

Attenuation of iminodipropionitrile induced behavioral syndrome by sodium salicylate in rats

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Abstract

Iminodipropionitrile (IDPN) produces irreversible behavioral abnormalities characterized by excitation with choreiform and circling movements (ECC) syndrome in rodents. Concomitant exposure to drugs or environmental chemicals has been shown to alter IDPN-induced neurobehavioral toxicity. This investigation was undertaken to study the effect of sodium salicylate (SS) on IDPN-induced behavioral abnormalities in rats. The animals were exposed to IDPN (100 mg/kg ip) daily for 8 days. SS was administered daily 30 min before IDPN in the doses of 50, 100 and 200 mg/kg ip for 12 days. The animals were observed for neurobehavioral abnormalities including dyskinetic head movements, circling, tail hanging, air righting reflex and contact inhibition of the righting reflex. Horizontal and vertical locomotor activities and forelimbs grip strength were also measured. After behavioral studies, the animals were sacrificed, and the cerebrum and temporal bones were collected for glutathione (GSH) analysis and inner ear histopathology, respectively. The onset of ECC syndrome was observed on Day 9 in the IDPN-alone group with 100% incidence on Day 12. Cotreatment with salicylate dose-dependently delayed the onset time and significantly attenuated the incidence and severity of IDPN-induced neurobehavioral signs. IDPN alone significantly increased horizontal motor activity and reduced vertical motor activity and forelimbs grip strength; these effects were significantly reversed by salicylate treatment. Treatment with salicylate also attenuated IDPN-induced depletion of GSH in the cerebrum, suggesting its free radical scavenging property. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Behavioral syndrome; Nitriles; Salicylate; Free radicals; Antioxidant

1. Introduction

The nitriles are extensively used for the manufacture of synthetic fibers, resins, plastics, dye stuffs and pharmaceuticals, hence, their occupational and environmental exposure is considered to be of potential relevance to human health (Ahmed and Trieff, 1983; Bergmark, 1997; Guirguis et al., 1984; Perbellini et al., 1998). Exposure to iminodipropionitrile (IDPN), propionitrile, dimethylaminopropionitrile, crotonitrile, allylnitrile and acrylonitrile may lead to neurobehavioral abnormalities in humans (Scolnick et al., 1993; Spencer and Schaumburg, 1983) and experimental animals (Gagnaire et al., 1998; Llorens et al., 1993b; Tani et al., 1989, 1991). IDPN is one of the most commonly used nitriles, which produces 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) or waltzing syndrome (Chou and Hartman, 1964). 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 activity 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 (Ivens, 1990; Schulze and Boysen, 1991).

A number of neurochemical, histopathological and pharmacological studies suggest the involvement of various target sites for IDPN. Evidence from neuropathological studies suggests that IDPN causes axonal swellings and neurofilamentous accumulation in the brainstem, spinal cord and peripheral nervous system (Chou and Hartman, 1964; Griffin et al., 1982; Yokahama et al., 1980). Furthermore,

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IDPN has been shown to damage areas of central nervous system, which was evident from dose-dependent elevation of glial fibrillary acidic protein (GFAP) in the pons medulla, midbrain, cerebral cortex and olfactory bulb (Llorens et al., 1993a). Peele et al. (1990) provided further support for a role of higher levels of the CNS in IDPN neurotoxicity by clearly showing learning and memory deficit in rodents treated with IDPN. Moreover, IDPN-induced neurobehavioral abnormalities are associated with changes in serotonin (5-HT), dopamine (DA), noradrenaline (NA) and gamma-aminobutyric acid (GABA) in different regions of the brain (Cadet et al., 1988a,b; Gianutsos and Suzdak, 1985; Ogawa et al., 1991). On the other hand, recent literature suggests a firm association between degeneration of vestibular sensory hair cells and behavioral toxicity in IDPN-treated rats (Al Deeb et al., 2000; Llorens et al., 1993b, 1994; Tariq et al., 1998).

The precise mechanism by which IDPN produces neuropathological changes and vestibular hair cell degeneration is far from clear. A large number of reports point towards a pivotal role of oxygen-derived free radicals (ODFR) in IDPN-induced neurobehavioral toxicity (Al Deeb et al., 1995; Lohr et al., 1998; Tariq et al., 1995b). Although the cells are equipped with various free radicals scavenging systems to combat oxidative stress, an excessive generation of ODFR may overwhelm the body's antioxidant defense resulting in membrane disruption and cellular damage (Halliwell and Gutteridge, 1989; Sies, 1985). If the free radical hypothesis of IDPN-induced pathogenesis is true, the drugs with the ability to scavenge ODFR might alter the course of its toxicity. Salicylates are among the most commonly used nonprescription drugs, which are used as antiinflammatory, antipyretic, analgesic and antithrombotic agents. They are potent scavengers of hydroxyl radical and are commonly used as trapping agent to determine the formation of these highly reactive species in various tissues including the brain (McCabe et al., 1997; Obata and Chiueh, 1992). The objective of this study was to examine the effect of sodium salicylate (SS) on IDPN-induced neurobehavioral abnormalities in rats.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats weighing 210 ± 10 g were housed in a temperature-controlled room and were maintained on 12-h light/dark cycles with free access to food and water. The experimental protocol of this study was approved by the Research and Ethics Committee, Armed Forces Hospital, Riyadh, Saudi Arabia.

2.2. Drugs

IDPN (Aldrich Chemical, USA) and SS (Riedel, Germany) were dissolved in normal saline and sterile water,

respectively. Both the drugs were administered intraperitoneally in a volume of 2 ml/kg body weight of the animals.

2.3. Dosing and testing

The animals were divided into five groups of eight animals each. The rats in Group 1 served as control and received vehicles only, whereas rats in Group 2 received IDPN (100 mg/kg ip) daily for 8 days. The animals in Groups 3, 4 and 5 were treated with IDPN similarly as in Group 2; in addition, they also received SS (ip) in the doses of 50, 100 and 200 mg/kg, respectively, daily 30 min before IDPN for 12 days (i.e. SS treatment continued for additional 4 days). A preliminary pilot study using few animals had revealed that even the high dose of SS (200 mg/kg) alone did not produce any behavioral abnormality in rats, thus, a drug-alone group was not included in study. The animals were observed daily for IDPN-induced characteristic neurobehavioral abnormalities (ECC syndrome) to monitor the onset of dyskinesia (Day 9 in this study) until a plateau (Day 13) was observed. In addition to the ECC syndrome, motor activity and grip strength were measured on Day 13 to determine if the animals with well-developed ECC syndrome also suffer a lack of normal activity and muscle strength. All behavioral observations were conducted in a blinded fashion.

2.4. ECC syndrome

The animals were observed 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 previously published behavioral testing battery (Al Deeb et al., 2000). 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 (Al Deeb et al., 2000).

2.5. Motor activity

Motor activity was measured using Optovarimex activity meter (Columbus Instruments, USA). The horizontal motor activity was detected by two perpendicular arrays of 15 infrared beams located 2.5 cm above the floor of the testing area. Each interruption of a beam on the *x* or *y* axis generated an electric impulse, which was presented on a digital counter. Similarly, the vertical motor activity was recorded using two additional rows of infrared sensors located 12 cm above the floor. Each animal was tested separately, and the motor activity was measured for a period of 2 min.

2.6. Grip strength

Forelimbs grip strength was measured using a grip strength meter (UGO Basile, Italy) consisted of a grasping

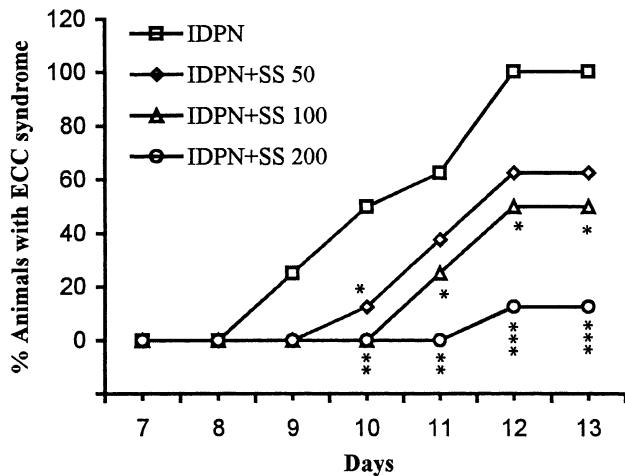


Fig. 1. Effect of different doses of SS on the onset and incidence (% of animals showing at least one clinical sign) of IDPN-induced behavioral syndrome in rats. * $P < .05$, ** $P < .01$ and *** $P < .001$ versus the IDPN-alone-treated group (chi-square test).

trapeze attached to a force transducer (Model 7105), a peak amplifier (Model 7108) and a multifunction printer (Model

2650). After adjusting the height of the grasping trapeze, the animal was allowed to grasp the trapeze and was then pulled by the tail. The peak pulling force (grip strength) was recorded from the digital display on the amplifier.

2.7. Inner ear histology

After behavioral studies on Day 13, rats from Groups 1, 2 and 5 ($n=1$ per group) were sacrificed for histopathological assessment of effect of salicylate on IDPN-induced vestibular toxicity. Rats were subjected to cardiac perfusion with 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 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 processor (Shandon Southern 2L Processor MkII, England). The specimens were embedded in paraffin blocks and sections of 5 μm in

Table 1
Effect of SS on IDPN-induced behavioral abnormalities

Treatment	Days				
	9	10	11	12	13
<i>Dyskinetic head movements (counts/2 min)</i>					
IDPN	1.000 \pm 0.68	3.625 \pm 1.51	8.000 \pm 2.75	10.250 \pm 2.56	14.250 \pm 1.78
IDPN + SS 50	0	1.000 \pm 1.00	4.625 \pm 1.82	5.750 \pm 2.23	6.750 \pm 2.40**
IDPN + SS 100	0	0**	2.750 \pm 1.35	5.500 \pm 2.16	4.875 \pm 1.89***
IDPN + SS 200	0	0**	0**	0.625 \pm 0.62***	1.375 \pm 1.11***
<i>Circling (counts/2 min)</i>					
IDPN	0.500 \pm 0.50	1.125 \pm 0.66	2.375 \pm 1.14	3.125 \pm 1.39	4.750 \pm 1.29
IDPN + SS 50	0	0.125 \pm 0.12	1.250 \pm 0.55	1.875 \pm 0.78	1.750 \pm 0.64**
IDPN + SS 100	0	0*	1.000 \pm 0.53	1.250 \pm 0.72	0.750 \pm 0.36***
IDPN + SS 200	0	0*	0*	0*	0***
<i>Tail hanging (severity score)</i>					
IDPN	0.250 \pm 0.16	0.750 \pm 0.31	1.125 \pm 0.35	1.625 \pm 0.18	1.750 \pm 0.16
IDPN + SS 50	0	0.125 \pm 0.12*	0.750 \pm 0.31	1.000 \pm 0.32	1.000 \pm 0.32
IDPN + SS 100	0	0**	0.625 \pm 0.32	0.875 \pm 0.35	0.750 \pm 0.31**
IDPN + SS 200	0	0**	0**	0.125 \pm 0.12***	0.125 \pm 0.12***
<i>Air righting reflex (severity score)</i>					
IDPN	0.125 \pm 0.12	0.750 \pm 0.31	1.125 \pm 0.35	1.625 \pm 0.18	1.875 \pm 0.12
IDPN + SS 50	0	0.125 \pm 0.12*	0.750 \pm 0.31	1.000 \pm 0.32	1.000 \pm 0.35
IDPN + SS 100	0	0**	0.625 \pm 0.32	0.875 \pm 0.35	0.875 \pm 0.35**
IDPN + SS 200	0	0**	0**	0.125 \pm 0.12***	0.125 \pm 0.12***
<i>Contact inhibition of righting reflex (severity score)</i>					
IDPN	0.125 \pm 0.12	0.750 \pm 0.31	1.000 \pm 0.37	1.625 \pm 0.18	1.625 \pm 0.18
IDPN + SS 50	0	0.250 \pm 0.25	0.750 \pm 0.31	1.000 \pm 0.32	0.875 \pm 0.29*
IDPN + SS 100	0	0*	0.500 \pm 0.26	0.625 \pm 0.32**	0.625 \pm 0.26**
IDPN + SS 200	0	0*	0*	0***	0***

The control group did not show any behavioral abnormality. Values are means \pm S.E.

* $P < .05$ versus the IDPN-alone group using Dunnett's test.

** $P < .01$ versus the IDPN-alone group using Dunnett's test.

*** $P < .001$ versus the IDPN-alone group using Dunnett's test.

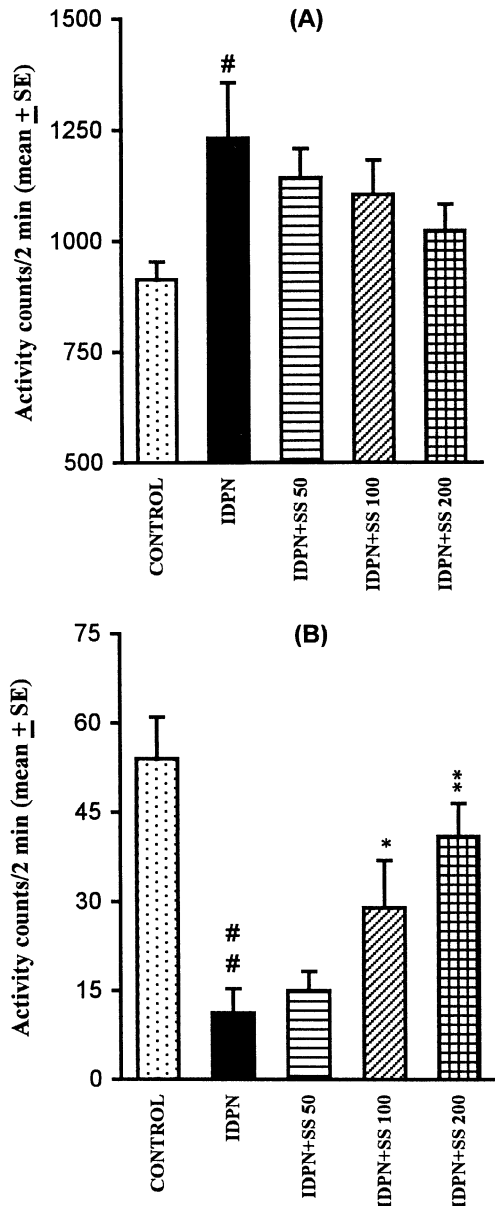


Fig. 2. Effect of IDPN alone and in combination with different doses of SS on the (A) horizontal and (B) vertical locomotor activity of rats. * $P < .05$ and ** $P < .01$ versus the IDPN-alone-treated group and # $P < .05$ and ## $P < .01$ versus the control group (Dunnett's test).

thickness were stained with 1% toluidine blue for light microscopy observations.

2.8. Glutathione (GSH) analysis

For GSH analysis, rats were sacrificed on Day 13, and brains were dissected and stored at -80°C . The measurement of GSH in the cerebrum was done enzymatically according to the modified procedure of Owen (1980). The tissue was homogenized in ice cold perchloric acid (0.2 M) containing 0.01% of EDTA. The homogenate was centrifuged at 4000 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 GSH reductase (all the above three 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.

2.9. Statistics

The incidence of behavioral syndrome was evaluated by χ^2 test using the EPI-INFO computer software. The results of severity scores of dyskinetic head movement, circling, tail hanging, righting reflex and contact inhibition of righting reflex were analyzed by MANOVA. One-way ANOVA was used to analyze the results of motor activity, grip strength and cerebral GSH levels using statistical software SPSS version 10. Dunnett's multiple comparison test was used to test the significance level between the groups. A value of $P < .05$ was considered as statistically significant.

3. Results

3.1. ECC syndrome

The onset of ECC syndrome was observed in some rats on Day 9 in the IDPN-alone group, and all the animals in this group became dyskinetic on Day 12 (Fig. 1). Cotreatment with salicylate dose-dependently delayed the onset and significantly reduced the incidence and severity of IDPN-

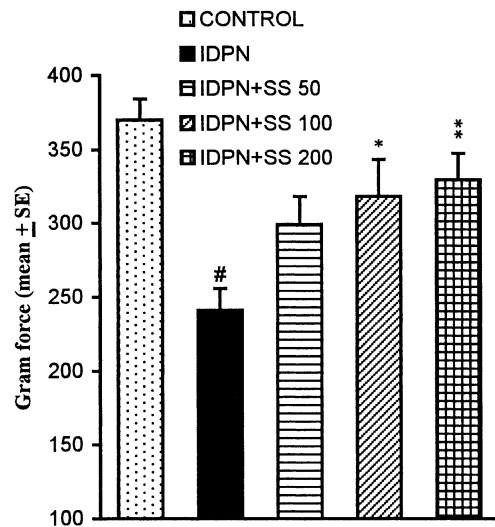


Fig. 3. Forelimbs grip strength of the rats in different treatment groups. * $P < .05$ and ** $P < .01$ vs. the IDPN-alone-treated group and # $P < .05$ versus the control group (Dunnett's test).

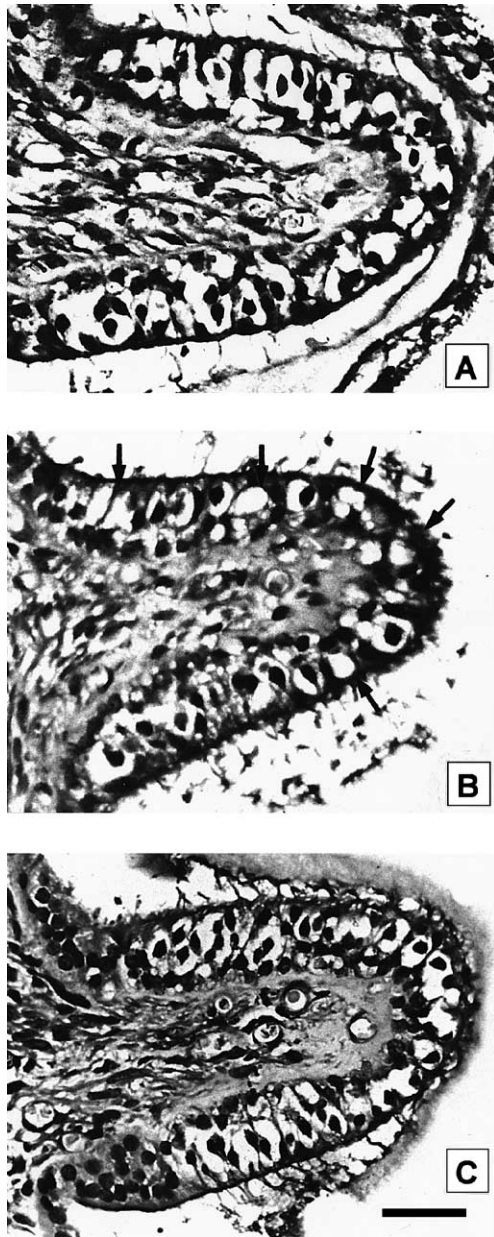


Fig. 4. Effect of SS on IDPN-induced hair cell degeneration in crista ampullaris. (A) control, (B) IDPN alone and (C) IDPN + SS, 200 mg/kg. Administration of SS attenuated the degeneration of hair cells in the sensory epithelium of IDPN-treated rats. IDPN alone produced severe loss of hair bundles, which was prevented by salicylate treatment. Arrows show degeneration of hair cells. Scale bar, 20 μ m.

induced ECC syndrome (Table 1) including head weaving [$F(4,175) = 26.34$, $P < .001$], circling [$F(4,175) = 15.10$, $P < .001$], tail hanging [$F(4,175) = 25.15$, $P < .001$], righting reflex [$F(4,175) = 24.97$, $P < .001$] and contact inhibition of righting reflex [$F(4,175) = 25.13$, $P < .001$]. A significant effect of Time for all clinical signs [$F(4,175) > 10.00$, $P < .001$] and Time \times Treatment interaction [$F(16,175) > 2.10$, $P < .01$, for head weaving, righting reflex and contact inhibition of righting reflex; $F(16,175) = 1.69$, $P = .052$, for

circling; $F(16,175) = 1.97$, $P < .05$ for tail hanging] were also observed.

3.2. Motor activity

Administration of IDPN alone increased horizontal motor activity [$F(4,35) = 2.36$] and reduced vertical motor activity [$F(4,35) = 9.53$, $P < .001$]. Concomitant treatment with salicylate significantly and dose-dependently protected rats against the effects of IDPN-induced changes in locomotor activity (Fig. 2).

3.3. Grip strength

The treatment of rats with IDPN alone resulted in a significant decrease in the forelimbs grip strength (Fig. 3). Administration of salicylate significantly and dose-dependently attenuated IDPN-induced deficiency in the grip strength [$F(4,35) = 5.32$, $P < .001$].

3.4. Histological observation

The crista ampullaris of the rat treated with IDPN alone showed severe degeneration of vestibular sensory hair cells (Fig. 4). Concomitant treatment with salicylate protected the animal against the toxic effects of IDPN on sensory hair cells.

3.5. Cerebral GSH

There was a significant depletion of cerebral GSH following IDPN treatment [$F(4,32) = 3.26$, $P < .05$, Fig. 5]. Cotreatment with salicylate (in all the three doses) significantly reversed the effect of IDPN on GSH depletion (Fig. 5).

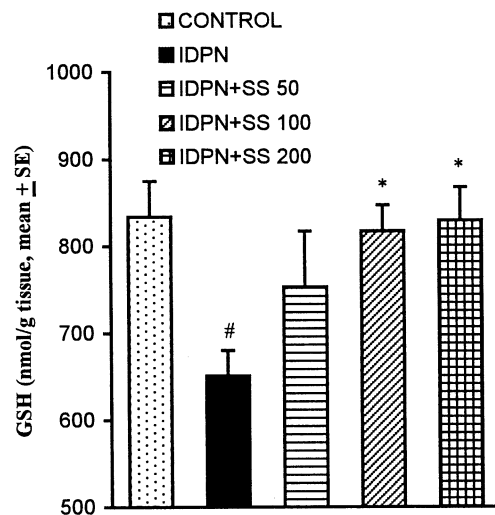


Fig. 5. Effect of SS on IDPN-induced changes in cerebral GSH levels in rats. * $P < .05$ versus the IDPN-alone-treated group and # $P < .05$ versus the control group (Dunnett's test).

4. Discussion

The results of this study clearly suggest the protective effect of salicylate against IDPN-induced ECC syndrome, which is evident from the delayed onset and reduced incidence and severity of behavioral abnormalities in drug treated animals (Fig. 1; Table 1). Treatment with salicylate also significantly attenuated IDPN-induced alterations in locomotor activity (Fig. 2) and deficiency in grip strength of the rats (Fig. 3). Our histopathological studies showed significant degenerative changes in sensory hair cells of crista ampullaris, which was markedly attenuated by cotreatment with salicylate (Fig. 4). Llorens et al. (1993b, 1994) claimed that IDPN-induced degeneration of vestibular sensory hair cells is the major contributing factor in IDPN-induced behavioral toxicity. Although high doses of salicylate alone may also produce reversible ototoxicity, its target (Dieler et al., 1991; Douek et al., 1983) and mechanism of action (Jung et al., 1990; Stypulkowski, 1993) appear to be quite different from IDPN (Al Deeb et al., 2000; Llorens et al., 1993b, 1994).

The results of our biochemical study showed a significant depletion of cerebral GSH in IDPN-treated rats, suggesting a role of oxidative stress in IDPN neurotoxicity (Fig. 5). Concomitant treatment with salicylate significantly protected the animals against IDPN-induced depletion of GSH. Recently, Aubin et al. (1998) have shown the protective effect of salicylate against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity in mice, which was attributed to hydroxyl radical scavenging activity of salicylate (Obata and Chiueh, 1992; Wei et al., 1998). As an OH-trapping agent, salicylate has been shown to play a neuroprotective role against NMDA and non-NMDA receptor-activated neurotoxicity in rodents treated with glutamate and kainic acid, respectively (Gepdiremen et al., 2000). The role of ODFR in IDPN-induced neurotoxicity is further supported by earlier studies showing the ability of antioxidants to reverse IDPN-induced neurobehavioral toxicity (Al Deeb et al., 1995; Lohr et al., 1998; Tariq et al., 1995b).

Nitric oxide (NO) is one of the endogenous free radicals that participate in a variety of physiological processes in human body (Moncada, 1992). NO may act as a second messenger, neurotransmitter and neuromodulator in CNS (Dawson et al., 1992). It plays a double-edged role in brain function and ischemic injury. Beckman (1991) suggested that NO, at lower concentrations, might be neuroprotective as it regulates thrombogenesis and vasodilation, while higher concentrations of NO might accelerate the formation of destructive species thereby intensifying cerebral injury. Recently, SS has been shown to significantly inhibit NO production (Chung et al., 2000). Furthermore, excessive increase in oxidative stress triggers a sequence of biochemical events leading to enhanced phospholipase A₂ (PLA₂) activity, release of potentially noxious platelet-aggregating factor (PAF) and altered arachidonic acid (AA) metabolism.

Imbalance of AA metabolites including vasodilator prostacyclin (PGI₂) and vasoconstrictor thromboxane (TXA₂) may adversely affect microcirculation and neuronal function (Dennis et al., 1991; Dorandeu et al., 1998; Farooqui et al., 1997). Recently, salicylate has been shown to inhibit the release of AA (Levine, 2000) and to decrease the level of TXA₂ without altering PGI₂ level (high PGI₂/TXA₂ ratio) leading to enhanced tissue perfusion, decreased fibrin formation and lowered platelet aggregation (Akagi et al., 1991; Caruso et al., 1995). Thus, by preventing excessive generation of ODFR and inhibiting the derangement of AA metabolism, salicylate may protect animals against IDPN-induced toxicity.

Furthermore, adenosine is an endogenous neuroprotective agent that is released in the brain during ischemia (Berne et al., 1974), epileptic seizures (Schultz and Lowenstein, 1978) and hypoxia (Winn et al., 1979) apparently as a natural protective mechanism. Tariq et al. (1995a) observed that adenosine transport inhibitor dipyridamole, which causes a several-fold increase in brain adenosine (Park and Gidday, 1990), protects rats against IDPN-induced neurotoxicity. The neuroprotective activity of adenosine is attributed to its vasodilator and calcium channel-blocking properties (Tonini et al., 1983). SS has been shown to enhance the hydrolysis of adenosine triphosphate resulting in a massive increase of adenosine levels (Cronstein et al., 1994). IDPN-induced neurotoxicity is also accompanied by increases in intracellular Ca²⁺ (Bangalore et al., 1991), and calcium channel blockers nifedipine, verapamil and diltiazem attenuate IDPN-induced neurobehavioral toxicity (Bangalore et al., 1991; Cadet et al., 1988a,b). SS acts as membrane stabilizer and maintains Ca²⁺ homeostasis by reducing the cellular inflow of Ca²⁺ (Iriño et al., 1985; Kimmel and Abramson, 1987; Northover, 1982). Thus, salicylate-induced release of adenosine and stabilization of Ca²⁺ movements across cellular membrane might contribute to neuroprotection against IDPN toxicity in rats.

Lastly, the possibility of salicylate–IDPN interaction and altered pharmacokinetics of IDPN may not be ruled out. Due to their high affinity to plasma proteins, salicylates have been shown to displace other protein-bound drugs and enhance their pharmacological/toxicological activity by increasing the bioavailability of these free drugs to target sites (Dasgupta and Timmerman, 1996; Dasgupta and Volk, 1996; Yu et al., 1990). Hence, the protective effect of salicylate against IDPN and/or its toxic metabolites (which have protein affinity) (Jacobson et al., 1987) is contrary to expected enhanced bioavailability of the neurotoxin.

In conclusion, this study clearly demonstrates the protective effect of SS against IDPN-induced neurobehavioral toxicity. A significant attenuation of IDPN-induced depletion of GSH clearly supports the hypothesis that hydroxyl radical scavenging activity of salicylate may contribute to its protective effect by boosting the cellular antioxidant defense system.

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