Reproductive Toxicology 43 (2014) 19–25

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Acute toxic effects of 3,3′-iminodipropionitrile on hypothalamic-pituitary-gonadal axis in male rats

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ARTICLE INFO

Article history:
Received 27 May 2013
Received in revised form 17 October 2013
Accepted 19 October 2013
Available online 27 October 2013

Keywords:
3,3′-Iminodipropionitrile
Anterior pituitary
GnRH neuron
Reproductive toxicity
Testis

ABSTRACT

Exposure to 3,3′-iminodipropionitrile (IDPN) causes persistent neurotoxicity, while its reproductive toxicity in female rats is transient, indicating that gonadotropin-releasing hormone (GnRH) neurons and gonadotrophs receive little or no damage from IDPN and that the transient gonadal toxicity may be also observed in males. To clarify these points, the acute toxic effects of IDPN on hypothalamic-pituitary-gonadal axis of male rats were examined histologically, biochemically and serologically. A single intraperitoneal injection of IDPN (1000 mg/kg body weight) induced signs of neurotoxicity within a day; nevertheless, GnRH neurons were not affected throughout the experimental period. Four days after IDPN treatment, the plasma level of testosterone but not gonadotropins decreased and active caspase 3-immunopositive spermatids increased; both parameters returned to normal levels afterwards. Data from our studies revealed that while IDPN had little or no toxic effect on GnRH neurons or gonadotrophs it was transiently toxic to gonads in both sexes.

1. Introduction

The neuroendocrine system hypothalamic-pituitary-gonadal axis (HPG axis) is critical for the development and regulation of the reproductive and immune systems. Gonadotropin-releasing hormone (GnRH) neurons in the preoptic area (POA) and anterior hypothalamus are responsible for the production and secretion of GnRH, and they form the final common pathway in the central regulation of fertility. Pulsatile GnRH release from axon terminals in the median eminence into the pituitary portal vasculature leads to the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from gonadotrophs of the anterior pituitary. Gonadotropins activate gametogenesis and steroid synthesis by the gonads, and these steroid hormones form both negative and positive feedback loops centrally to modulate GnRH neuron function and at the pituitary to regulate the response to GnRH [1].

Nitriles are commonly used as organic intermediates in the manufacture of synthetic fibers (nylon), resins, plastics, dyestuffs, pesticides, and pharmaceuticals [2–4], and they are easily absorbed regardless of exposure routes [5]. Among nitriles, 3,3′-iminodipropionitrile (IDPN) is used as a solvent of dyestuffs or a pesticide; therefore, occupational exposure to a relatively high concentration of nitriles, including IDPN, may occur through vapor inhalation or dermal absorption of the liquid at workplaces in which these compounds are produced or used. Exposure to IDPN and related compounds leads to acute and chronic toxicities in neurons [6,7] and induces neuropathy in humans [8,9] and experimental animals [10–14]. IDPN is one of the most suitable compounds for the validation of the functional observational battery (FOB) and motor deficits in screens of neurotoxic drugs [15]. In fact, the original FOB testing guidelines from the U.S. Environmental Protection Agency (1985, 1991) and the guidelines for Neurotoxicity Risk Assessment (1998) recommend IDPN as a positive control for these test procedures [15–17].

Recently, we showed the direct toxic effects of IDPN on rat ovary that involved a transient increase in follicular atresia [18,19]. However, the reproductive toxicities of IDPN in cells from the HPG axis other than female gonads, *i.e.* GnRH neurons in POA, gonadotrophs in the pars distalis and male gonads, have not been determined. The transient and recoverable *in vivo* toxicities of IDPN in the female reproductive system suggest that IDPN would have little or no effect on the neurons and gonadotrophs involved in the HPG axis, although motor and sensory neurons are severely and irreversibly

Abbreviations: DAB, 3,3′-diaminobenzidine; ELISA, enzyme-linked immunosorbent assay; FOB, functional observational battery; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; HPG axis, hypothalamic-pituitary-gonadal axis; IDPN, 3,3′-iminodipropionitrile; LH, luteinizing hormone; POA, preoptic area; RT-PCR, reverse transcription-polymerase chain reaction.

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http://dx.doi.org/10.1016/j.reprotox.2013.10.007

affected by IDPN. To clarify the spectrum of acute IDPN cytotoxicity in the HPG axis, we performed histological, biochemical and serological studies of the brain, adenohypophysis, testis and blood of adult male rats.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Wako Pure Chemical Industries (Tokyo, Japan), unless otherwise noted.

2.2. Animals

Acute, mid-term and chronic neurotoxicological doses of IDPN are 1000, 300–500 and 50–100 mg/kg body weight, respectively [6–17]. The administration of acute toxic dose of IDPN by intraperitoneal injection induces persistent neurobehavioral toxicity in rats of both sexes and transient reproductive toxicities in female rats within 4 days [6,18]. Therefore, in the present study, 12- to 14-week-old adult male Wistar-Imamichi rats (about 400–450 g; $n = 5$–7 at each sampling time point) received a single intraperitoneal injection of 1000 mg/kg IDPN (technical grade, 90%) with minimal distress and were then compared with untreated control males (0 mg/kg; $n = 4$–7 at each sampling time point). The SPF-certified animals were purchased from a domestic livestock dealer the Institute for Animal Reproduction (Tsukuba, Japan) and bred in a temperature- and light-controlled room (22 °C; 14 h light:10 h dark with lights on at 0600 h) with sterilized food and tap water available *ad libitum*. Plasma samples for enzyme-linked immunosorbent assay (ELISA) were collected from the caudal vein of animals at 1, 4, 8, 15 and 30 days after IDPN injection and stored at −80 °C until assayed. Blood samples were also used for hematology studies by spreading onto glass slides and staining with Giemsa's solution. Animals were then anesthetized with intraperitoneally injected pentobarbital and tissue samples were excised after perfusion with 200 ml of saline from the left ventricle, followed by 50 ml of 4% paraformaldehyde in phosphate-buffered saline as a fixative; the excised samples were kept in the same fixative overnight. Tissue samples from adult males treated with a lower dose of IDPN (300 mg/kg) were prepared on the fourth day after injections ($n = 4$). For RT-PCR of GnRH, POA including median eminence were collected and stored at −80 °C until RNA extraction ($n = 4$). All experimental protocols were approved by the St. Marianna University School of Medicine Animal Care and Use Committee.

2.3. Histology

Brain coronal cryosections (30-μm thick) and 6-μm serial paraffin sections of pituitaries and testes were prepared and immunostained. To identify GnRH neurons, brain sections were immunostained with mouse monoclonal antibody against mammalian GnRH (#HAC-MM02-MSM84; 1:2000 in dilution) provided from the Institute for Molecular and Cellular Regulation of Gunma University (Maebashi, Gunma, Japan). For the detection of apoptotic cells by immunohistochemistry, tissue samples were incubated with anti-active caspase 3 antibody (Abcam, Science Park, Cambridge, UK; 1:50) at room temperature overnight. Immunoreactivities were detected by the development of 3,3′-diaminobenzidine (DAB) by using a Histofine Simple stain rat MAX-PO kit (Nichirei, Tokyo, Japan). After counterstaining with Mayer's hematoxilin, sections were viewed by bright-field microscopy. When the primary antibodies were omitted, no signal was apparent except for erythrocytes with autonomous peroxidase activity. The effects of IDPN on gonadotroph and testis were assessed by examining the morphology and counting the number of adenohypophyseal cells and seminiferous tubules with active caspase 3 immunosignals.

2.4. RT-PCR

To monitor GnRH neurons by gene expression, RT-PCR for GnRH was performed. Total RNA (1 μg) extracted from POA was reverse-transcribed, and one tenth of the cDNA solution was used as a template for each PCR. Partial sequences of GnRH and beta-actin cDNAs were amplified with specific primer sets as follows: GnRH sense, gccagcactggtcctatggg; GnRH antisense, ttatgaaatctacgctgctggg; beta-actin sense, gacaacggctccggcatgtgca; and beta-actin antisense, tgaggatgcctctcttgctctg. These primer sets provide single PCR products at 336 bp for GnRH and 166 bp for beta-actin. PCR conditions are as follows: 28 (for beta-actin) or 31 (for GnRH) cycles of steps each with 30 s at 94 °C for denaturation, 63 °C for annealing and 72 °C for extension. Intensities of the PCR products were measured by using the ImageJ 1.40f program (NIH, http://rsb.info.nih.gov/ij/) and normalized with beta-actin mRNA levels.

2.5. ELISA

To quantify the plasma levels of gonadotropins, rat FSH and LH ELISA kits (Shibayagi, Gunma, Japan) were used according to the instruction manuals. To monitor the testicular function, the plasma testosterone level was also quantified by using a testosterone ELISA kit (#402510; Neogen, Lansing, MI, USA).

2.6. Statistics

All values are presented as mean ± S.E.M. Differences among groups or time points were analyzed by a nonparametric analysis of variance (Kruskal–Wallis-*H* test) followed by Dunn's *post hoc* comparison.

3. Results

3.1. Effects of IDPN treatment on neurons

A single injection of 1000 mg/kg IDPN into adult male rats induced the modulation of early neurological signs such as hypoactivity, polyuria and the presence of many acanthocytes and dacryocytes with a higher hematocrit level within a day (Fig. 1). Apparent movement disorders, such as tremors and faltering movements, began to occur the day after the injection. Gene expression levels of GnRH in POA were unchanged by IDPN treatment (Fig. 2A and B). Also, GnRH-immunopositive neurons were detected in POA from all treatment groups at all time points, even at 4 days after IDPN treatment when the maximal toxic effects were observed in other organs (Fig. 2C).

3.2. Effects of IDPN on anterior pituitary cells

IDPN treatments did not provide any remarkable changes in the morphology of pituitary cells for any of the doses examined. When IDPN was administrated at 1000 mg/kg, several active caspase 3-immunopositive cells were detected at 4 days but not at other time points after treatments (Fig. 3 and Table 1). Plasma FSH and LH levels were unchanged throughout the experimental period (Fig. 3E and F). Lower doses of IDPN (300 mg/kg) did not affect the immunoreactivity of active caspase 3 in pituitary cells (Table 1).