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Modulation of antioxidant defense system by the environmental fungicide carbendazim in Leydig cells of rats

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Abstract

Carbendazim (methyl-2-benzimidazole carbamate, MBC) a metabolite of benomyl is one of the most widespread environmental contaminant of major concern to human and animal reproductive health. The present investigation was undertaken to study the impact of carbendazim exposure on Leydig cell functions. Adult albino male rats of the Wistar strain were administered with carbendazim (25 mg/(kg (body weight)/day)) orally for 48 days. The control animals received vehicle (corn oil) alone. Another group of rats were treated with carbendazim and the same was withdrawn for a further period of 48 days. After the treatment period, rats were euthanized and blood was collected for the assay of serum hormones such as luteinizing hormone (LH), prolactin (PRL), testosterone and estradiol. Testes were immediately removed and Leydig cells were isolated in aseptic condition. Purified Leydig cells were used for quantification of steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD). Leydig cellular enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-*S*-transferase (GST), γ-glutamyl transpeptidase (γ-GT), glucose-6-phosphate dehydrogenase (G6PDH) and non-enzymatic antioxidants such as reduced glutathione (GSH), α-tocopherol (vitamin E), ascorbic acid (vitamin C) and β-carotene (vitamin A) were assayed. Lipid peroxidation (LPO) and reactive oxygen species (ROS) were also quantified. Carbendazim exposure had no effect on body weight, serum LH and prolactin. However, testis weight, serum testosterone and estradiol were significantly decreased. In addition to this, Leydig cellular activities of steroidogenic enzymes such as 3β-HSD, 17β-HSD, antioxidant enzymes SOD, CAT, GPx, GR, GST, γ-GT, G-6-PDH and non-enzymatic antioxidants such as GSH, vitamins E, C and A were significantly diminished, whereas LPO and ROS were markedly elevated. All these above-mentioned parameters from the animals after withdrawal of MBC treatment were similar to those of the control group. Thus, the present study suggests that chronic low dose treatment of MBC is capable of inducing reproductive toxicity through increased oxidative stress, but is transient and reversible upon withdrawal of treatment.
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1. Introduction

Many compounds introduced into the environment by human activity are capable of disrupting the endocrine system of animals, including fish, wild life and humans. Among these chemicals are pesticides, industrial chemicals, pharmaceuticals, phytochemicals and other anthropogenic products. Endocrine disruption can be profound because of the crucial role of hormones in controlling development [1]. Carbendazim (methyl-2-benzimidazole carbamate, MBC) is a systemic broad-spectrum fungicide controlling various fungal pathogens. It is also used as a preservative in paint, textile, papermaking, leather industry and warehousing practices, as well as a preservative of fruits [2]. Carbendazim is well absorbed (80–85%) after oral exposure and is subsequently metabolized into many compounds within the organisms. The main metabolites are 5-hydroxy-2-benzimidazole carbamate (5-HBC) and 5,6-hydroxy-2-benzimidazole carbamate-*N*-oxides (5,6-HOBC-*N*-oxides). Carbendazim is poorly catabolized and remains in tissues such as gonads, liver, adrenals, adipose tissue, skin and other organs [3].

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Evidences available support the deleterious effects of carbendazim on various aspects of male reproduction in hamsters, mice, rats and humans. The effects include the decreased mean testes weight, caput sperm numbers, mean seminiferous tubular diameter [4], total sperm count, motility, increased incidence of sperm abnormalities [5] and disruption of microtubule formation [6]. Carbendazim, an inhibitor of microtubule synthesis, directly alters testicular function *viz*. germ cell depletion, alterations of Sertoli and Leydig cellular functions [7]. In addition, it has been reported that lesions in the male tract cause blockage which may induce permanent testicular damage and a decrease in sperm production exposed to benomyl and carbendazim in adult rats [8]. But the mechanism by which carbendazim induces Leydig cellular dysfunction remains obscure.

Oxidative stress and lipid peroxidation (LPO) are established as a significant factor in the etiology of male infertility. Reactive oxygen species (ROS) formed in the body as a result of normal metabolic reactions, exposure to ionizing radiations, environmental pollution, alcohol toxicity, and by the influence of several xenobiotics are implicated in several diseases. Among the ROS, hydroxyl radical is the most reactive species that could damage both macromolecules and small molecules. ROS damage DNA, proteins, carbohydrates and lipids and affect enzyme activity and the genetic machinery. However, biological systems possess a number of mechanisms to remove free radicals, the integrated antioxidant system which scavenges free radicals [9]. Free radicals are produced as by-products of normal cellular metabolism, generated by chemicals in the environment, in air and food [10]. The risk of oxidative damage from lipid peroxidation is especially high for steroid synthesizing tissues, because these tissues, in addition to oxidative phosphorylation, use molecular oxygen for steroid biosynthesis, and all interactions of the cytochrome P 450 enzymes with their substrates (cholesterol and its metabolites) are additional sources of oxygen free radical (OFR) generation [11]. Indeed, it has been shown that free radicals inhibit steroidogenesis interfering with cholesterol transport to mitochondria and/or catalytic function of P 450 enzymes [12].

Leydig cells, which reside in the testicular interstitium are particularly susceptible to extracellular sources of ROS because of their close proximity to testicular interstitial macrophages [13]. ROS have been shown to inhibit steroidogenesis at the level of cholesterol transfer in MA-10 tumor Leydig cells [14]. The cytochrome P 450 enzymes of the steroidogenic pathway use molecular oxygen and electrons transferred from NADPH to hydroxylate the substrate. In this process, superoxide anion or other OFRs can be produced as a result of electron leakage in normal reactions or due to interaction of steroid products or other pseudosubstrates with the enzyme [15]. OFR enzymatic scavengers, such as SOD, CAT, GSH metabolism-regulating enzymes, such as γ-GT, GPx, GR, GST, G-6-PDH, may protect the cellular system from various deleterious effects of free radicals induced by various pesticides. The non-enzymatic antioxidants, α-tocopherol, ascorbate and β-carotene scavenge and quench free radicals, getting oxidized and inactivating the process. They often work synergistically to enhance the overall antioxidant capacity of the body [16]. Recently, the effects of carbendazim on the haematological, biochemical and histopathological changes in the liver, kidney and endocrine glands (pituitary, thyroid, parathyroid and adrenal) and their hormonal levels have been demonstrated in male rats [2,17]. However, the impact of carbendazim on the Leydig cellular antioxidant system and its link to steroidogenesis has to be elucidated. Therefore, the present investigation was undertaken to elucidate the effect of carbendazim on Leydig cellular enzymatic and non-enzymatic antioxidant system.

2. Materials and methods

2.1. Chemicals

Carbendazim (99% purity) was obtained from the International Marketing and Development Department, India. Dulbecco's modified Eagle's medium + Hams F-12 nutrient mixture (1:1) (DMEM-F12), bovine serum albumin (BSA), collagenase type IV, Percoll, trypan blue and vitamin E were purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were purchased from Sisco Research Laboratories (SRL), Mumbai, India and were of analytical grade.

2.2. Animals and experimental treatments

Healthy adult male albino rats of Wistar strain *Rattus norvegicus* weighing 150–200 g were housed in clean polypropylene cages and maintained in an air-conditioned animal house with constant 12 h/12 h dark and light cycle. The animals were maintained and handled as per the guidelines given by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and Institutional Animal Ethical Committee (IAEC). The animals were fed with standard rat pellet diet (Lipton India Ltd., Mumbai, India) and clean drinking water was made available *ad libitum*. The rats were divided into three groups. Each group consists of 10 animals. Group I: rats were given corn oil as vehicle orally, daily for 48 days. Group II: rats were treated with carbendazim dissolved in corn oil at a dose of 25 mg/kg body weight daily for 48 days, orally. Group III: rats were treated with carbendazim at a dose of 25 mg/kg body weight in corn oil orally, daily for 48 days and left untreated for another 48 days to see the withdrawal effects. The dosage and duration were selected as per from the previous publications [5].

2.3. Blood and tissue collection

Twenty-four hours after the last treatment, rats were killed by decapitation, blood were collected in clean, dry test tubes, and allowed to clot at room temperature. The sera were removed after centrifugation and stored at −20 °C until the assay of hormones. Testes were removed from the adhering connective tissues, washed in physiological saline extensively and weighed.

2.4. Radioiodination of peptide hormones

Iodination of peptide hormones were carried out by following the procedure of Greenwood et al. [18] using chloramine-T as the oxidizing agent.

2.4.1. Radioimmunoassay (RIA) of luteinizing hormone (LH) and prolactin (PRL)

The serum LH and PRL were quantified by liquid phase RIA using double antibody technique following the procedure of Sufi et al. [19]. The cross reactivity of LH to other peptide hormones were <0.02% for rFSH and <0.01% for rPRL. The intra- and inter-assay variation was 4.9–8.48% and 9.9%, respectively. Sensitivity of LH was 5 ng/ml. The cross reactivity of PRL antibody with other hormones were <0.1% for rFSH, and <0.03% for rLH, The intra- and inter-assay coefficient of variation was 3.5–6.8% and 9.9%, respectively. Sensitivity of PRL was 2 pg/ml.

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