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Thyroid disrupting pesticides impair the hypothalamic-pituitary-testicular axis of a wildlife bird, *Amandava amandava*

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ABSTRACT

The effect of two thyroid disrupting pesticides (TDPs) mancozeb (MCZ) and imidacloprid (IMI) on the hypothalamic-pituitary-gonadal/testicular (HPG) axis of a seasonally breeding bird, *Amandava amandava* has been evaluated. Male birds (n = 8/group) were exposed to each of the pesticide (0.25% LD_{50} of respective pesticide) as well as to their two equimixture doses (0.25% of LD_{50} of each and 0.5% LD_{50} of each) through food for 30d during pre-breeding stage of the reproductive cycle. Reduction in weight, volume and other histopathological features revealed testicular regression. Suppression of gonadotropin releasing hormone, increased expression of gonadotropin inhibitory hormone in the hypothalamus of exposed groups as well as impairment of plasma levels of the reproduction related hormones indicated the disruption of the HPG axis. The pesticides interference of the thyroid function during the critical phase of reproductive development impaired the HPG axis; more significantly in co-exposed groups suggesting the cumulative toxicity.

1. Introduction

The neuroendocrine pathway of reproduction, the hypothalamic-pituitary-gonadal (HPG) axis, is a conserved axis in vertebrates whose homeostatic regulation is under the control of hypothalamic neuropeptides (gonadotropin releasing hormone/GnRH and gonadotropin inhibitory hormone/GnIH), pituitary gonadotropins (luteinizing hormone/LH and follicle stimulating hormone/FSH) and gonadal steroid hormones androgens and estrogens [1,2]. Moreover, thyroid hormones (THs: thyroxine/T4 and triiodothyronine/T3) play crucial roles in maintenance of the reproductive axis. In mammals, THs role in control of Sertoli cell proliferation and maturation as well as Leydig cell differentiation and steroidogenesis have been elucidated [3,4]. Presence of thyroid hormone receptors in testis of rats suggests direct regulation of the testicular functions by the THs [3,5]. Their roles during spermatogenesis in rodents also have been suggested [6]. THs are key regulators of seasonal reproduction in both mammals and birds. Metabolism of THs in hypothalamus, the deiodinase enzyme 2 mediated conversion of T4 to T3, triggers the attainment of breeding status in both mammals and birds [7–9]. However, contradictory effects of THs have been reported in tropical/sub-tropical zone birds. Whereas in passerine birds (*Emberiza bruniceps, Passer domesticus, Ploceus philippinus* and *Acridotheres tristis)* THs have been linked to up-regulation of the gonadal development [10,11], in estrildid finches (*Amandava amandava/Estrilda amandava*, *Lonchura punctulata* and *Lonchura castaneothorax)* the opposite effects have been suggested [12–14].

Pesticides of diverse chemical nature such as organochlorines, organophosphates, neonicotinoids and dithiocarbamates, disrupt thyroid functioning interfering with hypothalamic-pituitary-thyroid (HPT) axis of both mammals and birds [15–17]. Thyroid disrupting pesticides (TDPs), such as dithiocarbamate fungicide mancozeb [18], azoles fungicides prochloraz, tebuconazole and epoxiconazole [19] have been reported to impair reproductive axis in mammals. However, studies on disruption of reproductive axis on exposure to any TDP is almost lacking in birds. We have reported the impairment of the pituitary-thyroid axis in a seasonally breeding tropical/sub-tropical wild life bird, red munia (*Amandava amandava*) exposing to dithiocarbamate mancozeb (MCZ) [manganese ethylene bis (polymeric) complex with zinc salt] and neonicotinoid imidacloprid (IMI) (1[(6-chloro-3-pyridinyl)

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methyl]-*N*-nitro-2-imidazolidinimine) as individual exposure [17] as well as their mixtures [20]. Both MCZ and IMI disrupt normal testicular functioning in rodents [18,21,22]. There has been a single study on exposure to MCZ along with other chemicals demonstrating morphological impairments in testis of a wildlife bird tree swallows [23], but studies are completely lacking with regard to IMI. In a view to explore the relationship between the thyroid and gonadal axis, presently the effects of these two TDPs (MCZ and IMI) on testicular axis of the bird *Amandava amandava* were studied. This bird is highly sensitive to both these pesticides through dietary exposure (granivorous as well as insectivorous). Specific action of MCZ and IMI, as fungicide and insecticide respectively, demands their simultaneous use in agricultural fields making the wild birds vulnerable to their co-exposure. Therefore, the disruption of hypothalamic-pituitary-gonadal/testicular (HPG) axis on co-exposures to both the pesticides was assessed in addition to evaluation of effect of individual exposure. The study was carried out during pre-breeding/preparatory phase of reproductive cycle when distinct tissue remodeling and differentiation takes place in gonads for attainment of breeding status. Considering the role of thyroid hormones in regulation of growth and differentiation, interference of the HPT axis during this stage may result in the impairment of reproductive development. Therefore, it is important to evaluate the effects of pesticide exposure on HPG axis during this phase of reproductive cycle.

2. Materials and methods

2.1. Experimental design

Male red munia were captured from a particular forest area (to avoid background exposure to pesticides) near to Allahabad (25°27′N 81°44′E), UP, India, in the beginning of pre-breeding (preparatory) stage of the reproductive cycle (first week of July). The pre-breeding phase is the stage of testicular remodeling and growth and is characterized by a complex set of developing secondary sexual characters as well as sexually motivated behaviors [24,25]. Birds were acclimatized in the open air aviaries ($1 \times 1\,m^2$ area for four birds) under natural conditions of temperature, humidity and photoperiod for 10 days (d). Food (grinded wheat grains; grown organically to avoid background exposures) and water were given *ad libitum*. Acclimatized male birds (reproductively healthy/active of 1–1.5 yr age and 8.5 ± 0.5 gm body weight/BW) were maintained in one control and four exposure groups: MCZ-, IMI-, MIX-I and MIX-II (n = 8/group). Commercial pesticides MCZ (75%w/w, Uthane M-45, United Phosphorous Ltd, Pune, India) and IMI (17.80% w/w, Confidor, Bayer Crop Science Ltd, Thane, India) were used for exposure. Food was mixed (coated) with pesticides using olive oil as vehicle and kept overnight. Dietary median LD_{50} of MCZ for bird (860 mg kg^{-1} BW) was taken as the reference dose [26]. For IMI, chronic median LD50 of Japanese quail (31 mg kg^{-1} BW) was taken as the reference dose [27]. The mixture doses were decided keeping in view the study of individual exposure of both the pesticides where disruption of the pituitary-thyroid axis was noted with their environmentally realistic doses i.e. 0.5% LD_{50} [17]. Accordingly, dose of each pesticide was reduced to half (0.25% LD_{50} of each: 0.14 mg of MCZ and 2.75 μl of IMI) to make a mixture dose (MIX-I) equivalent to that of environmentally relevant exposure level. The second mixture dose (MIX-II) was taken for the comparative evaluation (0.5% LD_{50} of each: 0.28 mg of MCZ and 5.5 μl of IMI). The dose of individual pesticides was considered as environmentally realistic; the environmental concentrations of MCZ and IMI have been reported in invertebrates, seeds/grains and crop fields [28–30]. Birds were exposed for 30 d in preparatory phase (mid July-mid August). Control birds were given food with vehicle only. Food consumption was monitored for the exposure period (see the supplementary Fig. 1). Body Weight was recorded every alternate day by TB-214 digital weight balance (Denver Instrument, NY, USA). Criteria of selection of pesticides, vehicle and precision of dose intake were maintained as described earlier [17]. Experimental protocols were approved by Institutional Animal Ethical Committee (IAEC) of the Department of Zoology, University of Allahabad. Guidelines of Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Ministry of Environment and Forests, Government of India were followed for maintenance and termination of the bird. Permission was taken from Principal Chief Conservator of Forests, Wildlife, U.P., India, by wide letter no. 3564/23-2-12(9) for use of wildlife birds.

2.2. Hormonal assays

Birds were terminated by decapitation and blood was immediately collected from cervical outflow as well as cardiac puncture in 0.1% EDTA treated vials, centrifuged at relative centrifugal force 2050*g* for 20 min, plasma was separated and pooled. Assays for LH, FSH, prolactin (PRL), testosterone and E2 were conducted immediately, without freezing the plasma, using commercially available ELISA kits for human following the manufacturer's protocol (LH: Diagnostics Systems Ltd., Russia, FSH, PRL, testosterone and E2: LDN GmbH & Co. KG, Germany). The validation of ELISA kits for this avian species was done by parallelism and immunohistochemistry. In brief, plasma sample was serially diluted for five times and ELISA for respective hormone was conducted taking equal volume (25 μm) from each aliquot. The optical density obtained was dilution dependent in each of the assay, thus validating the use of mammalian kits in this bird. The validity of use of human ELISA kit in this bird further supported by immunohistochemistry as pituitary hormone producing cell types of this bird showed specific immunoreactions against human antibodies (anti-hTSH, anti-h LH, anti-hPRL; National Hormone and Pituitary Program, Torrance, CA, USA) (see the Supplementary Fig. 2). Inter-assay and intra-assay coefficient of variations (%) were <10% for LH (4.36 and 4.06), FSH (4.75 and 3.4), PRL (7.6 and 6.9), testosterone (7.3 and 6.6) and E2 (6.2 and 4.6), respectively. The cross-reactivity of the PRL was almost absent i.e. 0.9% with FSH, 0.2% with LH, 0.02% with hCG and 0% with TSH (information provided by manufacturers' fact sheet). The samples were run in duplicate and optical density was measured by Bio-Rad iMark microplate reader (Bio-Rad Laboratories, CA, USA) at 590λ.

2.3. Testis weight, volume and histopathology

Testes were quickly dissected out, blotted, weighed before fixation in Bouin's fixatives for overnight followed by washing, dehydration in graded ethanol and paraffin embedding. Testis volume was calculated morphometrically (discussed below). Testis sections were cut serially (5–6 μm), stretched on sterilized glass slides. Standardized protocol of our laboratory was followed for eosin-haematoxylin staining [31]. Acridine orange/AO (Sigma-Aldrich, USA) and ethidium bromide/EB (Molychem, India) staining were used to differentiate normal, apoptotic and necrotic cells in testis. AO and EB stain nuclei green and red respectively by intercalating with DNA. In case of normal and healthy cells with intact plasma membrane, EB does not pass through membrane and cells stain with AO only to fluoresce green, but in case of apoptotic and necrotic cells, plasma membrane gets ruptured and cells stain with EB to fluoresce red [32]. Apoptotic cell have heterochromatinized pyknotic and fragmented nuclei of smaller size; necrotic cells have fragmented and swollen nuclei of larger size [33]. In brief, protocol followed for AO/EB staining was: paraffin embedded testis sections were first kept on a hot plate at 45–50 °C for