**INTRODUCTION**

Gentamicin (GEM) is widely applied in veterinary and human clinical practices for treatment of life-threatening gram negative infections.\(^1,2\) GEM is considered an "obligatory nephrotoxin" and even small doses have been reported by several authors to produce nephrotoxicity in man and animals.\(^3,4\) It is an aminoglycoside antibiotic that is still commonly used in the treatment of life-threatening infections. Their broad-spectrum activity, chemical stability and rapid bactericidal action have often made them first-line drugs in a variety of clinical situations.\(^2,5,6\) Their broad-spectrum activity, chemical stability and rapid bactericidal action have often made them first-line drugs in a variety of clinical situations.\(^2,5,6\)

However, high concentrations of these antibiotics are known to be nephrotoxic. In some cases, this side effect is so severe that the use of the drug must be discontinued. It has been estimated that up to 30\% of the patients treated with aminoglycosides for more than 7 days showed some signs of nephrotoxicity.\(^7\) GEM induced nephrotoxicity leads to disability in the excretion of the daily waste products (i.e., urea, uric acid, creatinine etc.) in the urine.\(^1,8\)

GEM has shown increase in the generation of reactive oxygen species (ROS) such as H\(_2\)O\(_2\) or •OH in cellular systems in both *in vitro* and *in vivo* studies.\(^8,9,11\) Generally, ROS and cytotoxins can cause cell death by necrosis or apoptosis, often in a dose-dependent manner. Mitochondria can play a vital role in both apoptosis and necrosis.\(^12-15\) The opening of mitochondrial permeability transition pores (MPTP) due to ROS and cytokines was first described in necrotic cell death. During the process of opening of MPTP, a cyclosporin A (CsA)-sensitive pore is opened resulting in a swelling of mitochondria and destruction of the outer mitochondrial membrane.\(^14,16-18\) Mitochondrial Ca\(^{2+}\) overload leads to mitochondrial membrane permeability transition, which is associated with the opening of a MPTP.\(^19,20\) The attack of ROS to membrane protein thiols produces cross-linking reaction that may open membrane pores upon Ca\(^{2+}\) binding.\(^21\) It is interesting to note that CsA is the most potent mitochondrial permeability transition inhibitor described till date.\(^18\)

Pharmacologically, many agents have been reported to attenuate GEM induced nephrotoxicity in experimental animals.\(^8,22-24\) Recently, various natural antioxidant were focused on the prevention of nephrotoxicity.\(^9,21,25,26\) Clinically, flunarizine has been widely used to treat vertigo,\(^27\) migraine,\(^28\) epilepsy\(^29\) and tinnitus.\(^30\) Some of the studies sug-
gested the possibility that flunarizine may act directly on mitochondria and result in closing of MPTP.\textsuperscript{31} Furthermore, it reduced the mitochondrial swelling and lipid peroxidation induced by \textit{Fe}_{2}SO_{4} and ascorbic acid.\textsuperscript{32} No drug treatment has been shown to limit the progression of renal damage and/or speed up recovery from nephrotoxin induced renal failure.

The clinical pattern of GEM induced kidney damage has been studied extensively in both man and animals.\textsuperscript{3,4} Recently, some factors have been identified to increase the risk of aminoglycoside induced nephrotoxicity that cannot be modified readily by the clinician (e.g., sex, obesity, preexisting liver or renal diseases and underlying diseases) and factors that the clinician may be able to modify (\textit{i.e.}, drug dosage/interval/duration, specific aminoglycoside, hypokalaemia, hypomagnesaemia, metabolic acidosis, volume depletion and concurrent medications).\textsuperscript{33-35} However, there is no unanimity in the literature regarding the possible mechanism of action and factors that can modulate the GEM induced nephrotoxicity. Hence, in the present study it is the first attempt to explore the role of flunarizine on GEM induced nephrotoxicity via inactivation of MPTP.

**MATERIALS AND METHODS**

**Animal**
Male Sparque Dawley rats (180–250 g) were procured from the Panjab University, Chandigarh. The animals were housed under standard laboratory conditions of temperature relative humidity with free access to food (Hindustan Lever Products, Kolkata, India) and water \textit{ad libitum}. A 12 h light-dark cycle was maintained throughout the experimental protocol. An acclimatization period of 7 days was allowed for the rats before experimentation. The experimental protocols and surgical procedures were approved by Institutional Animal Ethics Committee (IAEC) and care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No. 874/\textbullet{}ac/05/ CPCSEA).

**Chemicals**
Chemicals such as Folin-Ciocalteu’s Phenol reagent (Merck Limited, Mumbai), 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), bovine serum albumin (BSA), (Sisco Research Laboratories Pvt. Ltd. Mumbai and S. D. Fine Chemicals, Mumbai), thiobarbituric acid and 1,1,3,3-tetra methoxy propane (Loba Chem, Mumbai) were procured for the present study. GEM was obtained as a gift sample from Ranbaxy Pharmaceuticals, Mumbai. All the reagents used in the present study were of analytical grade.

**Experimental Design**
Six groups, each comprising of six rats (\textit{n} = 6), were included in the acute renal injury. Group I (Normal control group): Normal saline (0.5 ml, \textit{p.o.}) was given for 10 (day 0–9) consecutive days. Group II (GEM control group): Gentamicin (40 mg/kg, \textit{s.c.}) was given for 10 consecutive days. Group III: Rats were subjected to administration of cyclosporin A (CsA, 50 \textmu{}mol/kg, \textit{p.o}) before one hour of each GEM injection for 10 consecutive days. Group IV–VI: Rats were subjected to administration of flunarizine (100, 200 and 300 \textmu{}mol /kg, \textit{p.o}) before one hour of each GEM injection for 10 consecutive days.

In all the groups rats were weighed, before (day 0) and after (day 10) drug administration.

**Biochemical Estimation**
Rats were placed in the metabolic cages (on day 9) and urine samples were collected over the period of 24 hours. Rats were anesthetized with thiopental sodium (35 mg/kg, \textit{i.p.}, on day 10). Blood was collected from the inferior vena cava in plain plastic tubes with and without anticoagulant (sodium citrate), left to stand at 4°C for 1 h, and centrifuged at 900 g at 5°C for 15 min to separate serum and plasma respectively. The rats were sacrificed with euthanasia. The kidneys were removed from the rats and washed with ice-cold saline. A small piece from the left kidney was fixed in 10% buffered formalin. The medullary portion of the kidney was homogenized in ice-cold saline to produce 10% (w/v) tissue homogenate.

**Evaluation of Renal Function**
Blood urea nitrogen (BUN) was estimated in blood sample whereas, N-acetyl \textit{\beta}-d-glucosaminidase (NAG) was estimated in urine sample by using standard diagnostic kits (Span Diagnostics, Gujarat, India). Abnormal changes in BUN level served as an indicator of impaired glomerular function whereas, changes in NAG level served as a specific indicator of tubular damage. The fractional excretion of sodium (FrNa = Sodium\textsubscript{Urine} / Sodium\textsubscript{Plasma} \times Creatinine\textsubscript{Plasma} / Creatinine\textsubscript{Urine} \times 100) and the creatinine clearance (CrCl = Creatinine\textsubscript{Urine}/Creatinine\textsubscript{Plasma} \times Urine\textsubscript{Volume} / time) were measured as an index of renal function.

**Estimation of Total Tissue Protein Content**
Renal tissue protein content was estimated according to the method of Lowry et al.\(^{30}\) using bovine serum albumin (BSA) as a standard. The absorbance was determined spectrophotometrically at 750 nm.

**Estimation of Tissue TBARS Levels** Estimation of lipid peroxidation was performed by measuring the thiobarbituric acid reactive substances (TBARS) by the method of Okawa et al.\(^{37}\) A standard calibration curve was prepared by using 1-10 nM of 1,1,3,3-tetramethoxy propane. The concentration was expressed in terms of nanomoles of TBARS per mg of protein.

**Estimation of Tissue Reduced Glutathione** The reduced glutathione (GSH), content in the renal tissue was estimated using method of Ellman.\(^{38}\) A standard curve was plotted using 5-50 \(\mu\)mol of reduced form of glutathione. The concentration was expressed in terms of micromole of GSH per mg of protein.

**Estimation of Total Calcium** Total calcium levels were estimated in the renal tissue as described by Severnhaus and Ferree.\(^{39}\) Briefly, the renal tissue homogenate was mixed with 1 ml of trichloroacetic acid (4%) in the ice-cold condition and centrifuged at 1500 \(\times\) g for 10 min. The clear supernatant was used for estimating the total calcium levels by atomic emission spectroscopy at 556 nm.

**Preparation of Mitochondria** Renal mitochondria was isolated from rat kidney tissue as described by method of Stephan et al.\(^{40}\) with slight modification of Long et al.\(^{41}\) Briefly, tissues were washed with saline, weighed and put into ice-cold isolation buffer containing 0.25 M sucrose, 10 mM Tris base, 0.5 mM EDTA (pH 7.4). Tissues were homogenized in 2.5 volume of isolation buffer. The homogenate was adjusted to 8 volumes with isolation buffer and centrifuged at 1000 \(\times\) g for 4 min. The supernatant fraction was decanted and saved. The pellet was washed once with 2 volume of isolation buffer. The supernatant fractions were combined and centrifuged at 10000 \(\times\) g for 4 min. The mitochondrial pellet was washed twice with isolation buffer. All the above mentioned operations were carried out at 4°C. An aliquot was used for the estimation of mitochondria respiration chain function (i.e., cytochrome c oxidase activity) and adenosine triphosphate (ATP) content.

**Estimation of Cytochrome C Oxidase Activity and ATP Content** The activity of cytochrome c oxidase (Cyt-C oxidase; as an index of respiratory marker enzymes) was measured according to the method of Rustin et al.\(^{42}\) The enzymatic activity was measured by following the decrease in absorbance of reaction mixture at 550 nm with 580 nm as reference wavelength (\(e=19.1 \text{mM}^{-1} \text{cm}^{-1}\)). The activity of cytochrome c oxidase was measured at 25°C for 10 min and activity was expressed in terms of nM of cytochrome c oxidase per minute per mg of protein.

**Statistical Analysis** All the results were expressed as mean \(\pm\) standard error of means (S.E.M). The data of all biochemical estimation was statistically analyzed by one-way ANOVA followed by Tukey’s multiple range tests by using Sigmastat Version-2.0 software. The \(p\)-value<0.05 was considered to be statistically significant.

**RESULT**

**Effect of Flunarizine on Body Weight** The results demonstrated that GEM caused a marked decrease in total body weight as compared to normal control group. However, pretreatment with flunarizine (200 and 300 \(\mu\)mol/kg) and CsA had attenuated this change as compared to GEM treated group (Fig. 1).

**Effect of Flunarizine on Renal Functional Markers** The results demonstrated that GEM caused a marked increase in blood urea nitrogen (BUN), urinary \(N\)-acetyl \(\beta\)-d-glucosaminidase (NAG) and decrease in fractional sodium excretion (FrNa) and creatinine clearance (CrCl) as compared to sham control group. However, pretreatment with medium and high dose of flunarizine had significantly improved this alteration in the serum and urinary biomarker changes which were comparable to that of normal control and CsA treated groups (Table 1).

**Effect of Flunarizine on Tissue and Mitochondrial Biomarker Changes** The results demonstrated that GEM caused a marked increase in tissue thiobarbituric acid reactive substances (TBARS) and total calcium whereas, decrease in reduced glutathione (GSH), mitochondrial cytochrome c oxidase (Cyt-C oxidase) and ATP levels as compared to normal control group whereas, pretreatment with medium and high dose of flunarizine (200 and 300 \(\mu\)mol/kg) had
Fig. 1. Effect of Flunarizine on Body Weight
Values are mean ± S.E.M. of 6 animals. a $p<0.05$, as compared to normal control group; b $p<0.05$, flunarizine pretreated groups as compared to gentamicin control group, in parenthesis indicated the dose of CsA and flunarizine in $\mu$mol/kg.

Table 1. Effect of Flunarizine on Renal Functional Markers

<table>
<thead>
<tr>
<th>Groups</th>
<th>BUN (mg/dl)</th>
<th>NAG (U/min/l)</th>
<th>FrNa (%)</th>
<th>CrCl (ml/min/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Normal</td>
<td>19.92±0.72</td>
<td>21.1±1.92</td>
<td>62.3±2.1</td>
<td>0.73±0.12</td>
</tr>
<tr>
<td>II. GEM Control</td>
<td>31.41±0.69</td>
<td>89.5±3.15</td>
<td>14.74±3.6</td>
<td>0.11±0.05</td>
</tr>
<tr>
<td>III. CsA (50)</td>
<td>20.69±0.85</td>
<td>24.1±2.63</td>
<td>57.5±1.9</td>
<td>0.68±0.09</td>
</tr>
<tr>
<td>IV. Flunarizine (100)</td>
<td>29.36±0.93</td>
<td>79.2±3.67</td>
<td>18.26±3.5</td>
<td>0.17±0.09</td>
</tr>
<tr>
<td>V. Flunarizine (200)</td>
<td>22.52±1.03</td>
<td>45.6±2.48</td>
<td>49.32±1.8</td>
<td>0.45±0.04</td>
</tr>
<tr>
<td>VI. Flunarizine (300)</td>
<td>21.25±1.09</td>
<td>26.5±1.57</td>
<td>55.4±2.8</td>
<td>0.64±0.13</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. of 6 animals. a $p<0.05$, as compared to normal control group; b $p<0.05$, flunarizine pretreated groups as compared to GEM control group, in parenthesis indicated the dose of CsA and flunarizine in $\mu$mol/kg.

BUN, Blood urea nitrogen; Cr, Creatinine; NAG, N-acetyl-$\beta$-d-glucosaminidase; FrNa, Fractional excretion of sodium; CrCl, Creatinine clearance.

Table 2. Effect of Flunarizine on Tissue Biomarker Changes

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS (nmol/mg of protein)</th>
<th>GSH (m mol/mg of protein)</th>
<th>Total Calcium (m mol/g of dry weight)</th>
<th>Cyt-C oxidase (m mol/min of mg of protein)</th>
<th>ATP (m mol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Normal</td>
<td>26.13±2.61</td>
<td>10.04±0.32</td>
<td>11.16±2.71</td>
<td>0.35±0.03</td>
<td>3.86±0.12</td>
</tr>
<tr>
<td>II. GEM Control</td>
<td>79.52±3.14</td>
<td>2.71±0.21</td>
<td>124.15±4.36</td>
<td>0.18±0.01</td>
<td>2.29±0.18</td>
</tr>
<tr>
<td>III. CsA (50)</td>
<td>29.13±2.65</td>
<td>9.76±0.19</td>
<td>21.64±3.26</td>
<td>0.33±0.01</td>
<td>3.62±0.09</td>
</tr>
<tr>
<td>IV. Flunarizine (100)</td>
<td>72.92±2.63</td>
<td>3.75±0.42</td>
<td>106.32±2.73</td>
<td>0.22±0.02</td>
<td>2.49±0.14</td>
</tr>
<tr>
<td>V. Flunarizine (200)</td>
<td>47.8±2.69</td>
<td>6.92±0.31</td>
<td>53.67±2.94</td>
<td>0.31±0.03</td>
<td>3.38±0.21</td>
</tr>
<tr>
<td>VI. Flunarizine (300)</td>
<td>31.42±2.09</td>
<td>8.69±0.23</td>
<td>32.91±3.26</td>
<td>0.29±0.02</td>
<td>3.54±0.16</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. of 6 animals. a $p<0.05$, as compared to normal control group; b $p<0.05$, flunarizine pretreated groups as compared to GEM control group, in parenthesis indicated the dose of CsA and flunarizine in $\mu$mol/kg. TBARS, Thiobarbituric reactive substances; GSH, Reduced glutathione; Cyt-C oxidase, cytochrome c oxidase; ATP, Adenosine triphosphate.

significantly ameliorated GEM induced renal tissue biomarkers change which were comparable to that of normal control and CsA treated groups (Table 2).

**DISCUSSION**

In this study, we demonstrated that flunarizine
(200 and 300 \( \mu \text{mol/kg} \)) administration had significantly reduced serum BUN, urinary NAG, tissue TBARS and total calcium whereas, increased body weight, FrNa, CrCl, GSH, mitochondrial cytochrome c oxidase (Cyt-C) and ATP levels resulting in the attenuation of renal dysfunction in rats subjected to GEM induced nephrotoxicity. This is the first report, as far as we are aware, on the effect of flunarizine in GEM induced nephrotoxicity via inactivation of MPTP opening.

Administration of GEM has been resulted in the body weight reduction along with renal dysfunction, which had also been reported in the previous studies.\(^{10,45}\) The reduction in body weight following GEM treatment may possibly be due to the injured renal tubules and the subsequent loss of the tubular cells to reabsorb water, leading to dehydration and loss of body weight. The blood, urine and renal tissue sample analysis has confirmed that GEM caused significant increase in the serum BUN, urinary NAG and tissue total calcium levels whereas, it decreased the FrNa and CrCl levels. GEM induced nephrotoxicity involves generation of reactive oxygen species (ROS).\(^{25}\) GEM has been reported to inhibit the activity of antioxidant systems (i.e., superoxide dismutase, catalase, glutathione peroxidase) and deplete the cellular thiols and membrane lipid products.\(^{46-48}\)

Oxidative stress in kidney plays an important role in GEM induced renal damage as well as several antioxidants and thiol compounds have been shown to possess protective action against GEM induced nephrotoxicity.\(^{1,25,49}\) Calcium ions play role in diverse models of cell death induced by ROS or toxins.\(^{20,50}\) Further, GEM is known to possess MPTP opening action.\(^{19}\)

GEM induced renal damage associated with renal mitochondrial dysfunction, oxidative stress and modulation of functional marker such as BUN, NAG, TBARS and GSH are well documented.\(^{51,52}\) In physiological condition, mitochondrial functions are an index of uptake of oxygen, calcium handling, production of ATP, activity of the citrate cycle and the regulation of respiratory chain. In pathological condition, mitochondrial lipid peroxidation is well documented to alteration of membrane integrity and permeability of mitochondrial including sarcolemmal membranes.\(^{33,54}\) These alterations of GEM result in modulation of mitochondrial function (i.e., calcium overload, phospholipase activation, inhibition of respiratory chain function etc.) leads to ATP depletion and lipid peroxidation crucially irreversible injury.\(^{34,55}\)

Many authors reported that cyclosporin A (CsA) is a very potent selective inhibitor of MPTP due to its binding ability with cyclophilin D protein of MPTP.\(^{54}\) Therefore, in our present work cyclosporine was taken as reference drug to compare the effect of flunarizine in GEM induced acute renal failure. Further, flunarizine is known to have free radical scavenging activity,\(^{50,56,57}\) and it also possesses the property of decreasing lipid peroxidation process as well as inactivation of MPTP channel opening.\(^{31,50}\) Moreover, it has been reported that mitochondrial calcium overload induced lethal MPTP opening, and flunarizine (calcium channel blocker) was found to attenuate this effect in the present study. Flunarizine has also shown ameliorative effect on GEM induced biochemical changes in rats. Therefore, it can be concluded that dual (calcium channel blocker and direct MPTP inactivation) roles of flunarizine possess the renoprotective effect on GEM induced nephrotoxicity.

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