

# Gentamicin Effects on Renal Ischemia/Reperfusion Injury

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**This study assessed gentamicin's effects on ischemia/reperfusion renal injury to better understand when and how it worsens postischemic acute renal failure. Rats were subjected to 25 minutes of renal pedicle occlusion with and without preischemic (15-minute) or postischemic (15-minute or 8-hour) gentamicin treatment (100 mg/kg, by itself a subtoxic dose). Gentamicin's impact on hypoxia/reoxygenation injury to isolated rat proximal tubular segments was also assessed. Preischemic and postischemic gentamicin worsened the severity of acute renal failure to the same degree, suggesting that pretreatment induces its effect in the reperfusion period. Gentamicin paradoxically lessened hypoxic damage to proximal tubular segments (assessed by lactate dehydrogenase release), again implying no adverse impact on oxygen deprivation-induced tubular injury. From 0–4 hours of reperfusion, gentamicin approximately halved ATP/ADP ratios (due to increased ADP), indicating a drug-induced defect in cellular energetics. This abnormality temporally correlated with evolving morphological damage. Although antioxidants (deferoxamine and sodium benzoate) have been reported to protect against pure aminoglycoside nephrotoxicity, they did not mitigate gentamicin's adverse impact on postischemic acute renal failure. Gentamicin did not influence ischemia/immediate reperfusion deacylation/reacylation (assessed by renal free fatty acid content) despite its known antiphospholipase activity. Although in the normal kidney gentamicin preferentially accumulated in cortex, in the postischemic kidney, both cortex and outer medullary stripe developed striking (approximately threefold to fivefold) and comparable gentamicin increments. In conclusion, gentamicin appears to exacerbate postischemic acute renal failure by adversely influencing the reperfusion, not the ischemic injury, process. This may occur because increased gentamicin accumulation negatively impacts on reperfusion cellular energetics. (*Circulation Research* 1992;70:20–28)**

In a previous study, this laboratory demonstrated that a subtoxic amount of gentamicin administered during mild renal ischemia worsened the resultant damage, thereby causing severe acute renal failure (ARF).<sup>1</sup> Thus, it was concluded that aminoglycosides predispose to ischemic renal damage. Recently, however, Spiegel et al<sup>2</sup> reported that a subtoxic dose of gentamicin (100 mg/kg), when given 4 hours after sublethal renal ischemia, also induced severe ARF. Therefore, they concluded that the postischemic kidney is highly vulnerable to aminoglycoside administration. This observation has led us to question our previous conclusion that a true adverse gentamicin–ischemia interaction exists. Be-

cause gentamicin given just before or during ischemia is still present in the postischemic period, it is possible that in the previous study of this laboratory, gentamicin, when given during ischemia, adversely affected postischemic (reperfusion) not true ischemic (oxygen deprivation) renal damage. To understand this synergistic form of injury, it is critical to resolve whether gentamicin adversely affects the ischemic (oxygen deprivation) and/or the postischemic injury phase. The present study was undertaken to resolve this issue. In addition, the effects of gentamicin on specific determinants of ischemia/reperfusion injury, including adenine nucleotide depletion, oxidant stress, and membrane deacylation/reacylation, have been assessed.

## Materials and Methods

### *Time as a Variable in the Gentamicin–Ischemia Interaction*

The purpose of this experiment was to contrast the severity of postischemic ARF (plasma creatinine and histology) among rats receiving gentamicin either before or at variable times after an ischemic event. It

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was rationalized that if gentamicin worsens both ischemic and postischemic renal injury, more severe ARF would result from preischemic than from postischemic gentamicin treatment. Conversely, if the gentamicin effect occurs in the postischemic period, comparable renal damage should occur irrespective of preischemic or postischemic gentamicin administration. Female Sprague-Dawley rats (175–200 g; Bantin and Kingman, Inc., Fremont, Calif.) were anesthetized with pentobarbital (30–40 mg/kg) and subjected to 25 minutes of bilateral renal pedicle occlusion (RPO) at 37°C through a midline abdominal incision. Then the abdomen was sutured and the rats were allowed to recover from anesthesia, with free food and water access being provided. The rats were divided into four experimental groups. Group 1 ( $n=8$ ) received no gentamicin, whereas groups 2–4 received gentamicin (100 mg/kg i.m.; Elkins-Sinn, Inc., Cherry Hill, N.J.) either 15 minutes before RPO ( $n=8$ ) or 15 minutes ( $n=8$ ) or 8 hours ( $n=7$ ) after RPO. Of note, gentamicin given 15 minutes before RPO is virtually totally absorbed by the time of ischemia and leads to massive renal gentamicin accumulation (greater than 2,000  $\mu\text{g}/\text{mg}$  dry wt; pilot data). Twenty-four hours after RPO, all rats were reanesthetized and killed by aortic puncture. The plasma was used for creatinine (Cr) assay, the left kidneys were fixed for histology (in methyl Carnoy's solution), and the right kidneys were frozen for subsequent gentamicin assay (see below). Five rats subjected to gentamicin injection and sham RPO were treated in an identical fashion. All kidneys were paraffin-embedded, and 5- $\mu\text{m}$  sections were cut and stained with hematoxylin and eosin.

#### *Effect of Gentamicin on Early Ischemic Morphological Injury*

As an additional index of gentamicin effects on ischemic injury, 10 rats were subjected to RPO, five with and five without gentamicin pretreatment (15 minutes before clamping). Fifteen minutes after clamp release, the kidneys were fixed by in vivo perfusion with 1.25% glutaraldehyde.<sup>1</sup> The 15 minutes of reflow was permitted to allow sufficient time for ischemia-induced morphological changes to develop (since these are minimally expressed if no reflow is permitted). The right kidneys were postfixed in 10% formalin and processed for histology. The severity of injury in the gentamicin and control groups was then compared (see "Results").

#### *Gentamicin Effects on In Vitro Oxygen Deprivation Injury*

A third approach taken to gauge gentamicin's impact on oxygen-deprivation injury was to assess its influence on hypoxia/reoxygenation damage to isolated proximal tubular segments (PTSs). Rat PTSs were isolated from renal cortices according to the method of Weinberg<sup>3</sup> and as slightly modified by this laboratory.<sup>4</sup> Sixteen rats were used, each for a single preparation. Nine of the rats received gentamicin,

100 mg/kg, 15 minutes before PTS isolation to ensure gentamicin uptake by the kidneys; the remaining seven rats served as controls. After isolation, the PTSs were suspended in nutrient buffer (1.5–2.5 mg protein/ml)<sup>3</sup> and rewarmed from 4°C to 37°C for 15 minutes; then each preparation was divided into two aliquots: one was maintained under continuous oxygenated conditions (95% O<sub>2</sub>–5% CO<sub>2</sub>) for 1 hour and the other was subjected to 30 minutes of hypoxia (95% N<sub>2</sub>–5% CO<sub>2</sub>) followed by 30 minutes of reoxygenation (95% O<sub>2</sub>–5% CO<sub>2</sub>). Gentamicin, 1 mg/ml, was added to the nutrient buffer of those PTSs pretreated with gentamicin (maintaining the same buffer pH). Injury, assessed by calculating the percent lactate dehydrogenase (LDH) release,<sup>3–5</sup> was assessed at the start of each incubation and at 30 and 60 minutes thereafter. Hypoxia-specific cell injury was calculated as percent LDH released after N<sub>2</sub>/CO<sub>2</sub> incubation minus the time-matched percent LDH released under oxygenated conditions. Reoxygenation injury was assessed as the increment in percent LDH release from the end of N<sub>2</sub>/CO<sub>2</sub> incubation to the end of the reoxygenation period.

In the above PTS experiments, it was found that in vivo PTS gentamicin loading decreased PTS viability during O<sub>2</sub>/CO<sub>2</sub> incubation compared with the control PTS (see "Results"). Thus, to eliminate this variable from the data interpretation, PTSs were harvested from four normal rats and after 15 minutes of rewarming, each preparation was divided into four treatment groups: 1) O<sub>2</sub>/CO<sub>2</sub> incubation for 60 minutes, 2) O<sub>2</sub>/CO<sub>2</sub> incubation for 60 minutes with 1 mg/ml gentamicin added to the buffer, 3) N<sub>2</sub>/CO<sub>2</sub> incubation for 45 minutes followed by 15 minutes of reoxygenation, and 4) N<sub>2</sub>/CO<sub>2</sub> incubation for 45 minutes followed by 15 minutes of reoxygenation with gentamicin (1 mg/ml) added to the buffer. LDH release was determined after rewarming and at 45 and 60 minutes of each incubation. The results were analyzed as noted above.

#### *Effect of Gentamicin on Ischemia/Reperfusion Adenine Nucleotide Pools*

Aminoglycosides are known to inhibit state 3 mitochondrial respiration.<sup>6,7</sup> However, a single dose of gentamicin, by itself, does not alter in vivo renal cortical/outer medullary ATP, ADP, or AMP concentrations.<sup>1</sup> This experiment assessed whether gentamicin adversely influences renal adenylate high energy phosphate levels during ischemia or prevents their recovery during reperfusion, possibly explaining why it worsens the severity of postischemic ARF. Rats were subjected to 25 minutes of RPO with or without gentamicin pretreatment. The left kidneys were instantly frozen at liquid nitrogen temperature<sup>8</sup> either at the completion of ischemia or at 15 minutes, 4 hours, or 8 hours of reflow ( $n=5$ –7 kidneys for both the gentamicin and control groups at each time). Cortical/outer medullary tissues were assayed for adenine nucleotides by high-performance liquid chromatography (HPLC), as previously described,<sup>4,9</sup> the values being expressed as micro-

moles per gram tissue dry weight. ATP/ADP ratios were calculated as an indirect assessment of oxidative phosphorylation rates.<sup>10,11</sup> To correlate adenine nucleotide values with the severity of evolving renal injury, all 8-hour reflow rats had their right kidneys fixed in methyl Carnoy's solution for subsequent histological analysis.

To assess whether gentamicin, if administered after, not just before, renal ischemia alters renal reperfusion adenylate pools, five rats were subjected to RPO and 15 minutes later gentamicin was injected. The left kidneys were assayed for adenine nucleotides 3.75 hours later. The values were contrasted to those found in the above 4-hour reflow experiments.

#### *Effect of Gentamicin on Ischemia-Induced Deacylation/Postischemic Free Fatty Acid Disposal*

Membrane phospholipid deacylation is a prominent response to ischemic renal injury (e.g., see References 4 and 12), and it previously has been established that aminoglycosides inhibit renal tubular phospholipase activity.<sup>13–15</sup> Therefore, the effect of gentamicin on ischemic free fatty acid (FFA) accumulation, a marker of deacylation, was assessed. In addition, gentamicin's net impact on immediate post-ischemic FFA disposal, in part reflecting reacylation, was determined. Rats were subjected to RPO with and without prior gentamicin treatment. Renal cortical/outer medullary tissues were harvested from both groups either at the end of ischemia or after 15 minutes of reflow ( $n=4-6$  kidneys for each group at each time). The tissues were extracted in chloroform/methanol,<sup>16</sup> the FFAs were recovered by HPLC,<sup>17</sup> and the major FFAs (palmitic, stearic, linoleic, oleic, and arachidonic acids) were individually quantified by a second HPLC procedure<sup>18</sup> as previously described by this laboratory.<sup>4</sup>

#### *Effect of Antioxidant Interventions on the Severity of Gentamicin-Treated Ischemic Acute Renal Failure*

Walker and Shah<sup>19</sup> demonstrated that antioxidant agents (e.g., deferoxamine and/or hydroxyl radical scavengers [e.g., sodium benzoate]) can ameliorate gentamicin nephrotoxicity in otherwise normal rats. This experiment evaluated whether these same agents mitigate the gentamicin-ischemia interaction. If so, it would suggest that oxidant injury is a pathway by which gentamicin worsens postischemic ARF. Seven rats received deferoxamine, 100 mg/kg i.m.; in addition, four of these rats also received sodium benzoate, 150 mg/kg i.m., followed by gentamicin injection. Fifteen minutes later, all seven rats were subjected to 25 minutes of RPO. After clamp release, the rats were sutured and allowed to recover from anesthesia. Six hours later, the sodium benzoate-treated rats received a second dose of this agent. Twenty-four hours postischemia, the severity of ARF was assessed.

#### *Effect of Ischemia on Cortical/Outer Medullary Stripe Gentamicin Accumulation*

Although it has been shown that ischemic injury increases renal gentamicin uptake,<sup>1,2</sup> the influence of

ischemic damage on gentamicin accumulation in the outer medullary stripe (OMS), the principal site of ischemic renal injury and of the gentamicin-ischemia interaction,<sup>1,2</sup> has not been assessed. Therefore, kidneys obtained 24 hours postischemia (pregentamicin treatment and 15 minutes postgentamicin treatment) were sectioned to yield cortical and OMS tissues. They were homogenized in saline and then solubilized with Triton X-100, as previously described.<sup>20</sup> They were assayed for gentamicin by fluorescence polarization (TDX; Abbott Laboratories, North Chicago, Ill.) and the values expressed as milligrams per gram tissue dry weight. The relative amount of OMS versus cortical gentamicin uptake was assessed by calculating OMS/cortical gentamicin ratios. The five rats subjected to sham RPO and then injected with gentamicin served as controls.

To assess regional gentamicin uptake at an earlier point in time (during evolving tissue damage), eight rats were injected with gentamicin and 15 minutes later half underwent RPO or sham RPO. Four hours postgentamicin injection, one kidney from each rat was resected and analyzed for cortical and OMS gentamicin levels.

#### *Effect of Gentamicin on Glycerol-Induced Renal Injury*

The purpose of this experiment was to determine whether gentamicin's adverse influence on ischemic injury is a highly specific one or whether gentamicin can also adversely affect other forms of acute tubular damage. To this end, 10 nonfasted, nondehydrated rats were injected with glycerol (50%; 3.5 ml/kg into the right thigh). Half received gentamicin (100 mg/kg into the left thigh), with the other half undergoing a sham injection. The severity of Cr elevation was assessed 24 hours later.

#### *Statistics*

All values are given as mean  $\pm$  1 SEM. Statistical comparisons were by unpaired Student's *t* test unless otherwise stated. Histological damage was semiquantitatively graded on a 1+ to 5+ scale, based on 0–20%, 20–40%, 40–60%, 60–80%, and 80–100% OMS S<sub>3</sub> PTS damage (necrosis/brush border blebbing/cast formation). Histological scores were contrasted by the Wilcoxon rank sum test. Significance was judged as  $p < 0.05$ .

### **Results**

#### *Time as a Variable of the Gentamicin-Ischemia Interaction*

The plasma Cr concentrations 24 hours after RPO without gentamicin treatment ranged from 0.4 to 0.8 mg/dl ( $n=8$ ). (The normal rat plasma Cr for this laboratory is 0.3–0.5 mg/dl.<sup>5</sup>) By using these 24-hour values, a 95% confidence band for postischemic Cr after RPO alone was constructed (0.3–0.9 mg/dl). Each rat that received gentamicin preischemia or postischemia had a 24-hour Cr above this normal

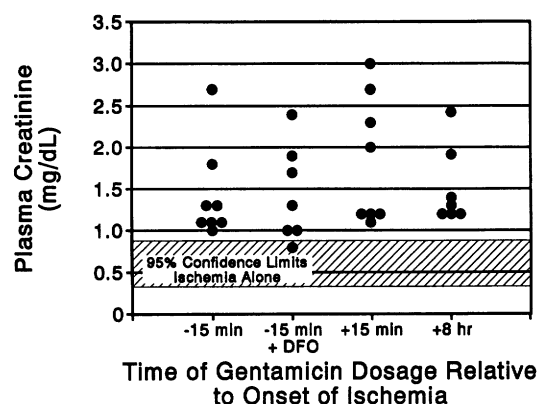


FIGURE 1. Individual plasma creatinine concentrations 24 hours after renal ischemia with gentamicin given 15 minutes before ischemia (–15 min) or 15 minutes or 8 hours after ischemia (+15 min and +8 hr). The shaded area is the 95% confidence band for plasma creatinine 24 hours after ischemia in the absence of gentamicin injection ( $n=8$  rats). The –15 min+DFO group represents those rats injected with both gentamicin and deferoxamine 15 minutes before renal ischemia. Four of these DFO rats were also treated with sodium benzoate (see text).

range (Figure 1), and the extent of Cr elevation for these groups did not significantly differ (by analysis of variance). Deferoxamine/sodium benzoate therapy conferred no protection, the mean Cr for these rats not significantly differing from that of the other gentamicin-treated ischemic groups. Gentamicin injection followed by sham RPO failed to induce an elevated Cr in any rat so tested (range, 0.3–0.5 mg/dl; mean, 0.4 mg/dl).

#### Effect of Gentamicin on Renal Morphological Injury

The major histological change noted at 15 minutes of reflow was proximal tubular cell blebbing and cast formation, which was observed in approximately 40–60% of OMS  $S_3$  segments (these changes were used for scoring purposes in Figure 2A). Overt necrosis was observed less commonly (approximately 10–20% of  $S_3$  segments). None of these abnormalities significantly differed between the gentamicin and control ischemic groups.

The 8-hour postischemic kidneys, taken for correlation with the 8-hour adenine nucleotide results, showed significantly greater OMS  $S_3$  tubular segment necrosis and cast formation in the gentamicin-treated ischemic than the control ischemic group ( $p<0.01$ ; Figure 2B). Thus, these findings, in concert with those above, indicated that gentamicin induced significant morphological damage between 15 minutes and 8 hours of the reperfusion period.

Renal histology at 24 hours confirmed that gentamicin had worsened the severity of renal damage (Figure 2C). Rats subjected to ischemia alone demonstrated mild OMS  $S_3$  proximal tubular necrosis and cast formation (scores, 1+ to 2+) with no cortical segment involvement. In contrast, gentamicin plus

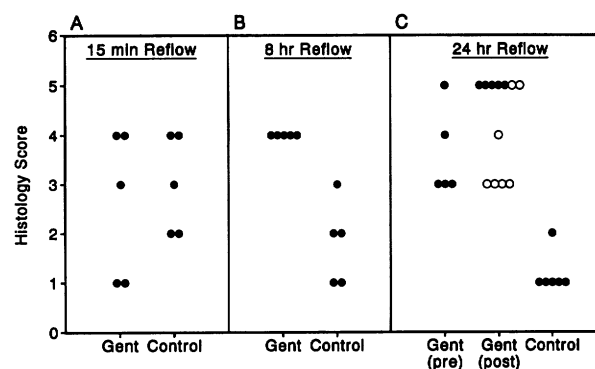


FIGURE 2. Histological injury scores for rats subjected to 25 minutes of ischemia with and without gentamicin (Gent) treatment. For the 15-minute and 8-hour reflow experiments, gentamicin was given only 15 minutes before ischemia, whereas for the 24-hour reflow experiment, gentamicin was given either 15 minutes preischemia or postischemia (●, 15 minutes postischemia; ○, 8 hours postischemia). At 15 minutes of reflow, the severity of morphological injury did not differ for the two groups. However, at 8 and 24 hours of reflow, all the gentamicin groups had significantly worse morphological damage than the postischemic controls ( $p<0.05$ ).

ischemia caused extensive OMS  $S_3$  necrosis/casts at 24 hours (scores, 3+ to 5+;  $p<0.01$  compared with ischemic controls) irrespective of the time of gentamicin treatment. Rats with 4+ or 5+ OMS scores also showed patchy cortical segment necrosis. Gentamicin in the absence of ischemia caused no morphological damage.

#### Gentamicin Effects on Proximal Tubular Segment Viability

PTSs harvested from gentamicin-treated rats and then incubated with gentamicin developed significantly greater percent LDH release under continuously oxygenated conditions than did the non-gentamicin-exposed controls (Table 1A). Despite this expression of *in vitro* gentamicin toxicity, these PTSs did not demonstrate greater vulnerability to hypoxia/reoxygenation versus their non-gentamicin-exposed counterparts. In fact, “hypoxia-specific cell injury” was paradoxically less in the gentamicin-exposed group ( $8\pm3\%$  versus  $20\pm3\%$ , respectively;  $p<0.05$ ; Table 1A).

When PTSs were exposed to gentamicin only *in vitro*, their viability under oxygenated conditions was not different from that of non-gentamicin-exposed PTSs (Table 1B). (Thus, their response to hypoxia/reoxygenation could be more easily compared with that of non-gentamicin-exposed controls than was possible in the above experiments.) Gentamicin exposure once again significantly decreased oxygen deprivation injury, both the absolute and hypoxia-specific injury being markedly lower than that observed in the controls. In both sets of PTS experiments, gentamicin tended to increase percent LDH

TABLE 1. Effect of Gentamicin on Percent Lactate Dehydrogenase Release From Proximal Tubular Segments

			Continuous O <sub>2</sub> /CO <sub>2</sub> incubation for		Hypoxia (30 min)	Reoxygenation (30 min)	Specific injury	
	<i>n</i>	Baseline	30 min	60 min			Hypoxia	Reoxygenation
A.								
Gentamicin	9	8±2	24±3*	28±3*	31±2	39±3	8±3*	9±2
Controls	7	7±2	14±2	19±2	32±2	38±3	20±3	7±1
			Continuous O <sub>2</sub> /CO <sub>2</sub> incubation for		Hypoxia (45 min)	Reoxygenation (15 min)	Specific injury	
	<i>n</i>	Baseline	45 min	60 min			Hypoxia	Reoxygenation
B.								
Gentamicin	4	8±2	9±1	10±1	27±4†	37±4*	18±5*	10±1
Controls	4	8±2	10±2	14±2	46±2	51±2	36±4	5±1

A: Effect of continuous O<sub>2</sub>/CO<sub>2</sub> incubation (30 and 60 minutes) and N<sub>2</sub>/CO<sub>2</sub> incubation (30 minutes) followed by 30 minutes of reoxygenation on percent lactate dehydrogenase release from proximal tubular segments harvested from gentamicin-treated and normal rats. Proximal tubular segments from gentamicin-treated rats also had gentamicin added to the proximal tubular segment buffer (1 mg/ml). All values are mean percent lactate dehydrogenase release ± 1 SEM. Hypoxia-specific injury is percent lactate dehydrogenase release after 30 minutes of N<sub>2</sub>/CO<sub>2</sub> incubation minus percent lactate dehydrogenase release after 30 minutes of continuous O<sub>2</sub>/CO<sub>2</sub> incubation. Reoxygenation injury is the increment in percent lactate dehydrogenase release from the end of N<sub>2</sub>/CO<sub>2</sub> incubation to the end of reoxygenation. All values were compared by unpaired Student's *t* test with Bonferroni correction for multiple comparisons. B: In these experiments, proximal tubular segments were harvested only from normal rats, gentamicin being added just to the proximal tubular segment buffer (1 mg/ml). (This permitted assessment of hypoxia/reoxygenation effects without having to account for gentamicin-induced lactate dehydrogenase release under oxygenated conditions.) In these experiments, a more severe hypoxic insult (45 minutes) followed by 15 minutes of reoxygenation was used. Gentamicin significantly decreased percent lactate dehydrogenase release in response to N<sub>2</sub>/CO<sub>2</sub> incubation irrespective of whether the data were analyzed as total amount of lactate dehydrogenase released after hypoxia or as "hypoxia-specific injury" (*p* < 0.05 by either paired or unpaired Student's *t* test, Bonferroni correction).

\**p* < 0.05, †*p* < 0.025 vs. controls.

release during reoxygenation, but this did not achieve statistical significance.

#### Effect of Gentamicin on Ischemia/Reperfusion Adenylate Pools

Gentamicin pretreatment did not alter end-ischemic ATP or AMP concentrations. However, it more than doubled ADP levels, thereby raising the total adenine nucleotide pool and lowering the ATP/ADP ratio (Table 2). At 15 minutes of reflow, both

ATP and ADP concentrations were higher in the gentamicin group. However, the ADP increment was greater, again lowering the ATP/ADP ratio. At 4 hours of reflow, ATP concentrations did not significantly differ between the groups. However, a significant gentamicin-induced ATP/ADP decrement (ADP increment) was still apparent. Postischemic (15 minutes) gentamicin injection reproduced the same perturbation in the 4-hour reperfusion ATP/ADP ratio (1.83±0.10) as did preischemic gentami-

TABLE 2. Adenine Nucleotide Values During Ischemia/Reflow With and Without Preischemic Gentamicin Treatment

	n	ATP	ADP	AMP	TAN	ATP/ADP
Normal values	13	8.85±0.30	3.30±0.12	0.75±0.05	12.9±0.35	3.2±0.29
25-Minute ischemia						
Gentamicin	6	0.39±0.04	1.93±0.20*	3.07±0.18	5.37±0.23†	0.21±0.01*
Controls	6	0.35±0.03	0.81±0.02	3.09±0.35	4.24±0.35	0.43±0.03
15-Minute reflow						
Gentamicin	5	4.80±0.31‡	3.10±0.20*	0.48±0.03	8.38±0.51*	1.55±0.06§
Controls	6	3.80±0.28	1.39±0.08	0.49±0.03	5.68±0.35	2.76±0.26
4-Hour reflow						
Gentamicin	5	5.00±0.50	2.95±0.40	0.62±0.05	8.57±0.86	1.74±0.13§
Controls	5	5.54±0.65	2.09±0.12	0.53±0.04	8.12±0.75	2.64±0.20
8-Hour reflow						
Gentamicin	6	6.55±0.43	2.38±0.23	0.61±0.12	9.54±0.54	2.89±0.36
Controls	7	7.36±0.65	2.53±0.21	0.63±0.10	10.53±0.82	2.97±0.23

All values are in micromoles per gram dry weight (mean±SEM). Normal values are from Reference 8. TAN, total adenine nucleotide (ATP+ADP+AMP).

\**p* < 0.001, †*p* < 0.02, ‡*p* < 0.05, §*p* < 0.01 vs. controls (by unpaired Student's *t* test).

**TABLE 3. Free Fatty Acid Levels in Kidneys During Ischemia/Reflow With and Without Preischemic Gentamicin Treatment**

	<i>n</i>	C16:0	C18:0	C18:1	C18:2	C20:4	Total
Normal values	5	146±11	127±5	53±4	46±5	128±12	499±26
25-Minute ischemia							
Gentamicin	4	1,438±43	859±18	418±16	440±11	640±6	3,794±78
Controls	4	1,520±27	870±14	423±7	436±12	620±24	3,868±54
15-Minute reflow							
Gentamicin	6	287±24	225±5	115±8	132±7	181±7	957±38
Controls	5	322±31	230±17	122±15	130±14	187±17	1,151±176

All values are in nanomoles per gram dry weight (mean±1 SEM). C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C20:4, arachadonic acid. Total=C16:0+C18:0+C18:1+C18:2+C20:4. Statistics were by unpaired Student's *t* test (no significant differences were found).

cin injection ( $p$ =NS). By 8 hours of reflow, differences in the adenylate pools were no longer observed, despite the fact that by this time the gentamicin group manifested substantially greater morphological damage (Figure 2C).

#### Deacylation/Free Fatty Acid Disposal During Ischemia/Reflow

Ischemia induced massive FFA release, the total of the five FFAs rising sevenfold compared with normal values (determined in five kidneys<sup>4</sup>) (see Table 3). However, the increments were virtually identical in the control and gentamicin-treated groups, indicating comparable phospholipase-mediated deacylation. After 15 minutes of reflow, both groups of kidneys sustained striking FFA decrements, so that FFA totals were only approximately two times higher than normal values. Again, no significant differences were observed between the control and gentamicin groups.

#### Regional Gentamicin Concentrations

In the absence of renal ischemia, gentamicin preferentially accumulated in the cortex compared with the OMS (OMS/cortical ratios of 0.57 and 0.49 at 4 and 24 hours, respectively) (Table 4). Gentamicin uptake in both regions was enhanced 2.5-fold to fivefold by the presence of ischemic renal damage,

whether the gentamicin was administered before or after the ischemic event (Table 4). Ischemia also changed the intrarenal distribution pattern since at 4 and 24 hours postischemia, the OMS/cortical gentamicin ratios were 0.99 and 0.62, respectively ( $p$ <0.001 compared with sham RPO/gentamicin groups; Table 4).

#### Gentamicin Effect on Glycerol-Induced Renal Injury

Glycerol alone failed to raise the serum Cr in any rat so tested (0.4–0.5 mg/dl). However, glycerol plus gentamicin caused an elevated Cr in four of five rats (0.9–2.9 mg/dl; mean,  $1.7\pm0.45$ ;  $p$ <0.05 versus glycerol alone), indicating its potential to severely exacerbate a nonischemic form of tubular injury.

### Discussion

Although it is recognized that gentamicin, when administered at the time of ischemia, can dramatically influence postischemic ARF,<sup>1</sup> it has been unclear as to whether it does so by predominantly affecting the ischemic or the postischemic injury process. Thus, the present study was conducted to assess gentamicin's net impact on both ischemic and reperfusion injury and to assess a few specific pathways by which these might be affected.

**TABLE 4. Renal Cortical and Outer Medullary Stripe Gentamicin Concentrations in the Presence and Absence of Concomitant Renal Ischemic Damage**

	<i>n</i>	Cortex	OMS	OMS/cortex
4-Hour gentamicin values				
With ischemia	4	5.12±0.66*	5.05±0.59†	0.99±0.03†
Without ischemia	4	1.79±0.14	1.01±0.13	0.57±0.05
24-Hour gentamicin values				
Ischemia+gentamicin (preischemia)	6	5.00±0.90	3.10±0.48	0.64±0.05
Ischemia+gentamicin (15 minutes postischemia)	8	4.97±0.79	2.98±0.47	0.62±0.03
Gentamicin alone	5	1.96±0.22‡	0.97±0.12‡	0.49±0.02§

All values are in milligrams of gentamicin per gram tissue dry weight (mean±1 SEM). Cortical and outer medullary stripe (OMS) values 4 and 24 hours after gentamicin injection are compared. For the 4-hour values, half the rats were subjected to renal ischemia or sham surgery 15 minutes after gentamicin injection. For the 24-hour values, the preischemic gentamicin and postischemic gentamicin injection values did not significantly differ; therefore, they were combined for the sake of statistical comparison with the gentamicin alone group.

\* $p$ <0.01, † $p$ <0.001 vs. without ischemia (unpaired Student's *t* test).

‡ $p$ <0.01, § $p$ <0.001 vs. combined values of preischemia plus gentamicin and postischemia plus gentamicin (unpaired Student's *t* test).

To gauge the net influence of gentamicin on ischemic and reperfusion injury, three approaches were taken. First, gentamicin was given before (15 minutes) or after (15 minutes or 8 hours) ischemia to determine when it influences the severity of postischemic ARF. It was reasoned that if gentamicin predominantly worsens ARF by altering the ischemic injury phase, more severe damage should result from preischemic than postischemic gentamicin administration. Alternatively, if gentamicin exerts its toxicity only during reperfusion, the severity of ARF should not be critically time dependent since gentamicin given preischemia is still present in the postischemic period. The second approach was to determine whether preischemic gentamicin exacerbates morphological damage at 15 minutes of vascular reperfusion with *in vivo* perfusion-fixed tissues. If no impact could be documented, this would speak against any exacerbation of the ischemic injury process. Lastly, to directly assess gentamicin's specific influence on oxygen deprivation injury, LDH release from PTS was quantified in the presence and absence of gentamicin treatment.

Each of the above studies indicates that it is during the reperfusion, not the ischemic, injury phase that gentamicin has its dominant adverse effects. First, the severity of ARF did not significantly differ depending on whether gentamicin was administered 15 minutes before or after the ischemic period. That gentamicin could exacerbate ARF even 8 hours postischemia is particularly noteworthy since by this time it is highly unlikely that gentamicin could have been acting on persistently ischemic tubular segments dictated by a "no-reflow" phenomenon. Certainly by 8 hours of reflow, any persistently ischemic segments would have succumbed to oxygen deprivation. Second, assessment of immediate morphological injury at 15 minutes of reflow failed to demonstrate an exacerbation of tubular damage. Thus, it is difficult to imply a worsening of the ischemic injury process. Third, a direct assessment of hypoxic PTS damage (LDH release) showed no adverse gentamicin effect. If anything, gentamicin induced cytoprotection against both a modest (30-minute) and a severe (45-minute) hypoxic insult. The reason for this surprising result remains to be defined. Nevertheless, this result supports the conclusion from the *in vivo* experiments that gentamicin does not adversely affect true oxygen deprivation-mediated tubular damage.

Because the data indicated that gentamicin predominantly worsens reperfusion, not ischemic, renal damage, gentamicin's impact on selected determinants of injury in the latter period was assessed next. Given gentamicin's ability to inhibit mitochondrial respiration,<sup>6,7</sup> its impact on reperfusion adenylate pools was extensively assessed. Even before reperfusion, an influence on cellular energetics was apparent: although end-ischemic ATP concentrations did not differ between the two groups, ADP was substantially higher with gentamicin treatment (suggesting decreased ADP breakdown and, hence, decreased

adenylate kinase activity). During reperfusion, seemingly paradoxical results were obtained: at 15 minutes, the gentamicin group had significantly higher ATP and ADP levels, indicating the drug's ability to conserve total reperfusion adenylate pools. The ATP increment could have been due to Na,K-ATPase inhibition,<sup>21</sup> while the elevated ADP again suggests decreased adenylate kinase activity. Perhaps more importantly, however, is that a failure to rephosphorylate ADP to ATP (thereby establishing a normal ATP/ADP ratio), strongly suggests a gentamicin-induced defect in mitochondrial oxidative phosphorylation.<sup>10,11</sup> That this defect, seen from 0–4 hours of reflow, temporally correlated with the period of evolving morphological damage (Figure 2) suggests pathogenetic relevance. Furthermore, that an equally depressed reperfusion ATP/ADP ratio could be produced by postischemic or preischemic gentamicin injection and that postischemic gentamicin also worsened ARF further suggests that this gentamicin-induced defect in bioenergetics had mechanistic relevance. That the depressed ATP/ADP ratio was not just a result of worse tubular injury, rather than being a potential mediator of it, is indicated by the appearance of comparable ATP/ADP ratios for the two groups at 8 hours of reflow, a time at which a pronounced difference in the extent of morphological injury was already apparent (Figure 2B).

Next, we assessed whether accelerated hydroxyl radical formation during reperfusion could explain gentamicin's adverse impact on postischemic ARF. Of note, Walker and Shah<sup>19</sup> have provided convincing evidence that hydroxyl radical is an important mediator of gentamicin nephrotoxicity in otherwise healthy rats. Thus, we tested whether deferoxamine and sodium benzoate, which attenuated pure gentamicin toxicity in their study, could induce a comparable protective influence on the postischemia-gentamicin interaction. However, no protection was observed, at least as assessed by 24-hour serum Cr concentrations. Of note, it has previously been shown that gentamicin plus ischemia fails to induce renal lipid peroxidation, as determined by malondialdehyde tissue assay.<sup>1</sup> Thus, although an appealing hypothesis, the available data do not support augmented reperfusion hydroxyl radical formation as a pathway by which gentamicin worsens postischemic renal damage.

A third pathway by which gentamicin could potentially influence postischemic ARF is through a perturbation in phospholipid deacylation during ischemia or by delayed FFA disposal during reperfusion. Of note, aminoglycosides can inhibit selected phospholipases,<sup>13–15</sup> and a drug-induced derangement in reperfusion bioenergetics might secondarily depress reperfusion reacylation rates.<sup>22</sup> Because FFAs can contribute to plasma membrane damage,<sup>12</sup> a failure of FFA disposal could theoretically worsen reperfusion injury. Thus, FFAs were quantified during ischemia and reperfusion in these experiments. By the end of the ischemia, striking deacylation had oc-



curred, but gentamicin did not appear to influence this process. By 15 minutes of reflow, most of the FFA burden had been disposed of (undoubtedly because of reacylation,  $\beta$ -oxidation, and renal venous efflux), but gentamicin again had no quantitative impact. Thus, it appears unlikely that potential gentamicin effects on deacylation/FFA disposal explains why the drug worsens postischemic renal damage.

Finally, in this study we have attempted to confirm that increased whole kidney<sup>1</sup> and renal cortical<sup>2</sup> gentamicin burdens, previously documented to occur in postischemic kidneys, also involve the OMS, the principal site of the ischemia-gentamicin interaction. Because under normal circumstances gentamicin preferentially accumulates in the cortex (e.g., Table 4), it is possible that values obtained from whole kidney and cortical gentamicin assays (e.g., References 1 and 2) did not accurately reflect the OMS drug burden. The present study lays this issue to rest since at 4 hours postgentamicin injection, a time of evolving renal injury (Figure 2), comparable cortical and OMS drug levels were observed. Interestingly, at 24 hours, the OMS had lost approximately 40% of its gentamicin, whereas cortical levels persisted largely unabated. This probably reflects the fact that the OMS, but not the cortex, developed widespread tubular cell necrosis, undoubtedly discharging its gentamicin into urine. The pathogenetic impact of a profound gentamicin load imposed on ischemia-damaged OMS proximal tubular cells can only be surmised. However, that gentamicin could exacerbate a totally dissimilar form of renal injury, that imposed by glycerol injection, suggests that aminoglycosides can induce profound nephrotoxicity if introduced in the setting of preexistent or concomitant tubular injury.

In conclusion, this study indicates that gentamicin, when delivered to the kidney just before vascular occlusion, worsens postischemic ARF predominantly by exacerbating the postischemic, rather than the ischemic, injury phase. This statement is based on observations that preischemic and postischemic gentamicin administration induce comparable degrees of renal damage, that immediate postischemic morphological injury is not altered by preischemic gentamicin treatment, and that in vitro hypoxic PTS injury is paradoxically lessened, not worsened, by gentamicin administration. Gentamicin can cause a modest preservation of ATP and ADP in an early (15-minute) postischemic kidney. However, a persistent lowering of the reperfusion ATP/ADP ratio, produced by either preischemic or postischemic gentamicin injection, indicates that the drug can negatively influence reperfusion cellular energetics. This latter finding correlates with and potentially could explain the exacerbation of tubular damage. Gentamicin does not appear to influence hydroxyl radical-mediated postischemic injury, at least as assessed at the whole organ level. Furthermore, gentamicin does not appear to alter ischemic deacylation or postischemic FFA disposal. Lastly, ischemia predisposes to pro-

found OMS gentamicin uptake. Because the OMS is the principal site of the ischemia-gentamicin interaction, this finding suggests that gentamicin worsens postischemic ARF by imposing a profound nephrotoxin burden on sublethally damaged proximal tubular segments. That gentamicin can exacerbate a totally dissimilar form of tubular injury, that imposed by glycerol injection, supports this possibility.

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