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Gentamicin Nephrotoxicity – A Comparison of *In Vitro* Findings with *In Vivo* Experiments in Equines

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ABSTRACT

The aminoglycoside gentamicin is often used in equine practice. Despite its clinical use, concerns remain regarding the potential toxic side-effects, such as nephrotoxicity, in equine patients, particularly after repeated dosing. The aim of the study was to investigate first *in vitro* the mechanisms contributing to the renal toxicity of gentamicin and to identify sensitive biomarkers indicating proximal tubule damage. To this end, the kidney-derived cell lines LLC-PK1 and MDCK were treated with gentamicin at different concentrations. Toxicity was assessed by measuring the release of gamma-glutamyl transferase (GGT), and the production of reactive oxygen species (ROS). Cell viability was measured using Alamar blue (AB) and Neutral red (NR) cytotoxicity assays. Gentamicin exerted a dose-dependent toxicity. Primarily, loss of brush border membrane integrity, indicated by GGT leakage, and an increased ROS production were observed. As GGT was found to be a sensitive marker for gentamicin-induced renal cell injury, in the subsequent *in vivo* experiments, in which ponies were given gentamicin (3.0 mg/kg bw three times daily and 4.5 mg/kg bw twice daily) for five consecutive days, plasma levels and the urinary excretion of GGT and creatinine were measured and the GGT:creatinine ratio was calculated. Elevated GGT levels in urine following gentamicin therapy were observed, but this enzyme leakage was transient and returned to baseline values after cessation of therapy. It could thus be concluded that even a conservative dose regimen of gentamicin did not result in significant renal toxicity in healthy ponies.

Keywords: dose regime, γ-glutamyl transferase, gentamicin, horses, nephrotoxicity

Abbreviations: AB, Almar blue; ANOVA, analysis of variance; ATCC, American Type Culture Collection; AUC, area under the curve; Cl, plasma clearance; C_{max}, maximum plasma concentration; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GGT, gamma-glutamyl transferase; H_2DCF, dichlorodihydrofluorescein diacetate; MDCK, Madin Darby canine kidney; MRT, mean residence time; NR, Neutral red; PBS, phosphate-buffered saline; ROS, reactive oxygen species; $t_{\frac{1}{2}\lambda_1}$, half-life of distribution; $t_{\frac{1}{2}\lambda_2}$, half-life of elimination; V_c, volume of the central compartment; V_{ss}, steady-state volume of distribution

INTRODUCTION

Gentamicin is a widely used aminoglycoside antibiotic, applied in equine medicine because of its bactericidal effects against Gram-negative and staphylococcal bacteria. Despite its frequent use, the potential nephrotoxicity of gentamicin has been a matter of concern in clinical pharmacotherapy. Renal toxicity of gentamicin is linked to its

accumulation in proximal tubule cells and is characterized by a mild rise in plasma creatinine levels, accompanied by an impairment of renal function in cases where prolonged therapy is needed. The relative toxicity of gentamicin appeared to correlate with the concentration in the renal cortex in experimental animals. Accumulation in renal tubule cells occurs following uptake via the organic anion transport system. As this is a saturable process, the degree of accumulation and toxicity is predominantly time-dependent and, to a lesser extent, depends on the individual dose given. This was exemplified in experiments demonstrating that a continuous infusion of gentamicin was more nephrotoxic than the same total dose given at intermittent time intervals (Powell *et al*., 1983).

Gentamicin-induced renal toxicity has been described in many experimental animal species, as well as in man (Frame *et al*., 1977; Riviere *et al*., 1983a,b; Houghton *et al*., 1986; Hinchcliff *et al*., 1989; Mattie *et al*., 1989; Gilbert, 1991; Prins *et al*., 1993). However, toxicity data in equines are scarce, as the majority of studies have described only the kinetics of gentamicin (Haddad *et al*., 1985; Hinchcliff *et al*., 1989; Clarke *et al*., 1992; Godber *et al*., 1995; Magdesian *et al*., 1998; Martin-Jimenez *et al*., 1998).

Hence, the aims of the present study were twofold. The first was to study the mechanisms of gentamicin toxicity in cells derived from either the proximal (LLC-PK1) or distal (MDCK) tubulus. Endpoints to describe gentamicin toxicity included the production of reactive oxygen species (ROS) (Trayner *et al*., 1995) and gamma-glutamyl transferase (GGT) release, as well as Alamar blue (AB) reduction and Neutral red (NR) uptake (Bull *et al*., 2001). Second, the toxicity and pharmacokinetics of gentamicicn were evaluated in the dose regime applied in clinical practice for post-surgery equine patients and early sins of nephrotoxicity were monitored by measuring GGT:creatinine ratio in urine during the course of gentamicin treatment and by postmortem histopathological examinations.

MATERIALS AND METHODS

In vitro *studies*

Phosphate-buffered saline (PBS), M199 and DMEM/F12 media, L-glutamine and fetal calf serum (FCS), were purchased from Invitrogen (Breda, The Netherlands). Neutral red, L-glutamyl-*p*-nitroanilide, gentamicin free base and gentamicin sulphate were obtained from Sigma (St Louis, MO, USA). Alamar blue (AB) was purchased from Biosource (Etten Leur, The Netherlands). Dichlorodihydrofluorescein diaecetate (H_2DCF-DA-D390) probe was purchased from Molecular Probes (Leiden, The Netherlands). All other chemicals used were of analytical grade.

Cell lines and culture conditions

Porcine kidney LLC-PK1 (ATCC number CL-101) cells, derived from the proximal tubule, and canine kidney MDCK (CCL-34) cells, isolated from the distal convoluted

tubule, were grown in Medium 199 (M199) and DMEM, respectively. Both cell lines were supplemented with 2 mmol/L L-glutamine, and either 5% or 10% FCS. Cells were maintained in a 5% CO_2 humidified atmosphere and were subcultured once weekly.

Determination of ROS production

The intracellular production of ROS was measured using the method described by Trayney and colleagues, (1995). In brief, LLC-PK1 and MDCK cells were seeded in 96-well plates (Corning Costar, Badhoevedorp, The Netherlands) using a seeding density of 8×10^3 cells per well. Following a 48 h incubation period, the cells were washed with warmed PBS. H_2DCF-DA-D390 (40 mmol/L), dissolved in serum-free DMEM/F12 without phenol red, was added to each well and the cells incubated for 2 h. The cells were then washed twice with warmed PBS and medium containing gentamicin sulphate or gentamicin free base (0–10 mmol/L) was added. ROS production was assessed fluorometrically (Cytofluor 2300 Fluorescence Measurement System, Millipore Corp., Bedford, MA, USA) after 24 h, using an excitation wavelength of 485 nm and emission wavelength of 538 nm. Data are expressed as the increase in fluorescence as compared to the fluorescence of controls, which was set at 100%.

Determination of gamma-glutamyl transferase

LLC-PK1 cells were plated in 24-well culture plates (Corning Costar, Badhoevedorp, The Netherlands) at 5×10^4 cells per well, and incubated for 48 h. Following exposure to gentamicin, either as sulphate or as free base (0–10 mmol/L) for 24 h, GGT release was measured. The supernatant was aliquoted into a 24-well plate and Tris-HCl buffer (0.1 mol/L, pH 8.6), supplemented with $MgCl_2$ (20 mmol/L), gly-gly (1 mmol/L) and L-glutamyl-*p*-nitroanilide (4 mmol/L) was added. After a 60 min incubation period the product, p-nitroanilide, was measured spectrophotometrically at 405 nm. Results are shown as a percentage of non-treated control cell values.

Determination of cell viability

Cell viability was assessed using a combined Alamar blue reduction assay in conjunction with the Neutral red uptake assay (Bull *et al.*, 2001).

Alamar blue reduction assay. LLC-PK1 and MDCK cells were plated in M199 or DMEM, respectively, in 24-well culture plates, using a seeding density of 5×10^4 cells per well, and incubated for 48 h to allow attachment. The medium was removed and replaced with medium containing either gentamicin sulphate or free base (0–10 mmol/L). Following a 24 h or 48 h incubation period, the chemical-containing medium was removed and the cells were washed twice with warmed PBS. Medium containing AB

(commercial stock diluted 1:10) and NR (50 μg/ml) was incubated with the cells at 37°C for 3 h. Resorufin, the fluorescent reduced AB product, was measured fluorometrically with excitation at 530 ± 15 nm and emission at 590 ± 10 nm. Results are expressed as (Fluorescence of treated cells/fluorescence of control cells) $\times$ 100.

Neutral red uptake. Following measurement of AB reduction, the AB/NR-containing medium was removed from the LLC-PK1 and MDCK cells, which were then washed with warmed PBS to remove any excess unincorporated dye. The NR incorporated in the cells was removed with 500 μl of de-stain solution (49% distilled water, 50% ethanol, 1% acetic acid). 200 μl was transferred to a 96-well plate and measured spectrophotometrically at 540 nm (Biorad 3550 microplate reader, Veenendaal, The Netherlands). Results are expressed as a percentage of the negative control.

In vivo *study*

All animal studies were conducted according to the principles of good clinical practice and were approved by the ethical committee for animal experiments of Utrecht University. For the study, 6 healthy mature male Shetland ponies weighing 138–200 kg were used, which were housed indoors and fed good-quality hay, carrots and water *ad libitum*. Prior to the study, all ponies underwent a clinical examination and renal function was evaluated by measuring serum GGT:creatinine ratio, serum urea nitrogen, total leukocyte counts and alkaline phosphatase. All ponies included in the study showed values within normal ranges for all parameters tested (results not shown). During the study, the ponies were treated for 5 days with gentamicin and blood and urine samples were collected. Sampling was continued for 3 (ponies 1–3) or 5 (ponies 4–6) days after the last gentamicin injection.

Gentamicin administration and plasma sampling

Gentamicin (gentamicin 5%) was administered for 5 consecutive days, starting the first day with 3.0 mg/kg body weight three times daily followed by 4.5 mg/kg body weight twice daily for the next 4 days according to the manufacturer's instructions (despite the recent recommendation to use a higher dose once daily, most pharmaceuticals licensed in Europe still suggest this dose regime). Before each injection and 3, 6, 12, 30, 60, 120 and 240 min after drug injection, blood samples were taken by jugular vein puncture using heparinized tubes. The last two samples (120 and 240 min) were taken only after the first and last gentamicin injection and after the start of the twice-daily regime. The plasma was separated by centrifugation (1000*g* for 10 min at 4°C) and stored at –20°C until analysis.

Analysis of plasma samples

Gentamicin concentrations were analysed in plasma using a fluorescence polarization immunoassay (TDX, Abbott Laboratories, Hoofddorp, The Netherlands), according to the manufacturer's instructions. The assay had a limited detection range (<10 μg/ml), so samples containing higher concentrations were diluted with equine serum. GGT and alkaline phosphatase were analysed using commercially available reagents (Synchron CX5Δ, Coulter/Beckman, Mijdrecht, The Netherlands).

Urine sampling

Urine was continuously collected with the help of a urine collector attached to the abdomen of the pony, and the volume of urine was recorded. During interim storage, sodium azide was added and samples were maintained at 4°C until analysis, which occurred daily. Prior to pooling of the urine samples collected at different time points, the sediment was examined for the presence of sperm cells, as their high GGT content impedes urinary GGT analysis. Urine samples containing sperm cells were discarded.

Analysis of the urine samples

Urine samples were centrifuged at room temperature for 10 min (1000*g*) and GGT and creatinine were measured by using commercially available reagents (Synchron CX5 D, Coulter/Beckman). The cell pellet after centrifugation was microscopically assessed for the presence of renal and epithelial cells.

Sampling and analysis of kidney tissue

Following premedication with romifidine hydrochloride (Sedivet, Boehringer Ingelheim, Germany), general anaesthesia was induced using midazolam (Dormicum, Roche, Mijdrecht, The Netherlands) and ketamine (Narketan, Chassat, Vught, The Netherlands) and maintained by halothane (Halothaan, Albic, Maasluis, The Netherlands) in oxygen. After the abdomen had been opened by midline laparotomy both renal arteries were transected to interrupt blood supply and both kidneys were removed immediately. Thereafter, all animals were euthanized with an appropriate dose of sodium pentobarbital (Euthesate, Ceva Sante Animale, Maasluis, The Netherlands).

The kidneys were cut into slices 4 mm thick and residual blood was removed by flushing the organ slices with physiological saline. The samples were wrapped in aluminium foil and stored at –80°C until analysis. Prior to analysis, the kidney samples were thawed and the cortex and medulla were separated.

Gentamicin was extracted from kidney samples according to the method described by Brown and colleagues (1990) and concentrations were measured using the fluorescence polarization immunoassay (TDX, Abbott Laboratories) as used for

serum analysis. Kidney sections of medulla and cortex were collected seperately to be fixed in buffered 10% formalin and subjected to histopathological examinations. Kidneys removed from an untreated Shetland pony served as controls in all assays.

Pharmacokinetic analysis of gentamicin plasma concentrations

Pharmacokinetic analysis was performed using non-linear regression analysis program adapted from Multi (Yamaoka *et al.*, 1981). On the basis of Akaike's information criterion (Yamaoka *et al.*, 1978), plasma concentrations were fitted to an equation corresponding to a bicompartmental model (Equation 1):

$$C(t) = Y_1 \quad \exp(-\lambda_1 t) + Y_2 \quad \exp(-\lambda_2 t) \tag{1}$$

where $C(t)$ is the plasma concentration at time t; Y_1, Y_2 are the pre-exponential constants expressed as concentrations; and λ_1 and λ_2 are the exponents (h^{-1}). The data points were fitted with the inverse of the squared fitted value.

The plasma half-lives of distribution $t_{\frac{1}{2}\lambda_1}$ and elimination $t_{\frac{1}{2}\lambda_2}$, the steady-state volume of distribution (V_{ss}), the volume of the central compartment (V_c), the plasma clearance (Cl) and the mean residence time (MRT) were calculated from the classical equations associated with compartmental analysis (Perrier and Gibaldi, 1982). Maximum plasma concentrations were obtained from the observed concentrations. The area under the plasma concentration–time curves over 24 h (AUC (0–24 h)) for the first and second dose regimes (3 mg/kg, three times daily, day 1; and 4.5 mg/kg twice daily, respectively) was calculated by non-compartmental analysis using the statistical moment approach.

Statistical analysis

Data were subjected to a one-way ANOVA with post-hoc Dunnets multiple comparison test. The gentamicin concentration of the cortex and medulla were analysed with a paired Student's *t*-test. Data were considered significantly different when $p < 0.05$.

RESULTS

In vitro *studies*

Following 24 h incubation of LLC-PK1 cells with 5 and 10 mmol/L gentamicin sulphate, ROS production was significantly increased (127% and 170%, respectively) (Figure 1). Similarly, MDCK cells also showed an increase in ROS production after 24 h following treatment with 5 and 10 mmol/L gentamicin sulphate (149% and 278% respectively) (Figure 1). No ROS production was measured in either cell line after gentamicin free base treatment (data not shown).

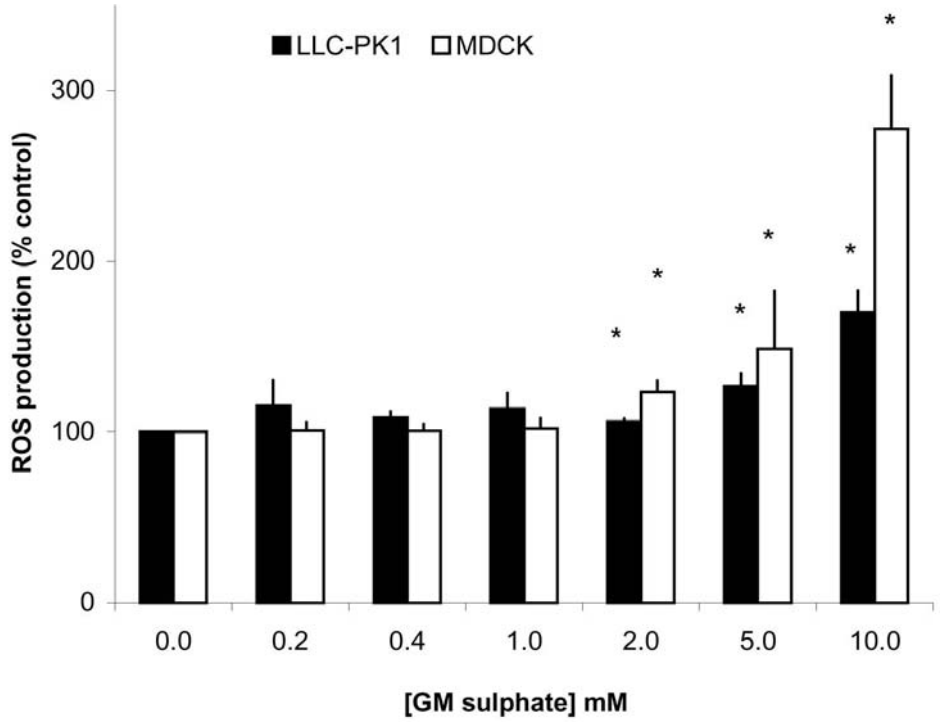

Figure 1. ROS production by LLC-PK1 and MDCK cells following exposure to gentamicin sulphate. Results expressed as mean $\pm$ SD of three independent experiments carried out in triplicate. *Results are considered significantly different from controls when $p < 0.05$

Following exposure to gentamicin sulphate (1 mmol/L), GGT release form LLC-PK1 cells occurred prior to a decrease in cell viability (Figure 2). In addition, incubation of LLC-PK1 cells with gentamicin free base showed a small but significant increase in the GGT release (data not shown).

The AB reduction was significantly decreased in the LLC-PK1 cells following treatment with 5 and 10 mmol/L gentamicin sulphate (49% and 36%, respectively) (Figure 2). Although no significant decrease in cell viability was seen in the MDCK cells after 24 h, following 48 h incubation decreases in AB reduction of 60% and 48% were seen after exposure to 5 and 10 mmol/L gentamicin sulphate, respectively. The free base failed to cause a decrease in cell viability in either cell line tested (data not shown).

A significant decrease in NR uptake (77%) in LLC-PK1 cells, but not in MDCK cells (Table I), after 24 h of incubation was measurable at a concentration of 10 mmol/L, whereas only a marginal effect was seen following exposure to gentamicin free base (data not shown). As mentioned above, GGT leakage occurred at lower concentration (1 mmol/L) than the increase in ROS production (5 mmol/L) or the loss of AB reduction and impairment of NR uptake (5 mmol/L).

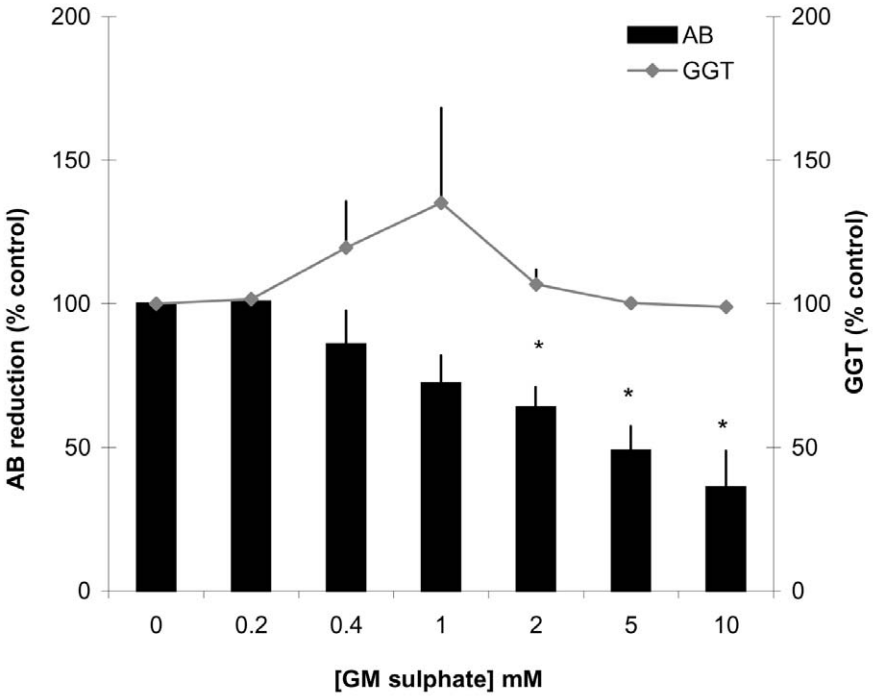

Figure 2. Cytotoxic effects of gentamicin sulphate in LLC-PK1 cells. Data are expressed as mean ± SD of three independent experiments carried out in triplicate. *Results are considered statistically significant from control values when $p < 0.05$

TABLE I
Effects of gentamicin sulphate on Neutral red uptake in LLC-PK1 and MDCK cells

	Neutral red uptake (% negative control) (mean ± SD)[a]	
Gentamicin mmol/L	LLC-PK1	MDCK
0	100 ± 6	100 ± 5
0.2	104 ± 6	106 ± 13
0.4	113 ± 5*	110 ± 14
1	110 ± 3	103 ± 7
2	108 ± 6	110 ± 8
5	96 ± 9	122 ± 7
10	77 ± 7*	109 ± 12

[a]Results expressed as mean ± SD of three independent experiments carried out in triplicate

*Data are statistically significant at $p < 0.05$

In vivo *study*

Following administration of gentamicin 3 mg/kg three times daily, maximum gentamicin plasma concentration was approximately 35 μg/ml, rising to approximately 60 μg/ml after 4.5 mg/ml twice daily treatment (Figure 3). Mean trough concentrations decreased from 1.28 ± 0.19 μg/ml to 0.95 ± 0.09 μg/ml. At the given dose regimen of 3 mg/kg three times daily or 4.5 mg/kg twice daily, accumulation of gentamicin was not evident and no significant increase in AUC (0–24 h) over time could be observed (data not shown).

The main pharmacokinetic parameters for gentamicin, including V_{ss}, MRT, Cl, $t_{\frac{1}{2}\lambda_1}$ and $t_{\frac{1}{2}\lambda_2}$ were calculated and are presented in Table II.

The GGT:creatinine ratio in the pooled urine samples collected daily showed a significant increase following gentamicin administration at days 5–7 compared to control values (mean ratio 7.8 ± 2.0) (Figure 4). The maximum ratio (40.7 ± 13.0) was achieved on day 7, two days after cessation of therapy. Thereafter, a decline was seen, resulting in a mean ratio of 11.6 ± 2.7 at day 10.

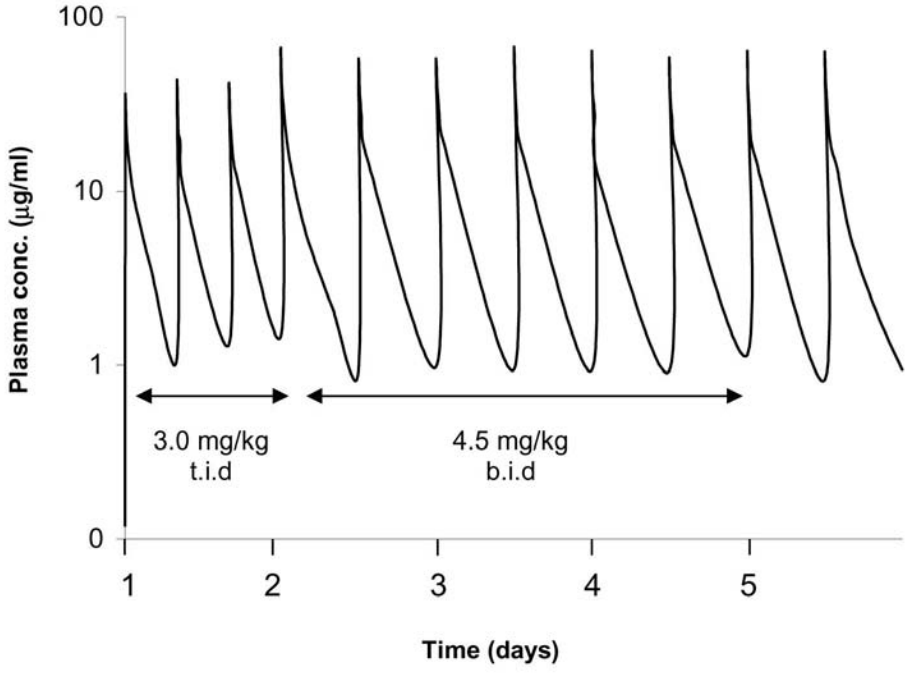

Figure 3. Gentamicin plasma concentrations after repeated dosing. Data are taken from one representative healthy pony, treated with gentamicin over five days. C_{max}, 60 μg/ml and C_{trough}, 0.95 μg/ml

TABLE II
Pharmacokinetic parameters of gentamicin in six healthy ponies following repeated intravenous administration (3 mg/kg three times daily for 1 day, followed by 4.5 mg/kg twice daily for 4 days)

Parameter	Value (mean ± SD)
CL ((ml/h)/kg)	0.98 ± 0.053
$t_{\frac{1}{2}\lambda_1}$ (min)	15 ± 0.5
$t_{\frac{1}{2}\lambda_2}$ (h)	2.8 ± 0.04
V_c (ml/kg)	79 ± 6.1
V_{ss} (ml/kg)	197 ± 17.7
MRT (h)	3.4 ± 0.18

Cl, plasma clearance; $t_{\frac{1}{2}\lambda_1}$, plasma half-life of distribution; $t_{\frac{1}{2}\lambda_2}$, plasma half-life of elimination; V_c, volume of the central compartment; V_{ss}, volume of distribution at steady state; MRT, mean residence time

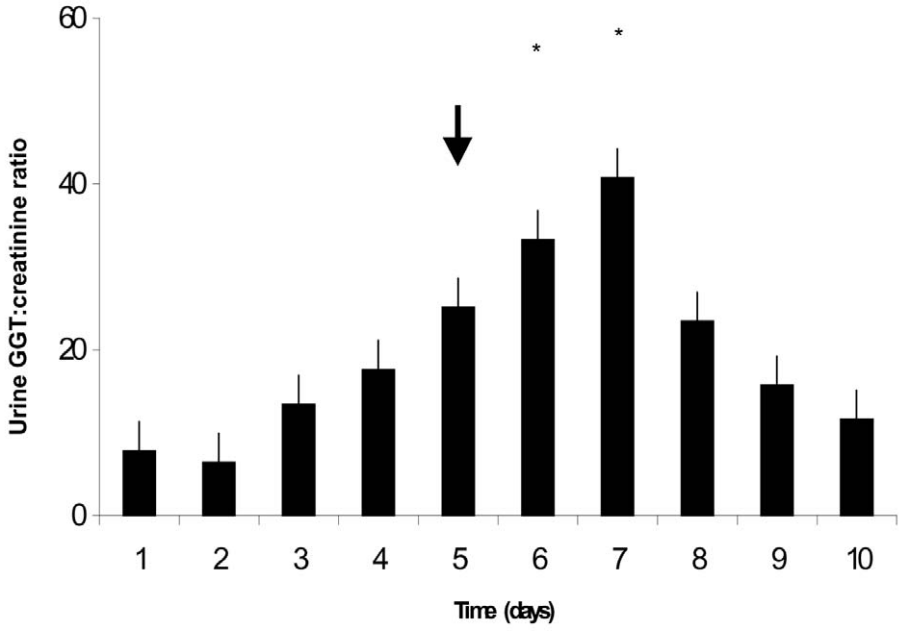

Figure 4. Measurement of urine GGT: creatinine ratio during gentamicin therapy. Results are expressed as mean ± SEM of six ponies (days 1–7) and three ponies (days 8–10). Arrow indicates cessation of drug administration. *Results are considered statistically significant from control values when $p < 0.05$

Significantly higher levels of gentamicin were found in the renal cortex in comparison with the medulla (Figure 5), which declined gradually after withdrawal of Gentamcin. The histopathological examination of the kidney samples revealed no microscopic changes caused by gentamicin treatment (data not shown).

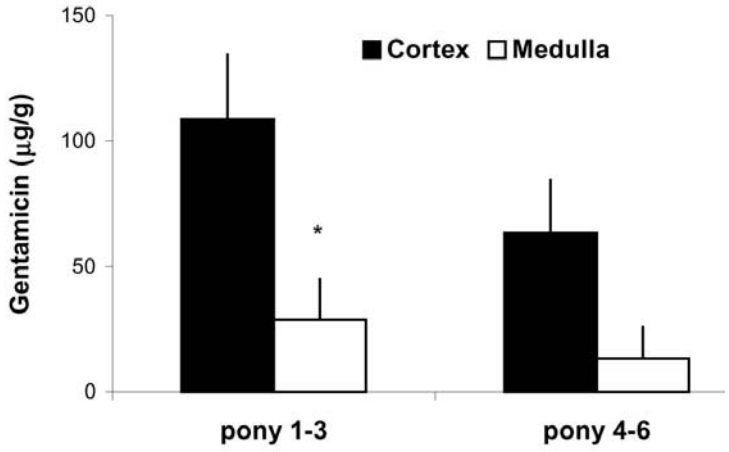

Figure 5. Comparison of GM concentrations in renal cortex and medulla. Data are expressed as mean ± SD of three ponies, treated with gentamicin for five consecutive days. Kidneys were collected from ponies 1–3 and 4–6 three or five days after cessation of therapy, respectively. *Results are considered statistically significant when $p < 0.05$

DISCUSSION

Numerous studies have referred to renal toxicity of gentamicin. The common understanding is that accumulation of the aminoglycoside, particularly in the renal proximal tubule cells, accounts for direct cytotoxicity. The *in vitro* data show that following gentamicin exposure *in vitro,* a dose-dependent loss of cell viability can be observed, as measured by the AB reduction assay. AB is a commonly used viability assay that indicates the oxidative status of the cell (Bull *et al.*, 2001); a decrease in reduction of the resazurin blue dye following exposure to 5–10 mmol/L gentamicin is indicative of disruption of the redox state of the cell due to a loss of mitochondrial activity. The modulation of membrane enzyme activities, changes in membrane fluidity, and the increase in cytosolic Ca^{2+}, all reported to be caused by gentamicin administration, alter mitochondrial membranes and thus contribute to the decrease in cell viability (El Mouedden *et al.*, 2000). In contrast, NR uptake was inhibited only by the highest concentration of gentamicin in LLC-PK1 cells and had no effect on MDCK cells, indicating that pinocytosis is largely unaffected. Studies carried out by Mukherjee and colleagues (1997) in cultured fibroblasts have shown gentamicin to be transported, via

pinocytosis, into fibroblasts, where it accumulates in the lysosomes. Accumulation leads to destabilization of lysosomal membranes, which ultimately results in cellular apoptosis owing to the release of lysosomal proteases and endonucleases (Choi *et al.*, 2000). In all experiments, the effects of both gentamicin sulphate and free base were tested. No significant decrease in NR uptake was found for the free base, which may be due to its lower solubility.

Gentamicin is known to accumulate in the cells of the proximal tubules (cortex); this was confirmed in the *in vivo* study, as the cortex contained significantly higher drug concentrations as compared to the medulla. Following accumulation, the gentamicin concentrations in proximal tubule cells can reach concentrations up to 30-fold higher than those seen in plasma (El Mouedden *et al.*, 2000). In the *in vivo* studies, a maximum plasma concentration of approximately 0.13 mmol/L (61.34 μg/ml) was measured, which translates into a local drug concentration of 3.9 mmol/L. Thus, the expected concentrations in the kidney *in vivo* and in cultured cells are of the same order of magnitude.

Various related mechanisms have been discussed for the mediation of gentamicin-induced renal toxicity. For example, gentamicin has been shown to enhance the generation of superoxide anion and hydrogen peroxide by mitochondria in the renal cortex (Walker *et al.*, 1999; Cuzzocrea *et al.*, 2002; Sener *et al.*, 2002); in the presence of metal catalysts, these will form hydroxyl radicals. In turn, scavengers of ROS and iron chelators, such as metallothionein or vitamin E, protect renal cells against gentamicin toxicity *in vivo* (Pedraza-Chaverri *et al.*, 1999; Walker *et al.*, 1999). Our results showed an increase in ROS production in both LLC-PK1 and MDCK cells following gentamicin exposure, and MDCK cells seemed to be more sensitive than LLC-PK1 cells. El Mouedden and colleagues (2000) also reported that MDCK cells, but not LLC-PK1 cells, showed lysosomal phospholipidosis following exposure to 2 mmol/L gentamicin, in addition to the presence of apoptotic bodies. As MDCK cells have shown to be more sensitive to other pro-oxidants as well, this may reflect a genuine lack of antioxidant capacity (Khand *et al.*, 2002).

GGT leakage was used as an early parameter of cell damage, indicating a loss of brush border integrity. The *in vitro* data showed increased GGT leakage at concentrations lower than those causing a decrease in AB reduction, indicating that cell integrity is affected prior to measurable cell death. However, these finding also indicated that GGT is a sensitive marker for early renal damage, and thus GGT measurement in urine was included in the *in vivo* protocol. Indeed, biochemical analysis of the urine in the *in vivo* studies revealed a significant increase of the GGT:creatinine ratio, indicating a certain degree of brush border damage. Similarly to the *in vitro* data, loss of cell membrane integrity *in vivo* preceded any visible morphological changes, as neither the histological examination of the kidney nor the analysis of cells in urine provided any evidence for loss of viability of the proximal tubule cells. The GGT:creatinine ratio returned to baseline values after 10 days, indicating a transient toxic effect.

To summarize, transient toxicity, as indicated by the increased GGT:creatinine ratio, was observed following the applied dose regimen for gentamicin in ponies of 3 mg/kg three times daily on the first day followed by 4.5 mg/kg twice daily for five consecutive days. The mechanism of action of aminoglycosides strongly supports a reassessment of

the current dose regimen towards a single undivided dose, given once daily (Tudor *et al.*, 1999). It can be assumed that this dose regimen has an equal or higher therapeutic value in terms of the bactericidal activity. In addition, it has been confirmed in different studies (Nicolau *et al.*, 1995; Freeman *et al.*, 1997) that a single higher dose, and subsequently higher C_{max} values, do not increase nephrotoxicity, as cellular uptake by renal tubule cells is a saturable process, which is time-dependent and, to a lesser extent, dose-dependent. Renal toxicity is thus related not to maximum plasma levels but to the time of persistent trough levels. However, if gentamicin is used for postoperative antibiosis, high C_{max} values, as measured following the once-daily administration of gentamicin, may interfere with neuromuscular transmission, since gentamicin is known to produce neuromuscular blockade, particularly when administered together with muscle-relaxing agents (Rutten *et al.*, 1980; Lippmann *et al.*, 1982; Jedeikin *et al.*, 1987).

To conclude, this study was designed to allow direct comparison of *in vitro* and *in vivo* findings on gentamicin toxicity in equines. In both studies, some degree of nephrotoxicity was observed following gentamicin administration. However, this nephrotoxicity was transient, with GGT:creatinine ratios returning to baseline values after cessation of therapy. Thus, it may be concluded that even a conservative dose regimen does not cause significant renal toxicity in healthy ponies. Further work should be carried out to assess the recent dose recommendations in healthy and renally compromised patients.

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