

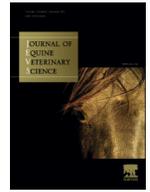


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Original Research

Vitamin C Ameliorates Gentamicin-Induced Acute Kidney Injury in Equines: An Experimental Study

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ABSTRACT

To date, there is no specific treatment used to protect against gentamicin (Genta)-induced nephrotoxicity. The main objective of the present study was to determine the potential protective effect of vitamin C against Genta-induced acute kidney injury (AKI) in equines using donkey as a model. Nine apparently healthy adult male donkeys (*Equus asinus*) were included in this study. All animals were clinically sound. Three donkeys were randomly selected to receive saline solution and served as controls. The other six donkeys (treated groups) were randomly assigned to receive either vitamin C at a dosage of 30 mg/kg (GVC-group; n = 3) or saline solution (G-group; n = 3) via IV route once daily for 14 days. Animals of treated groups have concomitantly received Genta at a dosage of 20 mg/kg IV thrice daily for 14 consecutive days. Blood and urine samples were simultaneously collected at day 14 of Genta administration. Blood samples were used for measuring selected acute-phase proteins, cytokines profile, and oxidative stress mediators; whereas, urine samples were used for measuring different urinary analytes. Acute kidney injury was confirmed by classic laboratory findings. Our results showed that the serum amyloid A, haptoglobin, fibrinogen, C-reactive protein, interleukin (IL)-1 β , IL-6, interferon gamma, IL-10, sialic acid, malondialdehyde, blood urea, serum creatinine, alkaline phosphatase, and serum gamma-glutamyl transpeptidase were significantly lower in GVC-group than those in G-group ($P < .05$). In contrast, total antioxidant capacity was much lower in G-group than that of GVC-group; however, they did not reach statistical significance. The results presented herein suggest that vitamin C could have a protective effect against Genta-induced AKI in equines. The ongoing trials orchestrated with improved diagnostic utilities can improve the outcomes of AKI in equines through prophylactic or early use of antioxidant therapy.

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1. Introduction

Acute kidney injury (AKI) is generally defined as a decline in kidney function resulting in accumulation of waste products in the bloodstream. The main causes of AKI

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are nephrotoxins, aminoglycosides, oxytetracycline, and nonsteroidal anti-inflammatory drugs (NSAIDs) [1].

Gentamicin (Genta) is an aminoglycoside antibiotic routinely used in every day equine clinical practices for the treatment of gram-negative infections either alone or in a combination with beta-lactam antibiotics [2]. Its low resistance rate and its reasonable cost make it the first antibiotic of choice for life-threatening diseases. Despite its frequent use in equine practices, its potential nephrotoxicity has been a real concern. Genta-induced

nephrotoxicity is characterized by morphologic and functional alterations of the renal tissue which lead to development of AKI [3]. The early detection of AKI is critical for the effective therapy as well as prognosis. However, the conventional laboratory approaches to detect AKI, such as measuring serum creatinine (S-Cr) and blood urea nitrogen (BUN) concentrations are not efficient for early detection of the cases as there is a time gap between kidney injury and the alterations in these indicators.

To date, there is no specific agent used to protect against Genta-induced nephrotoxicity. In this regard, various medications have been used concomitantly with Genta to prevent AKI in laboratory animal models [3–10]; however, in equine medicine, scarce reports are available on the protective therapies against AKI [11]. Several researchers have examined the potential therapeutic role of antioxidants in both experimental animal models and human studies of AKI in several clinical settings [3,5,6,8,9,12]. Among the different antioxidants tested, vitamin C exhibited a powerful scavenging property against free radicals and activated oxygen species. As an electron donor, vitamin C protects by neutralizing reactive oxygen species (ROS) and decreasing oxidative damages [13]. Vitamin C also exhibits anti-inflammatory effects, prevents endothelial dysfunction and apoptosis, and reduces the risk of cardiovascular diseases [14]. For example, vitamin C treatments improved kidney function in renal allograft recipients [15], decreased renal inflammation, and improved impaired renal function in salt-sensitive hypertensive rats [16]. In this view, the main objective of the present study was to determine the potential protective effects of vitamin C against Genta-induced AKI in equines using donkey as an experimental model.

2. Materials and Methods

2.1. Animals

The present study was conducted in accordance with principles of good clinical practice, and all the procedures were approved by the Ethical Committee for Animal Experiments at Mansoura University, Egypt. Nine apparently healthy adult male donkeys (*Equus asinus*), weighing 150–200 kg body weight (BW), were included in this study. All animals were clinically examined. Three donkeys were randomly selected to receive saline solution and served as controls, whereas the other six donkeys (treated groups) were randomly assigned to receive either vitamin C at a dosage of 30 mg/kg (GVC-group; $n = 3$) [17] or saline solution (G-group; $n = 3$) via IV route once daily for 14 days. Animals of treated groups have concomitantly received Genta at a dosage of 20 mg/kg IV thrice daily for 14 consecutive days after a previously described protocol [11]. Two weeks before the experiment, the selected donkeys were dewormed by using ivermectin (Equivectin; ADWIA, Egypt) at an oral dose of 0.2 mg/kg BW. The donkeys were fed on corn silage, bran, and hay. Food and water supply 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, and cecal and colon motility. Complete blood picture and renal function tests were carried out on all donkeys before the study (data not shown). The selected animals exhibited values within the normal reference range.

2.2. Sampling Protocol

Blood and urine samples were simultaneously collected from each donkey at the 14th day of Genta administration. Briefly, blood was collected from each donkey through jugular vein catheter. Half of the collected blood (10 mL) was added to a plain tube (without anticoagulant) and left at room temperature for 15 minutes to coagulate and then centrifuged at 3,000xg for 15 minutes for separation of blood sera. Only nonhemolyzed sera were collected and kept frozen at -20°C until required for the measurement of circulating cytokines, acute-phase proteins (APPs), and classic biochemical profile. The levels of different cytokines including interleukin (IL)-1 β , IL-6, interferon (INF)-gamma, and IL-10 were measured in undiluted sera according to the manufacturer's instructions by using commercially available equine enzyme-linked immunosorbent assay (ELISA) kits supplied by (Genorise Scientific, Inc, Paoli, PA). The plates were read at 450 nm and a correction wavelength of 550 nm using an automated microplate reader (Bio TEC). Values were expressed in pg/mL. Samples were run in duplicates for all examined cytokines. Serum amyloid A (SAA), haptoglobin (hp), and C-reactive protein (CRP) were measured by using equine ELISA kits supplied by (MyBioSource, San Diego, CA) and an automated microplate reader (Bio TEC) according to the manufacturer's instructions. Sialic acid (SA) was measured in the serum samples as previously described [18] by using Ehrlich reagent (made by adding 0.7 g of p-dimethyl aminobenzaldehyde [Sigma Aldrich] to 150 mL of concentrated HCl and 100 mL of distilled water). The absorbance of samples was measured at 525 nm by using Jenway 7305, UV-VIS spectrophotometer (Jenway Scientific Equipments, UK). Data were expressed as mmol/L. The following biochemical variables were also measured using commercially available kits according to the manufacturer's instructions, including alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (S-GGT), alanine amino transfease, total protein, albumin, S-Cr, and BUN. Globulin levels were also calculated. The other half of the blood sample was collected in a heparinized tube and was rapidly centrifuged at 3,000xg for 10 minutes for separation of blood plasma. The collected plasma was used for estimation of fibrinogen (Fb) and selected oxidative stress and antioxidant markers. Plasma Fb was measured by using commercially available ELISA kit supplied by (MyBioSource) according to the manufacturer's instructions. The kits used for the measurement of malondialdehyde (MDA), total antioxidant capacity (TAC), reduced glutathione (G-SH), and catalase (CAT) were obtained from Biodiagnostic, Egypt.

Urine (U) samples were collected from each donkey by urethral catheterization without sedation. Each urine sample was divided into two aliquots; one was used for microscopic examination and measuring of specific gravity

(U-SG); whereas, the other aliquot was centrifuged at 1,500xg for 10 minutes, and the supernatant was stored at -20°C and used within 1 week for estimation of urea (U-urea), Cr (U-Cr), GGT (U-GGT), Na (U-Na), and U-MDA. The U-SG was measured using (93067; Medi-Test Combi 10; Germany); whereas, the other urine biochemical variables were spectrophotometrically measured using commercially available kits according to the manufacturer's instructions. U-MDA was estimated by measuring thio-barbituric acid reactive substances, a method that has previously been proven to be efficient to measure MDA [19].

2.3. Statistical Analysis

Data were statistically analyzed by using SPSS statistical software package (version 15, SPSS Inc, Chicago). Means and standard deviation for each of the measured variables were calculated. Significant differences ($P < .05$) between groups were calculated by one-way analysis of variance and Duncan's post hoc test.

3. Results

An overview of significant clinical variables as well as biochemical and clinicopathologic profiles in donkeys with AKI and controls are presented in Tables 1–4 and Figs. 1 and 2. Clinically, the heart rate, respiratory rate, and rectal temperature showed no significant variations among all examined donkeys compared with controls. In general, all donkeys did not exhibit detectable clinical abnormalities throughout the study period and showed normal behavior, urination and defecation, hair coat, mucous membrane color, and cecal and colon motility. However, dental tar, ammonia breath, and increased water intake were observed in G-group and GVC-group (Table 1).

Biochemically, the inflammatory mediators including SAA, hp, CRP, Fb, IL-1 β , IL-6, INF- γ , IL-10, SA, and oxidative stress marker including MDA were significantly higher in G-group than those in GVC-group ($P < .05$; Table 2 and Figs. 1 and 2). Values of TAC were lower in both groups than

Table 1

Significant clinical variables in donkeys with experimentally induced acute kidney injury (GVC-group and G-group) compared with controls.

Variables	Control (n = 3)	G-Group (n = 3)	GVC-Group (n = 3)
Herat rate (b/min)	32.75 \pm 0.6 ^a	34.45 \pm 2.0 ^a	33.33 \pm 1.0 ^a
Respiratory rate (c/min)	12.02 \pm 1.0 ^a	12.66 \pm 0.4 ^a	12.40 \pm 0.5 ^a
Rectal temperature ($^{\circ}\text{C}$)	37.46 \pm 0.03 ^a	37.52 \pm 0.04 ^a	37.53 \pm 0.03 ^a
Visible mucous membranes	Moist, pink in color	Moist, pink in color	Moist, pink in color
Dental tar	Absent	Present	Present
Ammonia odor at the buccal cavity	Absent	Present	Present
Water intake	Normal	Increased	Increased

G-group represents animals received gentamicin plus saline solution; GVC-group represents animals received gentamicin plus vitamin C.

^a Variables with a similar superscript letter in the same row are not significantly different at $P < .05$.

Table 2

Mean values \pm standard deviation of selected inflammatory markers in donkeys with experimentally induced acute kidney injury compared with controls.

Analytes	Control (n = 3)	G-Group (n = 3)	GVC-Group (n = 3)
Serum amyloid A ($\mu\text{g/mL}$)	9.98 \pm 2.77 ^a	146 \pm 27 ^b	43.26 \pm 25 ^a
Haptoglobin (mg/dL)	47.56 \pm 10.77 ^a	267 \pm 47 ^b	118 \pm 36 ^c
C-reactive protein (mg/L)	4.96 \pm 0.72 ^a	8.46 \pm 0.41 ^b	5.30 \pm 0.26 ^a
Fibrinogen (mg/dL)	164 \pm 4.93 ^a	342 \pm 27 ^b	220 \pm 3.42 ^c
Sialic acid (mmol/L)	0.117 \pm 0.006 ^a	0.170 \pm 0.005 ^b	0.130 \pm 0.002 ^c

G-group represents animals received gentamicin plus saline solution; GVC-group represents animals received gentamicin plus vitamin C.

^{a,b,c} Variables with different superscript letters in the same row are significantly different at $P < .05$.

those in control group ($P < .05$). Of note, TAC was much lower in G-group than that of GVC-group; however, it did not reach statistical significance. On the contrary, the antioxidative parameters including G-SH and CAT were higher in G-group than those in GVC-group ($P < .05$; Fig. 2). The microscopic examination of urine samples of G-group and GVC-group revealed the existence of red blood cells, epithelial cells, granular casts, triple phosphate, and calcium oxalate crystals. while, in control group, traces of protein, amorphous phosphate, and calcium carbonate crystals were also observed.

Although values of serum ALP were higher in G-group than those in GVC-group, they did not reach to statistical significance (Table 3). However, S-GGT values were significantly increased in G-group compared with those in GVC-group ($P < .05$). BUN and S-Cr were significantly higher in G-group than those in GVC-group ($P < .05$). Total protein and globulin values were significantly higher not only in G-group and GVC-group than those in controls, but also in G-group compared with GVC-group. Albumin levels were however lower in G-group than those of GVC-group

Table 3

Mean values \pm standard deviation of selected clinicopathologic variables in donkeys with experimentally induced acute kidney injury compared with controls.

Variables	Control (n = 3)	G-group (n = 3)	GVC-group (n = 3)
ALP (U/L)	411 \pm 64.20 ^a	689 \pm 2.51 ^b	500 \pm 92 ^b
GGT (U/L)	20.66 \pm 3.05 ^a	33.0 \pm 0.01 ^b	22.3 \pm 0.57 ^a
ALT (U/L)	11.99 \pm 1.54 ^a	13.65 \pm 1.45 ^a	14.22 \pm 1.12 ^a
S-urea (mmol/L)	4.93 \pm 0.09 ^a	9.03 \pm 0.33 ^b	6.60 \pm 0.166 ^c
S-Cr ($\mu\text{mol/L}$)	84.27 \pm 16.42 ^a	157.35 \pm 7.07 ^b	122.27 \pm 3.53 ^c
Total protein (g/dL)	8.43 \pm 1.01 ^a	16.0 \pm 2.64 ^b	12.48 \pm 0.56 ^c
Albumin (g/dL)	4.41 \pm 0.19 ^a	3.76 \pm 0.15 ^b	4.30 \pm 0.10 ^a
Globulin (g/dL)	4.01 \pm 0.9 ^a	12.23 \pm 2.51 ^b	8.18 \pm 0.63 ^c

Abbreviations: ALP, alkaline phosphatase; ALT, alanine amino transferase; GGT, gamma-glutamyl transpeptidase; S-Cr, serum creatinine.

G-group represents animals received gentamicin plus saline solution; GVC-group represents animals received gentamicin plus vitamin C.

^{a,b,c} Variables with different superscript letters in the same row are significantly different at $P < .05$.

Table 4

Mean values \pm standard deviation of urine biochemical variables in treated (GVC-group) versus not treated (G-group) donkeys with experimentally induced acute kidney injury compared with controls.

Variables	Control	G-Group	GVC-Group
USG	1.032 \pm 0.002 ^a	1.008 \pm 0.003 ^b	1.014 \pm 0.005 ^c
U-GGT (U/L)	21.6 \pm 7.63 ^a	55.10 \pm 5.10 ^b	36.00 \pm 7.21 ^c
U-Cr (mmol/L)	17.36 \pm 1.45 ^a	4.0 \pm 1.0 ^b	7.30 \pm 1.52 ^c
U-GGT/Cr ratio	1.24	13.77	4.93
U-urea (mmol/L)	21.64 \pm 0.33 ^a	14.30 \pm 0.57 ^b	16.30 \pm 0.57 ^c
U-Na (mmol/L)	19.33 \pm 8.14 ^a	89.6 \pm 3.51 ^b	48.3 \pm 7.63 ^c

Abbreviations: USG, urine specific gravity; U-GGT, urine gamma-glutamyl transpeptidase; U-Cr, urine creatinine; U-Na, urine sodium.

^{a,b,c}Variables with different superscript letters in the same row are significantly different at $P < .05$.

and controls ($P < .05$; Table 3). Compared to GVC-group, G-group showed significantly increased values of U-GGT and U-sodium; whereas, values of U-SG, U-urea, and U-Cr were significantly decreased (Table 4).

4. Discussion

The findings of the present study suggest that vitamin C could have a potential protective effect against Genta-induced AKI in equines. In the past decade, most of the advances in the area of Genta-induced nephrotoxicity were focused on the molecular biology, genetic basis, and pathophysiology of nephrotoxicity [20–22]. However, several researchers have explored the potential therapeutic role of antioxidants in AKI in both experimental animal models and human clinical studies. In this regards, several trials have been performed to evaluate the protective effects of vitamin C on kidneys in humans and laboratory animal models [3,15,23]. However, such effect has not previously been evaluated in equines with AKI. We hypothesized that vitamin C might have a potential protective effect against Genta-induced nephrotoxicity in equines. In the present study, the examined donkeys did not exhibit any clinical

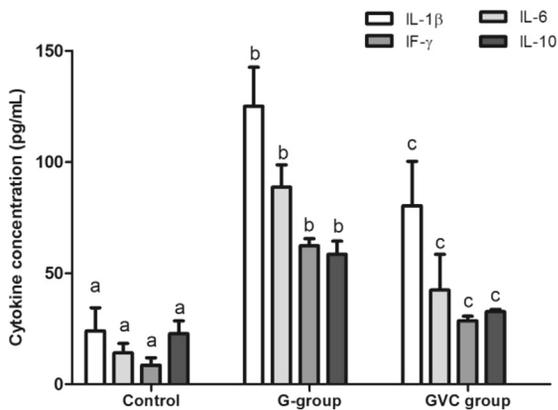


Fig. 1. Mean values \pm standard deviation of selected acute-phase cytokines in donkeys with experimentally induced acute kidney injury compared with controls. Bars labeled with different letters are statistically significant ($P < .05$). G-group represents animals received gentamicin plus saline solution; GVC-group represents animals received gentamicin plus vitamin C; IF- γ , interferon gamma; IL, interleukin.

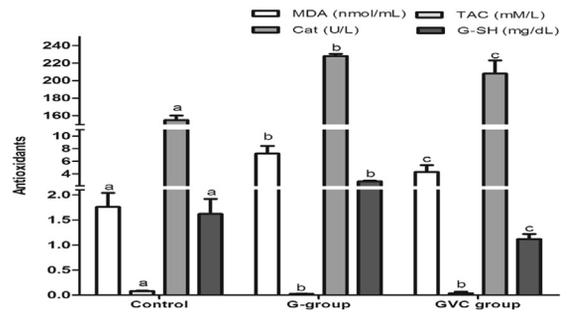


Fig. 2. Mean values \pm standard deviation of oxidative stress mediators in donkeys with experimentally induced acute kidney injury compared with controls. Bars labeled with different letters are statistically significant ($P < .05$). G-group represents animals received gentamicin plus saline solution; GVC-group represents animals received gentamicin plus vitamin C. Cat, catalase; G-SH, reduced glutathione; MDA, malondialdehyde; TAC, total antioxidant capacity.

illness throughout the 14-day study period. Similar finding was reported by Hinchcliff et al [24], where five ponies out of seven did not develop physical or behavioral abnormalities after 14 days of Genta administration, although the ultrastructural abnormalities of the proximal tubular epithelium were apparent in all ponies. Decreased specific gravity of the urine in both the G-group and GVC-group would suggest a loss of concentrating ability of the kidneys and would explain the increase in water intake. The Genta dose used in the present study is considered higher than the previously applied therapeutic dose where the conservative dose regimen of 3 mg/kg BW three times followed by 4.5 mg/kg BW twice daily for five consecutive days did not cause significant renal toxicity in healthy ponies [2]. Here, we aimed at developing a model of AKI as previously described [11].

The classical findings of serum and urinary biochemical profile confirmed the occurrence of AKI after 7 days of Genta exposure (data not shown) and became apparent after 14 days of Genta administration. The significant elevations of BUN and S-Cr concurrently with severe reduction in U-urea and U-Cr in Genta-treated animals are indicators of severe renal tubular necrosis. The increased GGT levels, a brush-border marker, suggest tubular damage and direct toxic injury. The observed changes in the proteinogram could be attributed to alterations in the acute-phase response and the associated systemic inflammatory response (as indicated by the results of APPs and circulating cytokines). These findings appear in part controversy to those previously obtained by other researchers [9]. Treatment with vitamin C resulted in less deterioration in the biochemical variables most likely because of its cytoprotective effect by inhibition of free radical production. Similar biochemical profiles have previously been reported in horses with NSAID-induced nephropathy and those with exertional rhabdomyolysis [25,26]. The development of AKI in the examined donkeys could be attributed to the toxic accumulation of Genta in the epithelial cells of the renal proximal tubules. It was previously suggested that the accumulation of Genta in the proximal tubules of the kidney might interfere with lysosomal, mitochondrial, and Na/K/ATPase functions [1].

In this study, G-group and GVC-group exhibited marked inflammatory and oxidative stress responses concomitantly with lower TAC and higher values of anti-oxidative parameters. However, the derangements in both inflammatory response and the oxidative status were more prominent in G-group than those in GVC-group. The increase in G-SH and CAT values in G-group could be a response toward increased ROS generation. The upregulation of antioxidant enzymes seems to render the cell more resistance to the subsequent oxidative damage. Our findings were in part similar to those reported in rats with CCl₄-induced oxidative damage [27]. However, our findings were opposite to those reported by other researchers who showed that G-SH and CAT were significantly decreased in rats with Genta-induced nephrotoxicity [12]. It is believed that the body has an effective defense mechanism to prevent and neutralize free radical-induced damage. This is accomplished by a set of endogenous antioxidant enzymes that constitute defensive team against ROS. It is suggested that administration of vitamin C could ameliorate the Genta-induced kidney damage. It was reported that vitamin C could protect against Genta-induced renal damage in rats [3]. Other investigators advocated that coadministration of moderate doses of vitamin C and vitamin E could protect against Genta-induced nephrotoxicity in rats [28]. Whereas, supplementation of vitamin E, either alone or in combination with vitamin C, was found to be effective in preserving the activity of superoxide dismutase in Genta-treated rats [29]. It was also recently suggested that ascorbic acid could provide a protection against contrast-induced AKI in humans [30].

Over the past years, we began to understand the underlying mechanisms behind the development of AKI. The generation of ROS and oxidative stress have been shown to play a key role in the pathogenesis of AKI in different clinical settings [25,26,31]. It was also hypothesized that Genta-induced AKI is associated with generation of free radicals [4,8,32,33], increase in intracellular calcium concentration, production of numerous inflammatory markers, and induction of apoptosis and necrosis [34]. In addition, Genta has been shown to induce iron release from renal cortical mitochondria [32], which is known to be a potent catalyst to generate free radicals. Moreover, Genta enhances the generation of ROS that is believed to cause nephrotoxicity [31] and cell death in many different pathologic states including glomerular disease and renal ischemia reperfusion injury [35–37]. Taken together, it could be suggested that Genta-induces nephrotoxicity is associated with disturbances of the renal tubular function, alterations in the antioxidant enzyme activities, and formation of lipid peroxides. Coadministration of vitamin C could have an ameliorative effect against Genta-induced AKI.

5. Conclusions

The data presented herein suggest that vitamin C has a potential protective effect against Genta-induced AKI in equines. However, further studies are required to explore this potential. The ongoing trials in concert with improved diagnostic utilities can improve the outcomes of AKI in

equines through prophylactic or early therapeutic use of antioxidants. Further studies are needed to evaluate the potential ameliorative effect of other agents in horses with AKI.

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