prior to the study (data not shown). The selected animals exhibited values within the normal reference range.

Ultrasoundographic examination

The kidneys and the liver were ultrasonographically examined while the animals were in standing position using 3.5MHz sector transducers (Chison Medical imaging Co. Ltd., Wuxi, China). For kidneys, the images were obtained by placing the sector transducer on the dorsal right and left 15th to 17th intercostal spaces; whereas, the liver was best scanned at the 6th to 14th intercostal spaces between the diaphragm and right dorsal colon.

GEN administration and sampling protocol

Six donkeys were randomly assigned to receive GEN 10% (Garavet, Memphis Co. for Pharmaceutical and Chemicals, Egypt) administered via intravenous injection at a dose of 20 mg kg⁻¹ BW thrice daily for 14 consecutive days, according to a previously described protocol [15]; while the other three donkeys received only saline solution and served as controls. Blood and urine samples were simultaneously collected from each donkey at the day 7 (D7) and the day 14 (D14) of GEN or saline injections.

Blood sampling and measurements

Ten mL blood sample was collected from each donkey through jugular vein catheter. The collected blood was divided into two aliquots. The first aliquot was collected in heparinized tube and rapidly centrifuged at 3000 x g for 10 min for separation of plasma that was used for estimation of fibrinogen (Fb) by using commercially available ELISA kit supplied by (My Bio Source, San Diego, California, USA) according to the manufacturer’s instructions. The second blood aliquot was added to a tube without anticoagulant for separation of blood sera. Serum samples were kept frozen at -20°C until needed for estimation of GGT (U-GGT), urea (U-urea), Cr (U-Cr), sodium (S-Na), total protein and albumin. The following biochemical variables were also measured using commercially available kits according to the manufacturer’s instructions; alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (S-GGT), alanine amino transferase (ALT), uric acid (U-urea), creatinine (S-Cr), sodium (S-Na), total protein and albumin.

Urine collection and measurements

Urine (U) samples were collected from each donkey by urethral catheterization without sedation. Each urine sample was divided into two aliquots. The first one was used for microscopic examination and measuring specific gravity (U-SG) using (Medi-Test Combi 10, 93067, Germany). Whereas, the other aliquot was centrifuged at 1,500 x g for 10 min and the supernatant was stored at -20°C until needed for estimation of GGT (U-GGT), urea (U-urea), Cr (U-Cr), and Na (U-Na) using commercially available kits according to the manufacturer’s instructions. Urine/Serum urea ratio (U/S urea), Urine/Serum Cr ratio (U/S Cr), and fractional excretion of sodium (FE, Na) were also calculated. FE, Na was calculated according to the equation (S-Cr X U-Na) / (S-Na X U-Cr).

Histopathological and Immunohistochemical (IHC) examinations

At D 14, all animals were necropsied. Kidney specimens were collected from all donkeys then fixed in 10% neutral buffered formalin. Fixed specimens were processed for routine histopathological examination. Paraffin sections of 5μm thickness were cut and picked up on uncoated slides, dried, deparaffinized, rehydrated with graded alcohol, washed and stained with H&E according to the standard protocols. IHC was applied on paraffin embedded kidney tissue from all groups using antibodies against caspase-3, α-smooth muscle actin (α-SMA), vimentin and transforming growth factor-β (TGF-β) (Santa Cruz, CA) (1:100). After being deparaffinized with xylene, renal sections were rehydrated in ascending grades of ethyl alcohol and treated with 3% H₂O₂ for 10 min. Sections were blocked with 2% animal-free serum for 30 min at 37°C. Following 1-hour overnight incubation at 4°C with primary antibodies, the sections were washed twice in PBS, and incubated with a peroxidase conjugated secondary IgG antibody for 30 min. Bound antibodies were detected using 3, 3’-diaminobenzidine tetra hydrochloride. The sections were counterstained with Mayer’s hematoxylin. All stained sections were visualized with a light microscopy (binocular, Olympus). Images were taken using digital camera (Canon 5 mega pixels, 3.2x optical zoom). The positive signal of caspase-3, α-SMA, vimentin and TGF-β was brown granular mass. A total of 15 fields per donkey (three fields per section and five sections per donkey) were randomly selected and examined. A semi-quantitative score from 0 to 3 was given to assess the intensity of this positive signal. Zero; when negative, 1; if the antigen was located infrequently, 2; if the antigen was moderately distributed in the field and 3.

Statistical analysis

Data were statistically analyzed by using statistical software program (SPSS, version 15, USA). Means and standard deviation (mean ± SD) for each of the measured variables were calculated. Significant differences (P<0.05) between groups were calculated by one-way ANOVA and Duncan’s post hoc test. Scores of IHC positive signal were tested by student t test to compare between control and GEN-treated donkeys. The means were considered significantly different when P<0.05.
Results

Clinically, the heart rate, respiratory rate, and rectal temperature showed no significant differences among all examined donkeys during the 14 day-study period (Table 1). All animals showed normal behavior, urination and defecation patterns, hair coat, mucous membrane color, cecal and colon motility. However, ammonia odor of the buccal breath and dental tar were detected at D 14.

Ultrasonically, the right and left kidney were located deep to the most caudal ribs (15° to 17°) and (16° to 17°), respectively. Occasionally, the kidneys could not be imaged because the bowel containing gas, interposed between the kidney and the body wall, interfered with the examination. In such case, re-examination was carried out for kidney imaging. In control animals, kidney showed several hypo echoic medullary pyramids that were surrounded by the uniform echogenicity of the cortex. There was also a distinct demarcation between the cortex and the medulla. The renal pelvis was visualized as a bright echogenic line in the center of the kidney (Figure 1). From the D 7 and onwards, the kidneys showed loss of their architecture without any demarcation between the cortex and the medulla (Figures 2 and 3). As with the spleen, the architecture of the liver was relatively homogenous with more vessels, and the liver was generally more echogenic than spleen; the portal vein was more echogenic than the hepatic veins. In one of the examined donkeys, the liver showed course echogenic deposits at D14 without any detectable abnormalities in the others.

Biochemically, values of SAA, Hp, Fb, IL-6, INF-γ, IL-10, SA, S-Urea, S-Cr, U-GGT, and FE were significantly higher at D 7 and D 14 compared to controls (P<0.05) (Tables 2 and 3) and (Figures 4 and 5). Moreover, values of IL-1β, SA, S-Urea and U-Na were higher at D 14 than those at D 7; however, they did not reach statistical significance (Tables 2 and 3) and (Figure 5). ALP, GGT, and ALT showed no significant differences during the experimental period (Table 2). Total protein values were significantly higher at D 14 than those of D 7 and controls (P<0.05), with no significant differences between D 7 and controls. Globulin levels were significantly higher at D 14 and D 7 than controls. Regarding albumin levels, they were lower at D 7 and D 14; however, they did not reach statistical significance (Table 2). Values of U-SG, U-Urea, U/S urea, U-Cr, and U/S Cr were significantly lower at D 7 and D 14 than those of controls (P<0.05) (Table 3). The microscopic examination of the urine samples revealed the appearance of red blood cells, epithelial cells and granular casts at D 7 and D 14 compared with those of controls. Traces of protein, amorphous phosphate as well as calcium carbonate crystals were observed in controls; while, triple phosphates as well as calcium oxalate crystals were detected at D 7 and D 14 in GEN-treated animals.

Histopathologically, the control group showed normal histological picture of the kidneys. However, kidneys of GEN-treated donkeys showed focal mononuclear cells aggregation, tubular dilatation, focal glomerular fibrosis and marked interstitial edema particularly in the medullary regions. Tubular epithelial individualization and desquamation were also shown (Figure 6 A–E). Positive IHC staining of caspase-3 was demonstrated in the glomeruli and tubular structures, a-SMA and vimentin were expressed in the glomeruli and interstitial renal tissue. Meanwhile, TGF-β was only visualized in treated donkeys in areas of interstitial macrophage aggregation (Figure 7 A–D). Statistical analysis of positive signals of caspase-3, a-SMA and vimentin showed significant higher scores in GEN-treated donkeys than in control ones (Figure 8).

Discussion

Our interest in the diagnosis of AKI in equines was motivated by the increase in numbers of clinical cases, indicating that it could be a more common problem than it is generally believed. In the present study, we hypothesized that GEN-induced AKI in equine could be associated with cytokine-mediated inflammatory processes. To test this hypothesis, we used draft donkeys as an experimental model for induction of AKI by using GEN as previously mentioned. In general, the examined donkeys did not exhibit clinical illness throughout...
Figure 3: Ultrasonogram of the right kidney at D14 post administration of gentamicin. The kidney (2) shows loss of its architecture and there was no demarcation between cortex and medulla. 1: abdominal wall; (3) liver; CR: cranial; Ca: caudal; L: lateral; M: medial.

Figure 4: Means ± SD of serum amyloid A (µg/mL), haptoglobin (mg/dL), C reactive protein (mg/L) and fibrinogen (mg/dL) in donkeys with AKI at D 7 and D 14 of GEN exposure compared with controls. Bars labeled with different letters are statistically significant (P < 0.05).

Figure 5: Means ± SD of selected inflammatory cytokines (pg/mL) in donkeys with AKI at D 7 and D 14 of GEN exposure compared with controls. Bars labeled with different letters are statistically significant (P < 0.05).

Figure 6: A. Kidney of the control group shows normal histological picture. B-E. Kidney of treated donkeys shows: B. Focal mononuclear cells aggregation in interstitial tissue (arrow) and tubular dilation (arrowheads) (H&E x 100). C. Focal glomerular fibrosis (arrow) (H&E x 200). D. Marked interstitial edema in the medulla (asterisks) (H&E x 100). E. Tubular epithelial desquamation (arrowheads) (H&E x 200).

Figure 8: A. Kidney of the control group shows normal histological picture. B-E. Kidney of treated donkeys shows: B. Focal mononuclear cells aggregation in interstitial tissue (arrow) and tubular dilation (arrowheads) (H&E x 100). C. Focal glomerular fibrosis (arrow) (H&E x 200). D. Marked interstitial edema in the medulla (asterisks) (H&E x 100). E. Tubular epithelial desquamation (arrowheads) (H&E x 200).

Figure 7: Kidney of the treated group shows positive IHC signal for A: caspase-3 in the glomeruli (arrowhead) and extremely damaged tubular structures (arrow) (IHC x 150); B. α- SMA in the glomeruli (arrow) and interstitial renal tissue (arrowhead) (IHC x 150); C. vimentin in the glomeruli (arrowhead) and interstitial renal tissue (arrow) (IHC x 150); D. TGF-β in areas of interstitial macrophage aggregation (arrow) (IHC x 200).
It has also been stated that more than two thirds of nephrons must be of the proximal tubular epithelium were apparent in all ponies [17]. Out of 7 did not develop physical or behavioral abnormalities after 14 part similar to a previous report in adult pony mares where 5 ponies sonography, laboratory, histopathologic and IHC findings confirmed the 14 day-study period following acute GEN exposure. However, and D 14 of GEN exposure compared with controls.

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<td>U-β2-microglobulin (mg/dl)</td>
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The development of AKI in the examined donkeys could be attributed to toxic accumulation of GEN in the epithelial cells of the renal proximal tubules that could interfere with lysosomal, mitochondrial, and Na/K/ATPase functions [1]. The acidic phospholipids were considered as binding sites for GEN in the brush-border membrane of proximal tubular cells [19]. Previous reports have described that transient nephrotoxicity was observed following GEN administration in ponies at 3 mg kg⁻¹ three times daily on the first day followed by 4.5 mg kg⁻¹ twice daily for five consecutive days [2]. However, in another study, acute renal failure (ARF) was achieved in ponies following a dose regimen of 20 mg kg⁻¹ three times daily for 14 days [15]. It seems likely that the GEN-induced nephrotoxicity is apparent with time as shown in the present study, where the maximum nephrotoxicity was evaluated at the 14th day of GEN exposure. The tested liver enzymes showed no statistical variation throughout the experiment. This finding suggests that nephrotoxicity is more liable to occur in donkeys after toxic GEN exposure.

The increased U-GGT levels could reflect the damage occurred to the brush borders of the proximal tubular epithelial cells [1]. Previous studies have suggested that U-GGT/Cr ratio could be an early predictor of AKI in horses with NSAID toxicity [20] and those with colic [21]. However, this ratio may be falsely elevated in sick animals through a decrease in Cr excretion resulting from reduction in glomerular filtration rate [20]. The observed changes in the proteinuria including hyperproteinemia, hypo albuminemia, and hyper globulinaemia could be attributed to acute phase changes associated with systemic inflammatory response in animals with renal failure. In contrast; other reports showed that nephritic rats had lower levels of serum total protein, albumin, and globulin than those of normal rats [22-24].

Recently, it has been suggested that the GEN nephrotoxicity in experimental animals could be associated with an inflammatory response [14] with increased cytokine production and increase capillary permeability. However, such response has not previously been evaluated in equines with AKI. Our findings clearly demonstrate that GEN-induced AKI was associated with higher levels of APPs (SAA, Hp, Fb, and CRP), as well as inflammatory markers (IL-1β, IL-6, INF-γ, IL-10 and SA). IL-10 is known to antagonize the cascade of pro-inflammatory cytokines that develop as a part of the APR. These data suggest that a disturbance of pro-inflammatory and anti-inflammatory cytokines may occur in parallel during AKI. The increased levels of APPs and cytokines in animals following GEN injection could suggest that these factors can play a role in AKI. It was previously reported that IL-10 was protective against ischemic and cisplatin-induced acute kidney injury in mice [25] and mediated rosiglitazone-induced kidney protection in cisplatin nephrotoxicity [26], implying that the inflammatory responses could play a role in the development of renal injury. Such role of cytokines was demonstrated in mice lacking intraceluar adhesion molecule -1, these mice were shown to be protected against acute renal ischemic injury [27].

In previous reports, it was hypothesized that ischemia, sepsis, and nephrotoxic models of AKI may result in morphological and/or functional changes in vascular endothelial cells and/or in tubular epithelium [6,14,28,29]. The endothelial and tissue injuries are associated with the release of specific mediators that may initiate

### Table 1: Clinico pathological variables in donkeys with AKI at D 7 and D 14 of GEN exposure compared with controls. * a,b Variables with different superscripts in the same raw are significantly different at P < 0.05. ALP: alkaline phosphatase; GGT: gamma-glutamyl transpeptidase; ALT: alanine amino transferase; S-Cr: serum creatinine; Na: sodium.

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the inflammatory cascades. These cascades might be augmented by generation of pro-inflammatory and chemotactic cytokines by the ischemic proximal tubule [14,28]. On the other hand, it is reasonable to think that the inflammatory response acts as an amplifying mechanism of damage. Initially, destruction of the cells by necrosis can initiate an inflammatory state. Tissue debris and cell content shed into the extracellular space would trigger inflammation [30]; while an exaggerated inflammation would contribute to further damage that in turn would exacerbate the inflammatory response [31]. This inflammation also activates the glomerular cells which in turn produce cytokines and growth factors that contribute to the pathophysiological process with different effects. It was found in a recent study that serum IL-6 was significantly elevated in both ischemic AKI and bilateral nephrectomized mice, and in AKI patients that could predict mortality [32]. It is worth mentioning that the link between IL-6 and mortality has previously been shown in human patients with chronic kidney disease [33,34]. Besides its possible role in AKI, the inflammatory response could also have potential systemic consequences including pulmonary and cardiac injuries [35,36].

The classic findings of various serum and urinary biochemical variables as well as histopathological findings and HIC confirmed the occurrence of AKI in the investigated donkeys. Structurally, GEN-related nephrotoxicity is associated with the edema of proximal tubular cells, glomerular hypertrophy, perivascular edema, inflammation, glomerular congestion, cellular desquamation, glomerular atrophy, tubular necrosis, and tubular fibrosis [37-43]. GEN causes macrophage infiltration and higher TGF-β which may lead to progression of tubule interstitial nephritis [37]. In previous report, it was stated that the histological scores of tubular degeneration, tubular necrosis and tubulo-interstitial nephritis increased significantly in GEN-treated rats compared to control group [44]. AKI as a result of GEN-induced tubular necrosis stimulates inflammatory events by recruiting intercellular adhesion molecule-1 and monocyte chemotactic protein-1 at the site of injury that enhance the migration of monocytes and macrophages to the site of tissue damage, ultimately leading to renal pathology [45]. The above mentioned data explained our histopathological findings while the HIC expression of caspase-3, vimentin, α-SMA and TGF-β in AKI were compatible with those previously described by several researchers [37,46,47].

Conclusion

It is clear that GEN-induced AKI in donkeys is associated with a marked acute phase response. It is possible that this inflammatory reaction could play a role in the development of AKI. Such findings can be useful to adopt new therapeutic strategies to restore inflammatory alterations associated with AKI and to improve its prognosis. Further studies are needed to evaluate the efficacy of these cytokines to predict the outcomes and extent of AKI in equines.

Disclosure

The authors of this paper have no financial or personal relationship with other people or organizations that could appropriately influence or bias the content of the paper.

Authors Contributions

Maged El-Ashker designed and coordinated the study, responsible for animal handling and examinations; performed sonographic examination, samples collection, take part in data analysis and interpretation, writing of the manuscript, review of the final manuscript, and responsible for all correspondence with the journal.

Engy Risha and Fatma Abdelhamid participated in laboratory and biochemical measurements, take part in data analysis and interpretation, writing of the manuscript, and review of the final manuscript.

Mohamed Salama performed SA measurements, participated in the other laboratory and biochemical measurements, data analyses and interpretation, participated in writing, graphs preparation, and edited the final version of the manuscript.

Mahmoud El-Sebaei participated in SA measurements, participated in laboratory analyses and measurements, take part in writing of the manuscript and review of the final manuscript.

Walaa Awadin is responsible for all processes of histopathology examinations and interpretation of the data. All authors approved the final version of the manuscript.

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