**Neurovestibular Toxicities of Acrylonitrile and Iminodipropionitrile in Rats: A Comparative Evaluation of Putative Mechanisms and Target Sites**

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Acrylonitrile (ACN) is a synthetic nitrile which is primarily used for the manufacture of synthetic fibers, resins, rubbers, plastics, adhesives and pesticides. It is one of the 50 most commonly used chemicals in United States. World consumption of ACN is over 4 million tons and is growing over 3% a year. The United States is the largest producer of ACN, with a production of almost 2 million tons a year. Asia is the largest consuming region of ACN, accounting for 39% of the world consumption. Occupational and environmental exposure of synthetic nitriles is of potential relevance to human health, especially after the discovery that several nitriles of industrial application including iminodipropionitrile (IDPN), ACN, crotonitrile, and allylnitrile are able to produce motor deficits in experimental animals (Balbuena and Llorens, 2001; Gagnaire et al., 1998; Khan et al., 2003, 2004; Tani et al., 1991). ACN also potentiates oxidant-induced DNA damage in rats (Murata et al., 2001) and exacerbates noise-induced hearing loss (Fechter et al., 2004; Pouyatos et al., 2005).

IDPN is a prototype nitrile compound that is known to produce a permanent behavioral syndrome in rodents, characterized by repetitive head movements, retropulsion, circling, hyperactivity, and swimming deficits (Delay et al., 1952; Selye, 1957). IDPN-induced behavioral syndrome has been designated as excitation with choreiform and circling movements (ECC) syndrome (Selye, 1957). Because of the unique array of neurobehavioral abnormalities, IDPN is considered as one of the most suitable compounds for validation of functional observational battery (FOB) and motor deficits for screening of neurotoxic drugs (Fukumura et al., 1998). In fact, the original FOB testing guidelines of U.S. Environmental Protection Agency (1985, 1991) recommended IDPN as a positive control for these test procedures (Crofton et al., 2004, 2008; Ivens, 1990; Schulze and Boysen, 1991).

The mechanism of nitriles toxicity is complex and multifactorial. Llorens et al. (1993a) have revealed a direct correlation between vestibular hair cell degeneration and the severity of IDPN- and related nitriles-induced behavioral deficits. On the other hand, neuropharmacological and biochemical studies implicated various neurotransmitters (Cadet...
et al., 1987; Diamond et al., 1986; Khan et al., 2004; Ogawa et al., 1991; Tariq et al., 1998, 1999) and oxygen-derived free radicals (ODFR) in the development of IDPN-induced neurotoxicity (Nomoto, 2004; Tariq et al., 2002, 2004; Wakata et al., 2000). Gagnaire et al. (1998) examined the effects of ACN together with four other nitriles on electrophysiological parameters including nerve conduction velocity and sensory/motor action potentials. They also performed a generalized behavioral study on ACN-treated rats showing salivation, hyperactivity and stereotypy however a specialized FOB was never applied to quantify the motor deficits specific to vestibular damage. Other investigators have studied the behavioral effects of allylnitrile in rats and their correlation with inner ear histology (Balbuena and Llorens, 2001) and brain serotonin levels (Tании et al., 1991). Structurally, ACN (CH$_2$=CH-CN) is slightly different from allylnitrile (CH$_2$=CH-CH$_2$-CN) but more different from IDPN ((CH$_2$-CH$_2$-CN)$_2$-NH) and therefore may have different target sites and mechanisms of toxicity than other industrial nitriles.

This investigation was therefore aimed to study the effect of ACN on motor deficits, oxidative stress, dopaminergic neurotoxicity, and vestibular pathology in rats. We used IDPN-treated rat as a standard model of neurobehavioral toxicity to compare the effects of ACN. In addition, we also evaluated the combined effects of these two nitrile compounds.

**MATERIALS AND METHODS**

**Animals and treatment groups.** Adult (â‰±10 weeks old) male Wistar rats, weighing 220 ± 20 g, obtained from the Animal Facility of the College of Pharmacy, King Saud University, Riyadh, Saudi Arabia, were used in the study. The animals were housed in polycarbonate cages with sawdust bedding. The animals were kept in a temperature-controlled (23°C) room and maintained on 12-h light/dark cycles, with free access to laboratory rodent chow pellets (Grain Silos and Flour Mills, Riyadh, Saudi Arabia) and nonchlorinated tap water. The rats were randomly divided into six groups of 8 animals each and then allowed to acclimatize (3 days) before starting the drug treatment. The protocol of animal studies was approved by Research and Ethics Committee of College of Science, King Saud University, Riyadh, Saudi Arabia.

**Drugs and dose regimens.** ACN (99%) and IDPN (90%) were purchased from Sigma-Aldrich (St. Louis, MO). Both the drugs were dissolved in normal saline and administered intraperitoneally (i.p.) in the volume of 2 ml/kg body weight of animal. Group 1 served as control and received vehicle only. The rats in the Groups 2, 3, and 4 were treated with ACN in the doses of 5 mg/kg (low), 15 mg/kg (medium), and 45 mg/kg (high), daily for 9 days, respectively. The animals in Group 5 (IDPN alone) and Group 6 (IDPN + ACN) received IDPN (100 mg/kg) daily for 8 days; in addition to IDPN. Group 6 also received the medium dose of ACN (15 mg/kg), 30 min after IDPN, aiming to study the combined effects of ACN and IDPN. Both ACN and IDPN treatments were started on the same day, however, IDPN was discontinued on day 9 because all the animals in Group 5 showed well-developed (plateau level) ECC syndrome on day 9; we assumed that further injections of IDPN at this stage would hamper the differentiation of behavioral symptoms between the Groups 5 and 6, on day 10.

**Behavioral analysis.** The animals were observed daily in the morning, before the drug administration, for the presence or absence of following signs: circling, dyskinetic head movements, tail hanging, air righting reflex and contact inhibition of the righting reflex using a previously published behavioral testing battery, specific to test the behavioral anomalies associated with vestibular dysfunction (Al Deeb et al., 2000; Khan et al., 2004). The animals were observed for a period of 2 min to assess the severity of dyskinetic head movements and abnormal circling behavior, whereas the tail hanging and the righting reflexes were tested at least three times for each animal for the grading of their severity. After behavioral analysis on day 10, one randomly selected animal from the each group was subjected to transcardiac perfusion with 10% neutral buffered formalin and fixation of the brains and temporal bones for tyrosine hydroxylase (TH) immunostaining and inner ear histology, respectively. The remaining seven animals from all the groups were also sacrificed, their brains dissected out and the specific regions (striatum, hippocampus and cortex) of the brains were stored at −80°C until analyzed.

**Analysis of glutathione.** The measurement of reduced glutathione (GSH) in the brain regions was performed according to the procedure reported by Owen (1980). The tissue was homogenized in ice-cold perchloric acid (0.2M) containing 0.01% of ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 9000 × g for 5 min, in a refrigerated centrifuge (4°C). The enzymatic reaction was started by adding 100 μl of clear supernatant in a spectrophotometric cuvette containing 800 μl of 0.3mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 100 μl of 6mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB), and 10 μl of 50 units/ml GSH reductase (all these reagents were freshly prepared in a phosphate buffer at pH 7.5). The absorbance was measured over a period of 3 min at 412 nm at 30°C. The GSH level was determined by comparing the rate of change of absorbance of the test solution with that of standard GSH.

**Tyrosine hydroxylase immunostaining.** TH immunostaining is a convenient and reliable method to demonstrate the presence and morphologic integrity of the dopaminergic neurons. The immuno-histological procedure reported earlier was used with some modifications (Sarre et al., 2004). The fixed brain specimens (3 days fixation) were processed overnight for dehydration with increasing concentrations of alcohol and clearing with acetone and chloroform using an automated tissue processor (Shandon Southern 2L Processor Mk II, UK). The specimens were embedded in paraffin blocks and coronal sections (4 μm) were made using a microtome (CUT 4050, Microm GmbH, Germany). The paraffin sections of the brain were deparaffinized by immersing the respective slides in xylene (5 min) followed by re-hydration using sequential treatment with decreasing concentrations of alcohol to water (100% alcohol, 95% alcohol, 80% alcohol, and distilled water). The re-hydrated specimens were immersed in citrate buffer (pH 6.0) and heated in the microwave for 5 + 5 min to free the binding sites for TH immunoreactivity. After cooling to room temperature, the brain sections were quenched with 3% H$_2$O$_2$ and allowed to react with specific monoclonal antibody against rat TH (Novacastra Laboratories, Ltd, UK) in 1:20 dilution for 30 min at ambient temperature. After rinsing with tris-buffered saline, the sections were sequentially incubated with biotinylated goat antibody, strept AB complex/horse radish peroxide, and chromogenic substrate for peroxidase, according to manufacturer’s instructions (Dako MS, Denmark). For each TH section, an adjacent section was stained with hematoxylin for structure identification using light microscopy.

**Inner ear histology.** The temporal bones were 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, Pittsburgh, PA) for 48 h. The specimens were then processed overnight for dehydration with increasing concentration of alcohol and clearing with acetone and chloroform using automatic 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.

**Statistics.** The incidence of ECC syndrome was evaluated by Fisher’s exact test using CalcFiher software (http://www.biometrica.tomsk.ru/programm_stat.htm). The effect of various treatments on animals’ body weight and the severity scores of ECC syndrome were analyzed by repeated-measures ANOVA whereas the results of GSH assay were evaluated by one-way
ANOVA (for individual brain regions) and two-way ANOVA (for full factorial and interaction analysis) using statistical software SPSS version 10 (Chicago, IL). Dunnett’s multiple comparison test was used to determine the significance level between the groups. *p values of less than 0.05 were considered as statistically significant.

RESULTS

Animal Body Weight

The control rats showed a steady weight gain during the course of study (Fig. 1). The animals treated with low and medium doses of ACN showed similar trends in the body weight gain, which was comparatively less than the control group. The body weight of the rats treated with high dose of ACN did not increase during the first 5 days post-treatment; however, a slight increase in body weight was observed on days 7 and 9. The administration of IDPN alone significantly and persistently reduced the body weight of the animals ($F_{5,206} = 18.77$, *p* < 0.001). There was also a significant interaction between the treatment and time ($F_{20,206} = 3.47$, *p* < 0.001).

Acute and Transient Behavioral Changes

The rats receiving vehicle (control group) or IDPN alone (without ACN) or low dose of ACN (5 mg/kg) did not show any acute behavioral effects related to the drug treatment. The treatment of rats with medium and high doses of ACN caused transient and dose-dependent excitation in the animals as shown by hyperactivity, tail shaking and erection and salivation (Table 1). However, all these symptoms were temporary and subsided quickly, on all the treatment days.

Subchronic and Permanent Behavioral Deficits (ECC Syndrome)

There was no dyskinetic behavioral abnormality in the animals treated with vehicle (control) or any of the three doses of ACN alone (Table 2). In the IDPN alone treated group, the onset of ECC syndrome was observed on day 7 when three out of eight animals showed behavioral abnormality; all the animals in this group developed behavioral deficits on day 9 (Table 2). Concomitant treatment of rats with ACN significantly attenuated the severity of IDPN-induced ECC syndrome ($F_{5,168} = 114.25$, *p* < 0.001). A significant effect of time ($F_{3,168} = 34.19$, *p* < 0.001) and treatment × time interaction ($F_{15,168} = 14.13$, *p* < 0.001) was also observed.

GSH in the Specific Brain Regions

Administration of ACN alone in medium (15 mg/kg) and high (45 mg/kg) doses significantly depleted GSH in all the specific brain regions.

TABLE 1

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Excitement at 5 min</th>
<th>Tail shaking at 5 min</th>
<th>Tail erection at 7 min</th>
<th>Salivation at 10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN 0 (Control)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ACN 5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ACN 15</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ACN 45</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>IDPN</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IDPN + ACN 15</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Note. Severity scores: no symptoms (–), low (+), moderate (++), severe (+++).

TABLE 2

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Days after the first dose</th>
</tr>
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<tr>
<td></td>
<td>7</td>
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Incidence of ECC syndrome (%)

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Incidence of ECC syndrome (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>ACN 5</td>
<td>0</td>
</tr>
<tr>
<td>ACN 15</td>
<td>0</td>
</tr>
<tr>
<td>ACN 45</td>
<td>0</td>
</tr>
<tr>
<td>IDPN</td>
<td>37.5±1.11</td>
</tr>
<tr>
<td>IDPN + ACN 15</td>
<td>0</td>
</tr>
</tbody>
</table>

Severity score (means ± standard error of means)

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Severity score (means ± standard error of means)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0* ± 0.32</td>
</tr>
<tr>
<td>ACN 5</td>
<td>0* ± 0.32</td>
</tr>
<tr>
<td>ACN 15</td>
<td>0* ± 0.32</td>
</tr>
<tr>
<td>ACN 45</td>
<td>0* ± 0.32</td>
</tr>
<tr>
<td>IDPN</td>
<td>2.25 ± 1.11</td>
</tr>
<tr>
<td>IDPN + ACN 15</td>
<td>2.25 ± 1.11</td>
</tr>
</tbody>
</table>

Note. #p < 0.001 and ##p < 0.0001 versus IDPN alone groups (Fisher’s exact test). *p < 0.05, **p < 0.01, and ***p < 0.001 versus IDPN alone group (Dunnett’s test).
three brain regions including striatum ($F_{3,36} = 7.87, p < 0.001$), hippocampus ($F_{3,36} = 6.96, p < 0.001$) and cerebral cortex ($F_{3,36} = 2.94, p < 0.05$) as compared to control group; the GSH depletion was dose-dependent in striatum and cortex but not in hippocampus (Fig. 2). Although the low dose (5 mg/kg) of ACN did not affect the GSH levels in striatum, it significantly reduced the GSH levels in hippocampus and insignificantly in cortex (Fig. 2). Administration of IDPN alone significantly reduced the GSH in striatum but insignificantly in hippocampus and cortex. IDPN treatment had no significant impact on ACN-induced GSH depletion (Fig. 2). However, the two-way ANOVA revealed significant effects of treatments ($F = 13.95, p < 0.001$), brain regions ($F = 8.99, p < 0.001$) and treatment×brain region interaction ($F = 3.01, p < 0.01$) for the GSH levels.

**Tyrosine Hydroxylase Immunoreactivity in the Striatum**

There was no difference in TH expression as observed by TH immunostaining of the medial striatum among different treatment groups (Fig. 3). However, the anterior striatum showed intense TH expression in IDPN alone treated rat as compared to control rat. Cotreatment with ACN reduced the intensity of TH immunostaining in IDPN-treated rats (Fig. 3). The results of TH immunostaining are based on one representative animal from each treatment group.

**Inner Ear Histopathology (Vestibular Hair Cell Degeneration)**

The crista ampullaris of control rats as well as the rats treated with any dose of ACN alone showed normal sensory epithelium with intact hair bundles (Fig. 4). Administration of IDPN alone caused massive loss of vestibular sensory hair cells in the crista ampullaris. The animals treated with combination of ACN (15 mg/kg) and IDPN showed comparatively less degeneration of sensory hair cells than IDPN alone group (Fig. 4). The results of vestibular histology are based on one representative animal from each treatment group.

**DISCUSSION**

Administration of ACN retarded the body weight gain in the rats however these weights were never below the baseline levels (Fig. 1). Whereas the IDPN treatment has significantly reduced the body weight of animals which is in agreement with earlier reports (Al Deeb et al., 2000; Llorens et al., 1993a; Moser and Boyes, 1993; Nace et al., 1997). The injections of ACN caused transient hyperactivity and salivation in rats (Table 1), which is supported by an earlier study (Gagnaire et al., 1998). Recently, orally administered ACN in the rats has been shown to produce neurobehavioral alterations related to locomotor activities, motor coordination, learning and memory (Rongzhu et al., 2007). However, we observed that none of the animals in ACN alone treated groups, irrespective of the doses, developed the symptoms of ECC syndrome (Table 2). On the other hand, all the animals in IDPN alone treated group developed peculiar behavioral deficits (ECC syndrome) on day 9. The onset and time-course progression of IDPN-induced behavioral syndrome (Table 2) was found to be consistent with our previous reports (Al Deeb et al., 2000; Khan et al., 2004; Tariq et al., 1998, 2000, 2004) suggesting the validity of IDPN as a reproducible positive control for neurovestibular toxicity studies in rats. Concomitant treatment with ACN increased the onset time and significantly reduced the severity of IDPN-induced behavioral syndrome (Table 2). These behavioral observations suggest the involvement of different mechanisms in the toxicities of ACN and IDPN.

We observed severe and highly significant depletions of GSH in different brain regions of the rats treated with ACN. The massive depletion of GSH by ACN treatment is mainly associated with the metabolism of this toxicant. There are two different routes of ACN catabolism in the body; the minor route is its conversion to cyanide whereas the major route (more than 2/3) proceeds via its conjugation with GSH catalyzed by GSH-$S$-alkenetransferases, to N-acetyl-$S$-(2-cyanoethyl)-cysteine as a final product (Kopecký et al., 1980a, b). Thus, the extensive utilization and depletion of GSH (a potent intracellular antioxidant) by ACN may lead to cellular oxidative stress. Exposure of ACN to rats has been shown to produce significant oxidative stress (Mahalakshmi et al., 2003) and lipid peroxidation in the rat brain (Nagasawa et al., 2003). Carrera et al. (2007) have used a cell culture model to study the effect of various antioxidants on the oxidative damage induced by ACN and concluded that only GSH seems to play a key role in ACN-derived toxicity (Carrera et al., 2007). Mahalakshmi et al. (2003) have shown that the antioxidant taurine significantly counteracts the oxidative stress induced by ACN by reducing the levels of peroxidation and enhancing the activities of enzymatic and nonenzymatic antioxidants.

Administration of IDPN alone caused significant depletion in GSH level in striatum only, although insignificant reductions were observed in hippocampus and cerebral cortex (Fig. 2).
indicating the excessive generation of ODFR in striatum than the other two brain regions studied, most probably due to the neurochemical differences among various brain regions. This notion is supported by several reports demonstrating brain region-specific differential effects of IDPN on neuropeptides (Kawada et al., 1995; Przedborski et al., 1989), monoamines (Langlais et al., 1975; Ogawa et al., 1991; Wakata et al., 2000), glial fibrillary acidic protein (Llorens et al., 1993b), gamma aminobutyric acid (Gianutsos and Suzdak, 1985), and antioxidant enzymes (Perumal et al., 1991) in rodent brains.

Our earlier studies have also shown depletion of GSH and increased oxidative stress in the rats exposed to IDPN (Khan et al., 2003, 2004; Tariq et al., 2002). The role of oxidative stress in IDPN-induced neurotoxicity is further supported by the findings showing significant reversal of IDPN-induced neurobehavioral deficits using the antioxidants (Lohr et al., 1988; Tariq et al., 1994).

The neurotoxic sequel of ACN is accompanied by significant increase in the brain glial fibrillary acidic protein (GFAP), which is a putative biomarker of astrogliosis (Enongene et al., 2000). Exposure to ACN has been shown to enhance lipid peroxidation in rat glial cells, as indicated by accumulation of malondialdehyde (MDA) and depletion of GSH levels (Esmat et al., 2007). An inverse relationship has been observed between brain GSH and GFAP levels in the rats treated with ACN with or without sodium thiosulfate (GSH replenishing

FIG. 3. Light microscopic observation of TH immunostaining of the striatal dopaminergic neurons. Capital and small letters denote the anterior and medial striata, respectively, of the same group. (A, a) Control, (B, b) ACN 45 mg/kg, (C, c) IDPN alone, and (D, d) IDPN + ACN 15 mg/kg. All the treatments showed almost similar staining intensity in medial striatum. IDPN alone treated rat showed intense TH expression in anterior striatum (C) as compared to control (A). Cotreatment with ACN reduced the intensity of TH immunostaining in IDPN-treated rats (D).
Earlier investigators have suggested a possible role of dopaminergic neurotransmission (Cadet et al., 1987; Ogawa et al., 1991; Tariq et al., 1999) in IDPN-induced neurotoxicity. We have observed intense TH immunostaining of dopaminergic neurons in the anterior striatum of IDPN-treated rats, though the medial region did not show any remarkable alteration irrespective of the treatment groups (Fig. 3). Our findings on TH immunostaining are based on one animal per group. Alterations in striatal neurotransmission have been associated with movement disorders. Fritschi et al. (2003) have suggested the involvement of striatal projection neurons in IDPN-induced dyskinesia. There are distinct variations in striatal anatomy (Maurin et al., 1999) that play important roles in controlling the typical behavioral patterns (Brown and Sharp, 1995; Michele and Jennifer, 1988). Interestingly, the activation fields have been found to be larger in anterior striatum than in posterior striatum (Brown and Sharp, 1995). The attenuation of IDPN-induced behavioral deficits by ACN may partly be attributed to comparatively reduced dopaminergic neurotransmission in the rats receiving the combined treatment (Fig. 3).

A definite role of vestibular hair cell degeneration has been reported in IDPN-induced behavioral deficits; IDPN has been shown to produce dose- and time-dependent degeneration of vestibular sensory hair cells in rats (Khan et al., 2003; Llorens et al., 1993a, 1994). We observed a massive loss of hair cells in the crista ampullaris of the rats treated with IDPN alone (Fig. 4). On the other hand, ACN did not show any toxic effect on vestibular sensory epithelium albeit it appeared to partially counteract IDPN-induced vestibular hair cell degeneration in rats (Fig. 4). We reiterate that our observations on vestibular histopathology are based on one animal per group. Tanii et al. (1999) have found that a single administration of allylnitrile (an analog of ACN) induces persistent behavioral abnormalities including head twitching, head weaving, circling, hyperactivity, backward pedaling, and somersaulting in rodents (akin to IDPN-induced behavioral deficits). The important roles of serotonin and dopamine neurotransmission and/or degeneration of vestibular sensory hair cells have been suggested in allylnitrile-induced behavioral toxicity (Tanii et al., 1999). On the other hand, exposure of ACN to rats in drinking water caused a significant alteration in the neurotransmitters with a possible implication in the neurobehavioral abnormalities (Lu et al., 2005).

In conclusion, our findings suggest that ACN produces more severe oxidative stress in rat brain as compared to IDPN. On the other hand, administration of IDPN (but not ACN) leads to the degeneration of vestibular sensory hair cells resulting in the development of ECC syndrome. Thus, the brain and vestibule of rats appear to be the major target sites of ACN and IDPN, respectively.

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