Exacerbation of iminodipropionitrile-induced behavioral toxicity, oxidative stress, and vestibular hair cell degeneration by gentamicin in rats

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Abstract

This study describes the effect of gentamicin, an aminoglycoside antibiotic on iminodipropionitrile (IDPN)-induced abnormal neurobehavioral syndrome in female Sprague–Dawley rats. The animals were exposed to IDPN in the dose of 100 mg/kg/day intraperitoneally for 7 days. Gentamicin (GM) was administered intraperitoneally daily 1 h before IDPN in the doses of 10, 40, and 80 mg/kg body weight in three different groups of rats. One more group of animals received gentamicin alone (80 mg/kg) and served as the gentamicin-alone group. The intensity of IDPN induced characteristic excitation with choreiform, and the circling movement (ECC) syndrome was examined using an observational test battery including dyskinetic head movements, circling, tail hanging, air righting reflex, and contact inhibition of the righting reflex on days 6, 8, 10, 12, 19, 26, and 33. The animals for histopathological observation were sacrificed on day 10, whereas the remaining animals that were used for long-term behavioral studies were sacrificed on day 35 for biochemical observations. The blood and brain samples were collected for the analysis of blood urea nitrogen (BUN), serum creatinine, cerebral malondialdehyde (MDA), conjugated dienes, and lipid hydroperoxides, whereas temporal bones were collected for inner ear histopathology. Our results showed that gentamicin significantly and dose dependently exacerbated the incidence and the severity of the IDPN-induced behavioral syndrome. The histopathology of the inner ear demonstrated more severe loss of sensory hair cells in the crista ampullaris of the rats treated with IDPN plus gentamicin compared to the IDPN-alone treated animals. Concomitant treatment with gentamicin also potentiated IDPN-induced increase in free radical indices, suggesting a possible role of oxidative stress in gentamicin-induced aggravation of IDPN toxicity. Further studies are warranted to determine the role of aminoglycosides in nitrile toxicity and drug-induced movement disorders. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Iminodipropionitrile; Gentamicin; Vestibular toxicity; Behavioral syndrome; Neurotoxicity; Free radicals; Oxidative stress

1. Introduction

Iminodipropionitrile (IDPN) is a neurotoxin that produces ECC syndrome (excitation with choreiform and circling movements) in rodents, characterized by repetitive head movements, retropulsion, circling, hyperactivity, and swimming deficits, that last throughout the lifetime of the animal without affecting its longevity [7,33]. Earlier investigators attributed IDPN-induced behavioral syndrome to its ability to cause proximal axonal swelling, ballooning with neurofilamentous accumulation [4,5] accompanied by slowing the axonal transport of neurofilaments, reduced nerve conduction velocity [18,38], and degeneration of distal elements [14]. However, the reversible nature of these pathological changes [13,19] are in contrast to the life-long permanence of the IDPN-induced behavioral syndrome [32]. Recently, Llorens and Rodriguez [23] have suggested that the vestibular toxicity, not the axonopathy, is responsible for IDPN-induced ECC syndrome. It has been suggested that IDPN and related nitriles produce behavioral toxicity through their toxic effect by time- and dose-dependent degeneration of vestibular hair cells, leading to the development of ECC syndrome [21–23]. The mechanism of IDPN-induced neurotoxicity, in particular its ability to produce vestibular hair cell degeneration, is far from clear. The role of oxidative stress in IDPN-induced neurotoxicity has been proposed by Lohr et al. [24]. A significant increase in oxygen-derived free radicals (ODFR) and depletion of antioxidant enzymes and vitamin E in different regions of brain of the rats following treatment with IDPN was observed by earlier studies [1,41,42]. On the other hand, the treatment of rats with antioxidants such as vitamin E and selenium significantly attenuated IDPN-induced behavioral syndrome,

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further confirming the role of oxidative stress in IDPN toxicity [1,41].

Gentamicin is an aminoglycoside used for treating a variety of infectious diseases, especially caused by Gram-negative bacteria, such as Pseudomonas aeruginosa, Enterobacter, and Klebsiella. The major side effects associated with gentamicin treatment are its renal toxicity and ototoxicity [2,11,16,31]. In some cases, gentamicin-induced labyrinthitis is used for the treatment of vertigo in the patients of Meniere’s disease [10,30]. Gentamicin-induced hair cell pathology in mammals is considered irreversible, while the vestibular syndrome can be reversible as animals compensate [45]. Aran et al. [2] have observed that gentamicin specifically penetrates into sensory hair cells of the inner ear from where its clearance is very slow (up to 11 months). Subsequent exposure to other ototoxic compounds, especially those causing sensory hair cell toxicity, may be hazardous [2]. The vestibular hair cell toxicity of gentamicin [27,34,40,43] was found to be remarkably similar to those described for IDPN [21–23]. In consideration of the above, the present investigation was undertaken to study the effect of gentamicin on IDPN-induced toxicity in rats.

2. Materials and methods

2.1. Animals

Adult female Sprague–Dawley rats weighing 200 ± 10 g were used in the study. The animals were housed in polycarbonate cages with sawdust bedding. The animals were kept in a temperature-controlled (23 ± 2°C) room and maintained on 12-h light/dark cycles, with free access to standard laboratory food (Grain Silos and Flour Mills, Riyadh, KSA) and tap water. The protocol of animal studies was approved by Research and Ethical Committee of Armed Forces Hospital, Riyadh, Saudi Arabia.

2.2. Drugs

IDPN and gentamicin were purchased from Aldrich Chemical Company (Milwaukee, WI) and Fujisawa, Inc., USA, respectively. Both the drugs were dissolved in normal saline (McGaw, Inc., USA), and administered intraperitoneally in the volume of 2 ml/kg body weight of the animals.

2.3. Dosing and testing

The animals were divided into six groups of 10 rats each. Eight animals from each group were used for neurobehavioral and biochemical studies, and the remaining two animals were used for histopathological study. The rats in group 1 served as control and received vehicle (saline) only, whereas animals in group 2 received IDPN (100 mg/kg, IP) daily for 7 days. The animals in groups 3, 4, and 5 were given IDPN in the same way as in group 2, but received gentamicin (IP) in the doses of 10, 40, and 80 mg/kg, respectively, 1 h before the IDPN for 7 days. The rats in group 6 were treated with a high dose of gentamicin (80 mg/kg) without IDPN treatment. The animals were carefully observed for any behavioral abnormalities before the daily administration of the drugs; however, a complete battery of tests for the assessment of behavioral toxicity were undertaken after the onset of clinical signs on days 6, 8, 10, 12, 19, 26, and 33 between 0800 and 1100 h.

2.4. Behavioral toxicity

The signs of behavioral toxicity were assessed using previously published procedures [6,22]. A battery of tests of vestibular function was used to test the clinical signs associated with ECC syndrome. The animals were observed for the presence or absence of following signs while the observer was blinded to various treatments.

2.4.1. Dyskinetic head movements and circling

The animals were placed individually in an observation chamber (50 × 50 cm), and were observed for dyskinetic head movements (head weaving) and circling for a period of 2 min.

2.4.2. Tail hanging

The rat was lifted by the tail and the response was carefully observed and rated as follows: 0 = straight body posture with extension of forelimbs towards the earth (normal), 1 = slightly bending the body ventrally (intermediate response), and 2 = persistently bending the body, sometimes crawling up towards its tail (severe response).

2.4.3. Air righting reflex

The animal was held supine and dropped from a height of 30–40 cm onto a foam cushion. The response was graded as follows: 0 = successful in righting and landing squarely on their feet (normal), 1 = poor righting or landing on side (intermediate response), and 2 = completely failed in righting and landing on back (severe response).

2.4.4. Contact inhibition of righting reflex

The rat was placed supine on a horizontal surface, and another horizontal surface was slightly placed in contact with the soles of the supine animal’s feet. The rating was performed as follows: 0 = animal rights successfully (normal), 1 = partial righting, animal does some efforts (intermediate response), and 2 = complete loss of righting, animal is facing up the feet and walking with respect to the upper surface (severe response).

2.5. Biochemical studies

The animals were sacrificed by decapitation on day 35, and the cerebrum was isolated and stored immediately at −80°C for the analysis of malondialdehyde (MDA), conjugated dienes, and lipid hydroperoxides. Blood was also collected for the analysis of serum blood urea nitrogen (BUN) and creatinine levels.

2.6. Analysis of malondialdehyde (MDA)

The level of MDA in the cerebrum was measured using the modified HPLC procedure [9]. One hundred milligrams
of cerebrum were taken in a Pyrex tube and 1 ml of 10% trichloroacetic acid (TCA) plus 50 μl of 500 ppm butylated hydroxytoluene (BHT) were added. After heating in a boiling water bath for 30 min, the sample was cooled and centrifuged at 300 × g for 10 min. Supernate (200 μl) was combined with 200 μl of 0.6% aqueous solution of thiobarbituric acid (TBA) and heated in a boiling water bath for 30 min. After cooling, the reaction mixture (200 μl) was extracted with 0.5 ml of n-butanol using a vortex mixer. An aliquot (100 μl) of the extract was mixed with 100 μl of methanol and 100 μl of the mobile phase (15% acetonitrile and 0.6% tetrahydrofuran in 5 mM phosphate buffer at pH 7). A 20-μl sample of the extract/methanol/mobile phase was diluted to 0.5 ml with an additional mobile phase, and 20 μl of this solution was injected onto the HPLC column.

The HPLC instrument from Waters Associate Inc. (Bedford, MA) consisted of a solvent delivery pump Model 510, an autoinjector Model 712, an Integrator Model 740, and a column (μBondapak C-18, 3.9 × 150 mm), whereas, the fluorescence detector Model RF-535 from Shimadzu, Japan, was used. The flow rate of mobile phase was adjusted at 1 ml per min, and the fluorescence was measured at Ex 515 and Em 550 nm. The level of MDA was calculated using 1,1,3,3-tetraethoxypropane as standard.

2.7. Analysis of conjugated dienes and lipid hydroperoxides

The level of conjugated dienes in the cerebrum was measured according to the method described by Handelman et al. [17]. Preminced cerebral tissue (100 mg) was homogenized with 1 ml of ice-cooled ethanol containing 1.2% pyrogallol at 4°C using Teflon homogenizer (Janke & Kunkel, Germany). The homogenate was saponified by adding 150 μl of 10 M potassium hydroxide, and acidified to pH 3 using 1 M hydrochloric acid. The acidified homogenate was extracted with 3 ml of n-hexane. A 1-ml aliquot of the n-hexane extract was evaporated under nitrogen and reconstituted with 2.5 ml of cyclohexane. The level of conjugated dienes was determined by measuring the absorbance at 233 nm using a quartz cell on a Perkin-Elmer (Model Lambda 40) spectrophotometer.

A second portion of a 1 ml aliquot of the n-hexane extract was evaporated under nitrogen and reconstituted with 1 ml of a mixture of glacial acetic acid and chloroform (3:2). One hundred microliters of 0.6 g/ml potassium iodide solution was added, and the tubes were kept in the dark for exactly 5 min. Then 3 ml of aqueous cadmium acetate (0.5%) were added and centrifuged at 3000 rpm for 10 min. The upper layer was collected, and absorbance was read at 353 nm for lipid hydroperoxides determination.

2.8. Analysis of serum BUN and creatinine

The commercial kits from United Medical Diagnostics, Saudi Arabia, were used for the analysis of BUN and serum creatinine levels.

2.9. Histology of Inner ear

For histopathology, the animals were sacrificed on day 10. The rats were subjected to cardiac perfusion with saline (20 ml) followed by 2.5% glutaraldehyde (100 ml) buffered with 0.2 M phosphate buffer solution (pH 7.4) under ethyl ether anesthesia. The temporal bones were quickly removed and postfixed in 10% neutral buffered formalin for 15 h. The bony labyrinth was decalcified by placing it in a decalcifying agent Cal-Ex (Fisher Scientific, USA) for 48 h. The specimens were then processed overnight for dehydration with increasing concentrations of alcohol and clearing with acetone and chloroform using an automatic tissue processor (Shandon Southern 2L Processor MkII, UK). The specimens were embedded in paraffin blocks and sections of 5 μm thickness were stained with 1% toluidine blue for light microscopy observations.

2.10. Statistics

The incidence of an ECC syndrome was evaluated by χ² test using EPI-INFO (version 5) computer software. The results of severity scores of the ECC syndrome, biochemistry, and animal body weight were first analyzed for homogeneity test followed by MANOVA (for body weight and behavioral parameters) and one-way ANOVA (for biochemical data) using SPSS (version 7.5) computer software. Dunnett’s multiple comparison test was used for comparing treatment means. A value of p < 0.05 was considered as statistically significant.

3. Results

3.1. Body weight

The administration of IDPN or gentamicin (80 mg/kg) individually significantly reduced the body weight during the first 12 days followed by a gradual recovery in the body weight gain (Fig. 1). Concomitant treatment of rats with gentamicin plus IDPN produced more loss of weight compared to their individual effect, F(5, 294) = 55.35, p < 0.001. The effect of time, F(6, 294) = 28.83, p < 0.001, and the interaction between treatment and time, F(30, 294) = 2.31, p < 0.01, were also significant.

3.2. ECC syndrome

There was no behavioral change in the animals treated with saline (control) or gentamicin alone. In the IDPN-alone–treated group, the onset of ECC syndrome was observed on day 10 when only one out of eight animals showed behavioral abnormality (Fig. 2). Concomitant treatment of rats with gentamicin significantly and dose dependently exacerbated the severity of IDPN-induced ECC syndrome with an early onset of clinical signs including head weaving, F(5, 294) = 103.59, p < 0.001, circling, F(5, 294) = 67.53, p < 0.001, tail hanging, F(5, 294) = 84.15, p < 0.001, air righting reflex, F(5, 294) = 77.54,
p < 0.001, and contact inhibition of righting reflex, \( F(5, 294) = 82.59, p < 0.001 \) (Table 1). A significant effect of time, \( F(6, 294) = 5.94, p < 0.001 \), and treatment \( \times \) time interaction, \( F(30, 294) = 1.64, p < 0.05 \), for circling, and \( F(30, 294) = 3.94, p < 0.001 \), for other tests was also observed.

### 3.3. Oxidative stress

There was an increase in cerebral MDA, \( F(5, 42) = 3.59, p < 0.01 \), conjugated dienes, \( F(5, 42) = 2.80, p < 0.05 \), and lipid hydroperoxides, \( F(5, 42) = 11.55, p < 0.001 \), of the rats treated with gentamicin or IDPN individually (Fig. 3).

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**Fig. 1.** Effect of different treatments on animal body weight. *p < 0.01 vs. IDPN alone and **p < 0.01 vs. control group using Dunnett’s test.

**Fig. 2.** Effect of different doses of gentamicin on the onset and incidence (% of animals showing at least one clinical sign) of IDPN-induced behavioral syndrome. *p < 0.05 and **p < 0.01 vs. IDPN alone group using chi-square test. There was no behavioral abnormality in saline (control) and gentamicin-alone treated rats (groups not shown in the figure).

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**Table 1**
The tests of vestibular dysfunction: effect of gentamicin on IDPN induced vestibular toxicity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>19</th>
<th>26</th>
<th>33</th>
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<td></td>
<td>0.00 ± 1.0</td>
<td>1.87 ± 1.3</td>
<td>1.75 ± 1.2</td>
<td>1.75 ± 1.2</td>
<td>1.75 ± 1.2</td>
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<td></td>
<td>1.37 ± 0.9</td>
<td>3.87 ± 2.5</td>
<td>2.87 ± 1.9</td>
<td>2.12 ± 1.4</td>
<td>1.75 ± 1.2</td>
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<tr>
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<td>7.62 ± 2.3</td>
<td>9.75 ± 2.3</td>
<td>10.12 ± 2.6</td>
<td>9.12 ± 2.3</td>
<td>8.37 ± 2.3</td>
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<tr>
<td>IDPN + GM 80</td>
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<td>1.43 ± 1.3</td>
<td>7.63 ± 1.5</td>
<td>10.12 ± 1.6</td>
<td>12.50 ± 0.8</td>
<td>14.16 ± 1.5</td>
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<th></th>
<th>Circling (counts/2 min.)</th>
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<tr>
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<td>0.50 ± 0.5</td>
<td>0.75 ± 0.7</td>
<td>0.25 ± 0.2</td>
<td>0.75 ± 0.5</td>
<td>1.12 ± 1.1</td>
<td>0.75 ± 0.7</td>
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<tr>
<td>IDPN + GM 10</td>
<td></td>
<td>2.12 ± 0.9</td>
<td>4.00 ± 1.3</td>
<td>3.12 ± 1.2</td>
<td>3.87 ± 1.7</td>
<td>2.87 ± 1.2</td>
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<tr>
<td>IDPN + GM 40</td>
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<td>0.87 ± 0.3</td>
<td>1.37 ± 0.3</td>
<td>1.37 ± 0.3</td>
<td>1.28 ± 0.3</td>
<td>1.00 ± 0.3</td>
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<tr>
<td>IDPN + GM 80</td>
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<td>2.32 ± 0.8</td>
<td>3.85 ± 0.9</td>
<td>5.66 ± 1.3</td>
<td>6.83 ± 1.8</td>
<td>3.33 ± 0.8</td>
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<td>0.37 ± 0.2</td>
<td>0.25 ± 0.1</td>
<td>0.25 ± 0.1</td>
<td>0.12 ± 0.1</td>
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<tr>
<td>IDPN + GM 10</td>
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<td>0.50 ± 0.3</td>
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<td>1.37 ± 0.3</td>
<td>1.37 ± 0.3</td>
<td>1.28 ± 0.3</td>
<td>1.00 ± 0.3</td>
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<td>IDPN + GM 80</td>
<td></td>
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<td>6.83 ± 1.8</td>
<td>3.33 ± 0.8</td>
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<th>Air righting reflex (severity score)</th>
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<td>0.37 ± 0.2</td>
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<tr>
<td>IDPN + GM 10</td>
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<td>0.25 ± 0.1</td>
<td>0.37 ± 0.2</td>
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<tr>
<td>IDPN + GM 40</td>
<td></td>
<td>0.87 ± 0.3</td>
<td>1.25 ± 0.3</td>
<td>1.25 ± 0.3</td>
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<tr>
<td>IDPN + GM 80</td>
<td></td>
<td>1.42 ± 0.2</td>
<td>1.85 ± 0.1</td>
<td>1.85 ± 0.1</td>
<td>1.66 ± 0.1</td>
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</table>

No vestibular dysfunction was observed in control and GM (80 mg/kg) alone treated rats. Values are mean ± SE.

*p < 0.05, **p < 0.01 and ***p < 0.001 vs. IDPN-alone group using Dunnett’s test.
Concomitant treatment with gentamicin, especially in the high dose (80 mg/kg), resulted in significantly higher level of these indices of oxidative stress (Fig. 3).

3.4. Serum creatinine and BUN

Administration of IDPN did not show any effect on BUN or creatinine levels compared to control group (Fig. 4). Gentamicin alone or in combination with IDPN also failed to produce any significant change in BUN levels, \( F(5, 42) = 1.62, p = 0.183 \). However, higher dose of gentamicin increased the level of serum creatinine, \( F(5, 42) = 5.44, p < 0.001 \), and cotreatment with IDPN enhanced gentamicin-induced renal malfunction (Fig. 4).

3.5. Histological observations

The sensory epithelium in the crista of control rats showed no loss of hair cells. The crista ampullaris from the rats treated with IDPN alone showed mild loss (<25% along the sensory epithelium) of vestibular sensory hair cells (Fig. 5). Cotreatment with gentamicin (80 mg/kg) along with IDPN produced severe loss (>75% along the sensory epithelium) of vestibular sensory hair cells.

4. Discussion

Administration of IDPN or gentamicin individually reduced the body weight of the rats for the first 12 days followed by a slow recovery (Fig. 1). Gentamicin and IDPN have been reported by several earlier investigators to reduce animal body weight [11,22,25,26]. Concomitant treatment of rats with IDPN and gentamicin had an additive effect on initial loss of weight. Our behavioral studies showed that administration of IDPN produced abnormal clinical signs associated with an ECC syndrome (Table 1). The severity of ECC syndrome observed in IDPN-treated female rats was comparatively milder than the behavioral response observed in male rats in our earlier studies [41,42]. This observation...
is in agreement with other investigators, who also reported lower toxicity of IDPN in female compared to male rats [25]. The absence of vestibular toxicity in gentamicin-alone–treated rats may be attributed to subeffective dose regimen used in this study. However, concomitant use of gentamicin significantly reduced the latency period for the onset of the IDPN-induced ECC syndrome (Fig. 2) and exacerbated the severity of abnormal clinical signs (Table 1).

The results of our histopathological studies showed that the treatment of rats with IDPN for 7 days produced a mild degeneration of vestibular sensory hair cells in crista ampullaris (Fig. 5). Our findings are in agreement with Llorens et al. [21–23], who suggested a close association between IDPN-induced neurobehavioral toxicity and degenerative changes in the crista ampullaris, including cytoplasmic vacuolation, detachment of hair cell-nerve terminal contacts, and loss of synaptic densification. Although gentamicin alone did not produce any degenerative change in the crista ampullaris, it produced more severe loss of vestibular sensory hair cells in crista ampullaris of IDPN-treated rats (Fig. 5).

The mechanism by which gentamicin enhances IDPN-induced structural and functional changes is far from clear. The role of ODFR in IDPN [1,24,41,42]- and gentamicin [12,29,36,37]-induced toxicity has been suggested by several recent studies. Pretreatment of animals with antioxidants has been shown to protect animals against IDPN [1,24,41] as well as gentamicin toxicity [12,37]. Despite the emphasis on vestibular and peripheral nervous system as the primary targets of neurotoxic effects of IDPN, evidence suggests that IDPN-induced cerebropathy may also contribute to its neurobehavioral toxicity [20]. The brain is particularly sensitive to oxidative damage because of its high concentration of polyunsaturated fatty acids, high rate of oxygen consumption, higher iron levels, and poor antioxidant defence system [28]. The free radical oxidation of unsaturated fatty acid side chains results in the formation of conjugated dienes that further react with O2 to produce peroxyl radicals (ROO•), leading to the propagation (chain reaction) of lipid peroxidation. The ROO• radical combines with hydrogen atom to give lipid hydroperoxide (ROOH). The fragmentation of ROOH produces several types of aldehydes, including MDA, which is one of the end products of lipid peroxidation process. Thus, conjugated dienes, lipid hydroperoxides, and MDA, which are produced at different
stages of lipid peroxidation process, are considered as good markers of lipid peroxidation [15]. The results of our biochemical studies showed a nonsignificant increase in the levels of cerebral conjugated dienes, lipid hydroperoxides, and MDA on the 35th day in IDPN (alone)-treated rats (Fig. 3). Earlier we observed significantly higher levels of these indices on the 12th day following IDPN treatment [1,41]. These findings suggest a recovery of biochemical parameters after stopping the IDPN. However, the levels of free radical indices remained significantly high on the 35th day in rats concomitantly treated with gentamicin and IDPN (Fig. 3). These findings are in agreement with other investigators [35], suggesting the ability of certain toxicants to trigger the chain of free radical reactions that maintain higher level of oxidative stress over a prolonged period after stopping the treatment.

The adverse interaction between gentamicin and IDPN observed in this study may, to some extent, be attributed to the renal toxicity of gentamicin. There was a significant increase in serum creatinine level of the rats treated with gentamicin alone or gentamicin plus IDPN (Fig. 4), clearly suggesting renal function impairment in these animals. As the IDPN and its toxic metabolites are mainly excreted through the kidney [8,44], their bioavailability may be altered in really impaired rats. Moreover, the risk of ototoxicity of gentamicin is also enhanced following kidney malfunction [3]. Furthermore, altered bioavailability of IDPN and gentamicin could also explain prolonged oxidative stress in the rats concomitantly treated with these drugs. However, our body weight data suggest that general toxicity of the drugs does not progress beyond the first week after the last dose (Fig. 1). Other investigators have also observed a lack of correlation between animal body weight and the extent of oxidative stress following long-term (30 days) administration of toxicant [39].

In conclusion, this study clearly demonstrates the ability of gentamicin to potentiate IDPN-induced vestibular toxicity in rats. Our results also suggest a possible role of oxidative stress in gentamicin-induced aggravation of IDPN toxicity. Further studies are deemed necessary to elucidate the role of aminoglycosides in nitriles-induced behavioral toxicity.

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