

# Alpha-linolenic acid protects against gentamicin induced toxicity

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**Background:** Recent studies indicate that reactive oxygen species are the major culprits behind the renal damage induced by gentamicin, an aminoglycoside antibiotic used to treat serious and life threatening Gram-negative infections. Experimental evidence suggests a protective role of alpha-linolenic acid supplementation against oxidative stress. The aim of the present study was to investigate the possible beneficial role of alpha-linolenic acid against gentamicin induced renal distress.

**Methods:** Male Wistar rats were divided into three groups of eight rats each, with the first group serving as a control. The other groups were treated intraperitoneally with gentamicin 100 mg/kg body weight per day for 10 days  $\pm$  alpha-linolenic acid and vitamin E (each given as 250 mg/kg body weight per day). Concentrations of creatinine, urea, cholesterol, inorganic phosphate in serum, malondialdehyde and total sulfhydryl levels, and glutathione-S-transferase, superoxide dismutase, and catalase activity in kidney tissues were determined.

**Results:** Administration of gentamicin to rats induced marked renal failure, characterized by a profound increase in serum creatinine, urea, and cholesterol concentrations, accompanied by significant lowering of renal alkaline phosphatase and acid phosphatase activity, an increase in malondialdehyde, a decline in total sulfhydryl levels, and lowered superoxide dismutase, catalase, and glutathione-S-transferase activity. Cotreatment with alpha-linolenic acid produced amelioration in these biochemical indices of nephrotoxicity in serum as well as in tissue. Further histopathological and human studies are necessary to demonstrate the beneficial effects of alpha-linolenic acid in renal disease.

**Conclusion:** Alpha-linolenic acid may represent a nontoxic and effective intervention strategy in gentamicin induced nephrotoxicity.

**Keywords:** aminoglycosides, nephrotoxicity, oxidative stress, omega 3 fatty acids, alpha linolenic acid, plant omega 3

## Introduction

Aminoglycoside antibiotics have remained the mainstay of treatment for Gram-negative infections for more than 40 years, despite their associated nephrotoxicity.<sup>1</sup> Gentamicin is a very effective antibiotic belonging to this class of agents but, unfortunately, causes renal failure in 10%–20% of therapeutic courses, with many patients developing at least some signs of nephrotoxicity when treated for more than 7 days.<sup>2</sup> The mechanism of nephrotoxicity for gentamicin is apparently related to its accumulation in the renal proximal convoluted tubules, causing a number of morphological, metabolic, and functional alterations.<sup>3</sup> Gentamicin induced oxidative stress causing deficiency in intrinsic antioxidant enzymes is considered to be the main mediator of its nephrotoxic effects.<sup>4</sup> Various strategies and agents used or under exploration for targeting the nephrotoxic

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effects of gentamicin have not been completely successful.<sup>2,4,5</sup> A large body of scientific evidence from randomized controlled trials has been amassed, indicating a myriad of health benefits from the omega-3 polyunsaturated fatty acids, eicosapentaenoic acid and docosahexaenoic acid, especially when derived from fish.<sup>5</sup> Plant omega-3 alpha-linolenic acid (abundant in flaxseeds, canola seeds, and walnuts) is their essential dietary precursor.<sup>6</sup> For alpha-linolenic acid, the relationship with health is less clear although a few recent studies have documented its cardioprotective, anticancer, anti-inflammatory, antioxidant, and antithrombotic activity in various disease models.<sup>7–11</sup> However, there is no literature reporting the efficacy of alpha-linolenic acid in the management of toxic insult caused by antibiotics.

With the continued and increasing use of aminoglycoside antibiotics and the numerous health benefits of omega-3 polyunsaturated fatty acids, and persistent lacuna on studies analyzing the effects of alpha-linolenic acid, the present work was designed to study the possible protective role of alpha-linolenic acid against gentamicin induced nephrotoxicity. The results indicate that alpha-linolenic acid ameliorates gentamicin induced nephrotoxicity almost completely, and reverses deranged kidney function and antioxidant status to normal. A potential therapeutic role for alpha-linolenic acid supplementation in drug induced nephropathies is anticipated.

## Materials and methods

Gentamicin was purchased from Nicholas Piramal India Ltd (Mumbai, India). All other chemicals used in the study were sourced from either Sigma (St Louis, MO) or SRL (Mumbai, India). The animal experiments were conducted according to the guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals, the Ministry of Environment and Forests, Government of India, and institutional animal ethics committee guidelines.

Healthy adult male Wistar rats weighing 150–200 g (3–4 months old) purchased from the Animal Facility Center, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India, were used in the study. The animals were acclimatized to the animal facility for a week on standard rat chow (Aashirwaad Industries, Chandigarh, India) and water ad libitum under controlled conditions of temperature, relative humidity, and a normal photoperiod (12-hour dark and light cycle). The rats were separated into three groups (8–10 rats per group). Gentamicin 100 mg/kg body weight per day was injected intraperitoneally in one daily dose for 10 days in the gentamicin group. Rats in the control group received the same

volume of 0.9% saline intraperitoneally. The third group was additionally administered an oral dose of alpha-linolenic acid and vitamin E (each 250 mg/kg body weight per day) for the same time period. At the end of experimentation period, the animals were sacrificed under light ether anesthesia. Blood was withdrawn from the left jugular vein, and the serum was separated by centrifugation at  $2000 \times g$  for 10 minutes. The kidneys were harvested and processed for preparation of homogenates as described below.

## Preparation of homogenate

The decapsulated kidneys were kept in ice-cold buffered saline (154 mM NaCl, 5 mM Tris–HEPES, pH 7.5). A 10% (w/v) homogenate was prepared in 0.1 M Tris–HCl buffer, pH 7.5, using a Potter–Elvehjem homogenizer (Remi Motors, Mumbai, India) and passing five pulses. The homogenate was centrifuged at  $3000 \times g$  at  $4^{\circ}\text{C}$  for 15 minutes to remove the cell debris, and the supernatant was saved in aliquots and stored at  $-20^{\circ}\text{C}$  for assaying the kidney marker enzymes, alkaline phosphatase and acid phosphatase as well as free-radical scavenging enzymes, and for estimation of total sulfhydryl and lipid peroxidation.

## Enzyme assays

The activities of the kidney biomarker and lysosomal enzymes were determined using a spectrophotometer (Cintra 5, GBC Scientific Equipment Pty, Victoria, Australia), with standard methods as described in previous studies.<sup>5,12</sup> Protein concentrations were determined using the method reported by Lowry et al.<sup>13</sup> Serum parameters were analyzed as described previously.<sup>5,12</sup>

## Assay of enzymatic and nonenzymatic antioxidants

Superoxide dismutase was assayed using the method of Marklund and Marklund.<sup>14</sup> Catalase and glutathione-S-transferase were determined by the method of Giri et al<sup>15</sup> and Habig et al,<sup>16</sup> respectively. Total sulfhydryl was determined using the method of Sedlak and Lindsay<sup>17</sup> and lipid peroxidation by the method of Ohkawa et al.<sup>18</sup>

## Statistical analysis

All data are expressed as the mean  $\pm$  standard error of mean. Statistical evaluation was conducted by one-way analysis of variance and using the unpaired Student's *t*-test with Statistical Package for the Social Sciences version 7.5 software (SPSS Inc, Chicago, IL). A probability level of  $P < 0.05$  was selected as indicating statistical significance.

## Results and discussion

Aminoglycoside antibiotics including gentamicin, are very important clinically in the treatment of serious Gram-negative infections, and are potential therapeutic agents in diseases characterized by premature stop mutations. However, their use is associated with a high incidence of acute renal failure.<sup>1</sup> Various approaches have been attempted to reduce gentamicin nephrotoxicity in experimental animals, although none has been found to be safe and/or suitable for use in clinical practice.<sup>5</sup> Omega 3 polyunsaturated fatty acids from marine sources have recently been shown to slow progression of various forms of cancers, depression, arthritis, and asthma, and to protect against antibiotic nephrotoxicity.<sup>5</sup> Plant-derived alpha-linolenic acid may constitute an attractive renoprotective alternative to fish-derived omega-3 polyunsaturated fatty acids. However, its effects on antibiotic induced nephrotoxicity remain unknown.

The beneficial effects of alpha-linolenic acid on gentamicin mediated nephrotoxic insult were studied using three different modes of alpha-linolenic acid supplementation (before, with, and after intraperitoneal gentamicin). However, the maximum potency was shown in the cotreatment mode. All animal groups consumed similar amounts of rat pellets (20 g/day per rat). There was no significant difference in body weight or food intake in the animals as a result of treatment with gentamicin or alpha-linolenic acid (data not shown). A control group, treated with alpha-linolenic acid and vitamin E alone, was also included in this study. The results did not vary significantly from normal control values.

In the present study, gentamicin produced a typical pattern of nephrotoxicity, with an elevation in serum urea from  $4.4 \pm 0.19$  mg/dL to  $22.3 \pm 1.1$  mg/dL and in serum creatinine from  $0.6 \pm 0.09$  mg/dL to  $1.2 \pm 0.28$  mg/dL (Table 1) after 10 days of injection when compared with normal control rats. Administration of gentamicin to rats also caused an increase in serum cholesterol levels from  $92.76 \pm 4.2$  mg/dL to  $132.32 \pm 6.8$  mg/dL and a decline in serum inorganic phosphate levels from  $1.76 \pm 0.05$   $\mu$ mol/mL to  $1.30 \pm 0.01$

$\mu$ mol/mL as compared with the control group (Table 1). These results are consistent with earlier studies.<sup>2,5</sup> As suggested by Abdel-Raheem et al,<sup>2</sup> the increase in serum creatinine and urea might be due to gentamicin induced glomerular damage. Administration of alpha-linolenic acid to gentamicin treated rats resulted in significant attenuation of various deleterious gentamicin elicited effects on serum parameters. Alpha-linolenic acid prevented any gentamicin induced increase of serum creatinine, urea, and cholesterol, and was associated with a decrease in serum inorganic phosphate (Table 1). Administration of alpha-linolenic acid alone had no appreciable effect on the above parameters.

Because brush border membrane and lysosomes of the renal proximal tubules are shown to be specific targets for gentamicin, their integrity was assessed by the status of the biomarker enzymes, alkaline phosphatase and acid phosphatase. Treatment with gentamicin in the control rats resulted in a significant reduction in the specific activity of the above enzymes. The specific activity of alkaline phosphatase declined from  $21 \pm 1.1$   $\mu$ mol/mg protein per minute to  $11 \pm 0.95$   $\mu$ mol/mg protein per minute and that of acid phosphatase from  $8 \pm 0.88$   $\mu$ mol/mg protein per minute to  $4 \pm 0.17$   $\mu$ mol/mg protein per minute (Table 2). The results are again consistent with those of other studies<sup>5</sup> and demonstrate tubular damage caused by gentamicin. Alpha-linolenic acid, when given along with gentamicin, protected against the gentamicin induced reduction of activity in these biomarker enzymes. The activity of alkaline phosphatase remained higher in rats treated with the combination of alpha-linolenic acid and gentamicin. It has been shown that dietary fatty acids incorporated in the cellular membranes alter the structural integrity and functional capacity of the plasma membrane and other organelles, leading to altered cellular metabolic activity.<sup>19</sup> Present observations could afford a similar justification. The integrity of the brush border membrane when challenged by this antibiotic causes phospholipidosis, which might be responsible for the decreased alkaline phosphatase activity. Fish oil (rich

**Table 1** Effect of alpha-linolenic acid on serum parameters in rats treated with gentamicin

Group	Creatinine (mg/dL)	Urea (mg/dL)	Cholesterol (mg/dL)	Inorganic phosphate ( $\mu$ mol/mL)
Normal control	$0.60 \pm 0.09$	$4.41 \pm 0.19$	$92.76 \pm 4.2$	$1.76 \pm 0.05$
Gentamicin	$1.18 \pm 0.28^{\dagger}$ (+96%)	$22.3 \pm 1.1^{\dagger}$ (+405.6%)	$132.32 \pm 6.8^{\dagger}$ (+42.6%)	$1.30 \pm 0.01^{\dagger}$ (-26%)
$\alpha$ LNA + GM	$0.59 \pm 0.09^{*}$ (-1.6%)	$6.98 \pm 0.9^{\dagger,*}$ (+58.2%)	$84.69 \pm 3.7^{*}$ (-8.6%)	$1.82 \pm 0.08^{*}$ (+3.4%)

**Notes:** Results are mean  $\pm$  standard error of the mean for eight different preparations; values in parentheses represent percent change from control; <sup>†</sup>significantly different from control at  $P < 0.05$  by one way analysis of variance; \*significantly different from gentamicin treated at  $P < 0.05$  by one-way analysis of variance.

**Abbreviations:**  $\alpha$  LNA, alpha-linolenic acid; GM, gentamicin.

**Table 2** Effect of alpha-linolenic acid on levels of renal biomarker enzymes and antioxidant parameters in kidney homogenates of rats treated with gentamicin

Parameter	Normal control	GM	$\alpha$ LNA + GM
<b>Renal biomarker enzymes</b>			
Alkaline phosphatase	21.01 $\pm$ 1.1	11.17 $\pm$ 0.95 <sup>†</sup>	27.77 $\pm$ 1.01 <sup>†*</sup>
Acid phosphatase	8.34 $\pm$ 0.88	4.88 $\pm$ 0.17 <sup>†</sup>	6.90 $\pm$ 0.98 <sup>†*</sup>
<b>Enzymatic and nonenzymatic antioxidants</b>			
MDA	180 $\pm$ 4.42	257 $\pm$ 8.48 <sup>†</sup>	164 $\pm$ 3.98*
Total-SH	6.89 $\pm$ 0.57	4.28 $\pm$ 0.09 <sup>†</sup>	7.00 $\pm$ 0.29*
Catalase	136.18 $\pm$ 4.3	61.33 $\pm$ 0.85 <sup>†</sup>	116.44 $\pm$ 2.8 <sup>†*</sup>
SOD	14.44 $\pm$ 1.3	4.37 $\pm$ 0.59 <sup>†</sup>	19.24 $\pm$ 1.01 <sup>†*</sup>
GST	1.92 $\pm$ 0.08	0.82 $\pm$ 0.005 <sup>†</sup>	2.31 $\pm$ 0.04 <sup>†*</sup>

**Notes:** Results are shown the mean  $\pm$  standard error of the mean for eight different preparations; activity of alkaline and acid phosphatase are expressed as  $\mu$ mol/mg protein per minute, catalase as  $\mu$ mol/mg protein/mL, superoxide dismutase as units/mg protein, glutathione-S-transferase as units/mg protein per minute, units for malondialdehyde and total-SH are nmol/g tissue and  $\mu$ mol/g tissue, respectively; <sup>†</sup>significantly different from control at  $P < 0.05$  by one-way analysis of variance; \*significantly different from gentamicin treated at  $P < 0.05$  by one-way analysis of variance.

**Abbreviations:**  $\alpha$  LNA, alpha-linolenic acid; GM, gentamicin; SH, sulfhydryl; MDA, malondialdehyde; SOD, superoxide dismutase; GST, glutathione-S-transferase.

in omega 3 polyunsaturated fatty acids) has been shown to protect the membranes of cells in the proximal tubule against antibiotic induced damage.<sup>5</sup>

Substantial in vivo and in vitro evidence indicates that partially reduced oxygen metabolites are important mediators of the nephrotoxicity of gentamicin.<sup>2</sup> Similarly, in the present report, gentamicin suppressed antioxidant enzymes and increased lipid peroxidation and decreased total sulfhydryl in renal tissue (Table 2). Gentamicin produced a decrease in total sulfhydryl levels from  $6.89 \pm 0.57 \mu\text{mol/g}$  tissue to  $4.28 \pm 0.09 \mu\text{mol/g}$  tissue. Alpha-linolenic acid prevented any gentamicin induced decrease in total sulfhydryl content and restored its normal level to  $7.00 \pm 0.29 \mu\text{mol/g}$  tissue. Reactive oxygen species induce lipid peroxidation in cells, and malondialdehyde formed during this oxidative process is accepted as an indicator of lipid peroxidation.<sup>20</sup> The gentamicin induced increase in malondialdehyde (+42%) was normalized by treatment with alpha-linolenic acid (−8%). Treatment with gentamicin caused a marked decrease in superoxide dismutase, catalase, and glutathione-S-transferase activity (Table 2). Alpha-linolenic acid alone slightly increased the activity of these antioxidant enzymes, as well as the total sulfhydryl content (data not shown).

Alpha-linolenic acid significantly attenuated the severity of gentamicin induced oxidative stress in renal tissue. The activity of all the enzymes studied returned back to near control values (Table 2). Protection against the adverse effects of gentamicin by alpha-linolenic acid can be attributed to the latter's intrinsic biochemical and natural antioxidant properties. Recently, it has been shown that ingestion of alpha-linolenic acid markedly attenuates myocardial ischemia-reperfusion injury via anti-inflammatory and antioxidative effects.<sup>8</sup> The mechanism of action of alpha-linolenic acid might have been

the same in the present case. Dietary omega 3 polyunsaturated fatty acid supplements derived from marine sources has also been shown to strengthen antioxidant defense mechanisms in the plasma of normal rats.<sup>21</sup>

Current research has shown that oral intake of alpha-linolenic acid is beneficial in experimental colitis, myocardial infarction, arterial thrombus formation, and osteoporosis.<sup>8–11</sup> Our results provide support for the notion that omega 3 polyunsaturated fatty acids are effective nutritional interventions. They also provide early evidence of the protective role of plant omega 3 against nephrotoxicity induced by gentamicin. The ability of alpha-linolenic acid to regulate the expression of nuclear factor  $\kappa$ B, tumor necrosis factor, and inflammatory interleukins may account for its protective effects.<sup>8–11</sup> Rigorous research is required to clarify the actual mechanism involved.

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## Disclosure

The authors declare no conflicts of interest in this work.

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