A direct association between aging and drug-induced dyskinesia has been reported by several investigators. Iminodipropionitrile (IDPN), a prototype nitrile compound produces a motor syndrome in rodents, which resembles neuroleptic drug induced dyskinesia. In this investigation attempt has been made to study the effect of age on IDPN induced vestibular hair cell degeneration and resulting dyskinetic syndrome. Male Wistar rats aged 3, 6 and 12 weeks received IDPN in the doses of 0, 200 and 400 mg/kg, intraperitoneally for 3 consecutive days. IDPN-induced dyskinesia was assessed using a behavioral testing battery on days 3, 4, 5, 6, 7, 14, 21 and 28. The rats were sacrificed on day 28; temporal bones were excised for vestibular histopathology and sera were collected for measuring the indices of oxidative stress (glutathione and conjugated dienes). IDPN in the dose of 200 mg/kg produced dyskinesia in 12 weeks old rats, but failed to do so in 3 and 6 weeks old rats. The high dose of IDPN (400 mg/kg) caused dyskinesia in all age groups, however, its onset and severity were age-dependent. Older rats showed an early onset and significantly high incidence of dyskinesia as compared to younger rats. The susceptibility of rats to IDPN-induced behavioral deficits was proportional to oxidative stress and degeneration of sensory hair cells in the crista ampullaris.

Introduction

Tardive dyskinesia (TD) is a potentially irreversible movement disorder associated with long-term administration of neuroleptic drugs (Kane 1999). However, a higher incidence of tardive dyskinesia in the early stages of even low dose treatment with typical neuroleptics has been observed in older patients suggesting a direct association between drug toxicity and aging (Jeste et al. 1999; Pollock and Mulsant 1995). Animal studies have also shown an increased susceptibility to various neurotoxins in older animals (Bosso et al. 1993; Geula et al. 1998). These observations are in accordance with the fact that significantly lower doses of reserpine are required in older rats to produce dyskinesia as compared to younger ones (Bergamo et al. 1997).

Iminodipropionitrile (IDPN) is a neurotoxic compound that produces motor syndrome in rodents, which resembles human idiopathic dyskinesia and has been used as an experimental model for drug-induced dyskinesia by several investigators (Al Deeb et al. 2000; Cadet et al. 1988; Iida et al. 1998; Ogawa et al. 1991; Tariq et al. 1995, 2002). IDPN-induced dyskinesia is also termed as ECC (excitation, chorea, circling) syndrome and characterized by repetitive head movements, retropulsion, circling, hyperactivity and swimming deficits. Earlier studies have shown that IDPN produces age-dependent deleterious effects on rat’s eye (Schneider et al. 1980) and olfactory mucosa (Genter and Ali 1998); however, no attempt has been made to study age-related effects of IDPN on vestibular pathology and associated behavioral abnormalities. This investigation was undertaken to study the effect of age on IDPN-induced vestibular hair cell degeneration and pattern of dyskinesia in rats.
Material and methods

Animals: Male Wistar rats aged 3, 6 and 12 weeks were obtained from Animal Care and Breeding facility of Armed Forces Hospital, Riyadh, Saudi Arabia. The animals were housed in a temperature-controlled room and maintained on 12 h light/dark cycles with free access to standard laboratory animals’ food and tap water. The protocol of animal studies was approved by Research and Ethical Committee of Riyadh Armed Forces Hospital, Saudi Arabia.

Drug and dosing: The rats grouped in the age categories of 3, 6, and 12 weeks were divided into 9 subgroups of 6 animals each (3 subgroups for each age category). After 1 week acclimation to home cages, the rats from each age category were treated with intraperitoneal injections of IDPN (Aldrich Chemical Company, USA) dissolved in normal saline (McGaw Inc., USA) in the doses of 0 mg/kg (control), 200 mg/kg and 400 mg/kg, daily for 3 consecutive days.

Behavioral studies: Assessment of IDPN-induced dyskinesia was carried out using a previously published behavioral testing battery (Ar. Deeb et al. 2000). All the animals were carefully monitored daily for any behavioral abnormality until the onset (day 3) of dyskinesia. Complete behavioral studies were undertaken on days 3, 4, 5, 6, 7, 14, 21, and 28. The animals were observed for the presence or absence of various behavioral abnormalities including: circling, dyskinetic head movements, tail hanging, air righting reflex, and contact inhibition of the righting reflex. The individual rats in each group 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 3 times for each animal for the grading of their severity. Each animal was given a rating score of 0 (normal behavior), 1 (intermediate response), and 2 (presence of expected anomalous behavior) for each parameter associated with ECC syndrome. The score of each behavioral parameter was added and a maximum score of 10 was indicative of all signs of the ECC syndrome in the animal.

After behavioral studies on day 28, the animals were sacrificed for the collection of blood for biochemical studies and of vestibular labyrinth for histopathology.

Histopathology of inner ear: For histopathological examination of sensory epithelium in crista ampullaris, the rats were subjected to cardiac perfusion with saline (20 ml) followed by 2.5% glutaraldehyde buffered with 0.2 M phosphate buffer solution (pH 7.4) under ether anesthesia. The temporal bones were quickly removed and postfixed in 10% neutral buffered formaline 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.

Analysis of Serum Glutathione (GSH): The measurement of GSH in serum was done enzymatically according to the procedure described by Owen (1980). The serum was homogenized with ice-cold perchloric acid (0.2 M) containing 0.01% of EDTA. The mixture was centrifuged at 4,000 rpm for 10 min. The enzymatic reaction was started by adding 100 µl of clear supernatant in a spectrophotometric cuvette containing 800 µl of 0.3 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 100 µl of 6 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and 10 µl of 50 units/ml glutathione reductase (all these reagents were freshly prepared in phosphate buffer at pH 7.5). The absorbance was measured over a period of 4 min at 412 nm at 30 °C. The GSH level was determined by comparing the change of absorbance (ΔA) of test solution with the ΔA of standard GSH.

Analysis of Conjugated Dienes (CDs): The level of CDs in the serum was measured according to the method described by Handelman et al. (1988). Serum 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 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 CDs was determined by measuring the absorbance at 233 nm using a quartz cell on a Perkin-Elmer (Model Lambda 40) spectrophotometer.

Statistics: The incidence of behavioral syndrome was evaluated by χ² test using EPI-INFO computer software. The results of severity scores of IDPN-induced dyskinesia were analyzed by multiple analysis of variance (MANOVA). One-way ANOVA was used to analyze the results of biochemical studies using statistical software SPSS version 10. Dunnnett’s multiple comparison test was used to test the significance level between the groups. A value of P < 0.05 was considered as statistically significant.

Results

Administration of IDPN in the dose of 200 mg/kg daily for 3 consecutive days failed to produce dyskinesia in 3 weeks and 6 weeks old rats, whereas, this dose regimen effectively produced dyskinesia in 12 weeks old rats (fig. 1). The first symptoms of dyskinesia following 200 mg/kg IDPN were observed on day 4 in 50% of 12 weeks old rats and all the rats in this group became irreversibly dyskinetic on day 5. The dose of 400 mg/kg of IDPN was effective in producing ECC syndrome in all age groups of rats; however, the onset latency and incidence of dyskinesia were strictly age-dependent. Older rats showed an early onset and significantly higher incidence of dyskinesia as compared to younger rats (fig. 1).

There was a highly significant difference in the severity of ECC syndrome among various treatment groups (MANOVA F₈,360 = 302.75, P < 0.0001). Post-hoc analysis using Dunnett’s multiple comparison test showed that
On the other hand, there was a gradual increase followed by slight decrease in IDPN (400 mg/kg)-induced ECC syndrome in 3 weeks old rats. This time-course difference in severity of ECC syndrome in different age groups is defined by a significant interaction between test days and age (MANOVA $F_{14,360} = 4.90, P < 0.0001$).

The crista ampullaris of control rats from all the 3 age groups showed normal sensory epithelium with intact hair bundles (fig. 2). Administration of IDPN in the dose of 200 mg/kg caused no apparent histopathological changes in crista ampullaris of 3 and 6 weeks old rats, whereas, this dose regimen produced severe loss of hair cells in crista of 12 weeks old rats. The dose of 400 mg/kg of IDPN caused mild degeneration of sensory hair cells and the detachment of hair bundles in 3 weeks old rats, whereas, the same IDPN dose regimen adverse-

**Fig. 1.** Incidence (line graphs) and severity (bar graphs) of ECC syndrome induced by IDPN (200 and 400 mg/kg, daily for 3 consecutive days) in 3, 6 and 12 weeks old rats. *P < 0.05, **P < 0.01 and ***P < 0.001 as compared to 3 weeks old rats using Chi-square test (for incidence) or Dunnett’s multiple range test (for severity of ECC syndrome).
ly damaged the sensory epithelium and hair bundles in the crista of 6 weeks old rats. Administration of 400 mg/kg of IDPN in 12 weeks old rats resulted in total loss of hair bundles, hair cells and the integrity of sensory epithelium leading to complete disappearance of sensory structure of crista (fig. 2).

There was no significant difference in the serum levels of markers of oxidative stress (glutathione and conjugated dienes) among the control rats from different age groups (fig. 3). Administration of IDPN dose-dependently reduced glutathione levels (ANOVA $F_{8,36} = 5.25$, $P < 0.001$) and increased conjugated dienes levels.

Fig. 2. Examples of crista ampullaris of 3, 6 and 12 weeks old rats treated with 0, 200 and 400 mg/kg of IDPN. Administration of IDPN produced age and dose-dependent toxic effects on sensory epithelium. Severe loss of hair cells was observed in 6 and 12 weeks old rats treated with 400 and 200 mg/kg of IDPN respectively, whereas, total disappearance of sensory epithelium was observed in 12 weeks old rats treated with 400 mg/kg of IDPN. Scale bar = 20 µm.
ANOV A $F_{8,36} = 4.91$, $P < 0.001$ in serum of rats, however, these effects were statistically significant only in 6 and 12 weeks old rats treated with 400 mg/kg of IDPN (fig. 3).

**Discussion**

The results of our behavioral studies demonstrated that IDPN induced dyskinesia is directly proportional to the age of animals and doses of the drug (fig. 1). The mechanism behind higher magnitude of IDPN-induced dyskinesia in older animals is far from clear. IDPN induced ECC syndrome has been attributed to its ability to produce degenerative changes in sensory hair cells of crista ampullaris (AL DEEB et al. 2000; LLORENS et al. 1993, 1994; TARIQ et al. 2002). In fact, surgically induced vestibular lesion also produced somewhat similar dyskinesia in rats as observed after IDPN treatment (GEISLER et al. 1997; LLORENS et al. 1993). The result of our histopathological studies showed that IDPN-induced structural damage to crista ampullaris was both age- and dose-dependent (fig. 2). A linear correlation between cochlear hair cell degeneration and advancing age has been reported in experimental animals which in turn may enhance the susceptibility of these animals to ototoxic compounds (BHATTACHARYYA and DAYAL 1985; BLOOM and HULTERANTZ 1994; NAKAYAMA et al. 1994). Our findings are also supported by the results of earlier studies showing that exposure to neurotoxins might aggravate age-related neurodegeneration by predisposing neurons to premature death and thus hastening the appearance of age-related functional deficit (GRALEWICZ et al. 1995).

The results of our biochemical studies on serum markers of oxidative stress showed that IDPN induced depletion of glutathione (GSH) and elevation of conjugated dienes (CDs) is directly linked with the age of animals (fig. 3). Both GSH and CDs levels in serum or plasma have been suggested as important biomarkers of oxidative stress (IKAGAMI et al. 1994; JONES et al. 1995; LIU et al. 1997; PURUCKER et al. 1995; SKRZYDLEWSKA and FARBISZEWSKI 1998). Oxidative stress has been shown to play a vital role in IDPN induced neurotoxicity (LOHR et al. 1988; TARIQ et al. 1995, 2002) as well as in aging process (GUTTERIDGE 1992; PAOISSO et al. 1988). The free radical oxidation of unsaturated fatty acids in cell membrane results in cellular degeneration and the formation of CDs that further react with oxygen to produce peroxyl radicals, leading to propagation (chain reaction) of lipid peroxidation (HALDWELL and GUTTERIDGE 1989). On the other hand, GSH is a potent scavenger of oxygen-derived free radicals (ODFR) and serves as a natural protectant against free radical damage. GSH has been implicated as an important factor in detoxification of various xenobiotics (HOGARTH et al. 1996; JONES et al. 1995). The findings of this study suggest that excessive oxidative stress with aging might enhance the susceptibility of animals to IDPN induced vestibular toxicity and dyskinesia.

In conclusion, this study clearly showed an increased susceptibility of aged rats to neurotoxic effects of IDPN possibly mediated by oxidative stress. Thus, maintaining proper antioxidant defenses in elderly might be helpful in combating adverse effects of neurotoxic drugs. Further studies are warranted to explore the prophylactic/therapeutic potential of antioxidants in preventing or alleviating the severity of drug-induced dyskinesia in elderly.

Fig. 3. Effect of IDPN on markers of oxidative stress in rats from different age groups. *$P < 0.05$ compared to respective age-matched control group using Dunnett’s test.
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References