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Reproductive and possible hormonal effects of carbendazim ☆

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ABSTRACT

This study aimed to better elucidate reproductive and possible hormonal effects of the fungicide carbendazim (CBZ) through a review of published toxicological studies as well as an evaluation of this fungicide in the Hershberger and uterotrophic assays, which are designed to detect *in vivo* effects of the sex hormones. The literature review indicates that CBZ induces reproductive and developmental toxicity through alteration of many key events which are important to spermatogenesis. The lower dose of CBZ (100 mg/kg) evaluated in the Hershberger test increased prostate weight compared to control group but did not alter the weight of other testosterone-dependent tissues. In the uterotrophic assay, CBZ did not induce an estrogenic or an antiestrogenic effect. In the literature, it has been reported that CBZ may: (1) alter the levels of various hormones (testosterone, LH, FSH, GnRH); (2) negatively influence testicular steroidogenesis; (3) have androgenic effects acting directly in the androgenic receptors and/or increasing the expression of androgen receptors. Despite the contradictory results reported by the different studies that investigated a possible endocrine mode of action of CBZ, it seems that this fungicide may influence the hypothalamus–pituitary–gonad axis in addition to being a testicular toxicant.

1. Introduction

Carbendazim (CBZ) is a fungicide from the benzimidazole group used in many countries. In Brazil, CBZ was the tenth most sold active ingredient of pesticides in 2009 (IBAMA, 2010). In addition, the report from the Brazilian Monitoring Program of Pesticide Residues in Food (PARA) published by the Brazilian National Health Surveillance Agency (ANVISA) reveals that CBZ is the main pesticide used irregularly, i.e., it is both detected in unauthorized crops as well as above the maximum allowed residues levels (ANVISA, 2011).

CBZ exerts its antifungal action inhibiting microtubule polymerization and, consequently, cell division in fungi. Microtubules are formed by heterodimers, the α and β-tubulin, which interact by non-covalent bindings and have a key role in cell division, being responsible for chromosome segregation in mitosis and meiosis. Furthermore, they are responsible for organizing, transporting and placement of organelles. The CBZ-induced inhibition of microtubule polymerization has been attributed to its interaction with β-tubulin at the tyrosine residue 167, Tyr-167 in *Saccharomyces cerevisiae* (Li et al., 1996) and to the inhibition of the binding of guanosine triphosphate (GTP) to β-tubulin in rats (Winder et al., 2001).

The tubulin structure is quite conserved in eukaryotes and 75% of its amino acid sequence is identical in human and fungi. Most of the sequence variations are in the C-terminal portion, which predominantly affects microtubule association with accessory proteins but not the polymerization (Alberts et al., 2002). Thus, it is expected that substances that interfere with microtubule polymerization have this effect in many different eukaryotic organisms due to the high conservation of these structures in different species. In fact, CBZ presents colchicine-like mechanism in rats, leading to adverse reproductive effects during spermatogenesis (Markelewicz et al., 2004).

Since the mid-1990s, it came into discussion the importance to include in the battery of studies designed to determine the toxicity of pesticides, assays able to screen for endocrine activity. Endocrine activity is a mode of action that may lead to reproductive and developmental toxicity as well as neurotoxicity and carcinogenicity since the endocrine glands tightly regulate critical physiological processes. Even though there is a report from the California Environmental Protection Agency (Cal/EPA, 1999) concluding that an endocrine mode of action did not appear to be involved in the

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reproductive effects of CBZ, it should be taken into account that new studies have been published since this report. Some evidences suggesting that CBZ could have endocrine actions have been reported (Barlas et al., 2002; Goldman et al., 1989; Lu et al., 2004; Morinaga et al., 2004; Rehnberg et al., 1989; Yu et al., 2009).

Considering that in many countries, including Brazil, pesticides with hormonal action must not be marketed, this study aimed to review published toxicological studies concerning the reproductive effects and endocrine action in order to evaluate evidences of adverse health effects of CBZ. Moreover, CBZ was evaluated in the uterotrophic and Hershberger tests, which are part of the Endocrine Disruption Screening Battery for Endocrine Disruptor Chemicals published by the Environmental Protection Agency (EPA) and designed to detect *in vivo* effects of the sex hormones. It should be mentioned that, to the best of our knowledge, this is the first study that proposes to conduct a literature review focusing on a possible endocrine mode of action and also to evaluate CBZ in the uterotrophic and Hershberger tests.

2. Materials and methods

2.1. Literature review and data analysis

Articles were scanned through the PubMed database using the keyword "carbendazim" associated with "toxicity", "endocrine", "reproduction". No language restriction was applied. Articles not related to mode of actions that could explain reproductive toxicity, developmental toxicity or endocrine disruption were excluded, as well as those describing therapeutic treatments against the effects caused by CBZ and the ones related to concomitant exposure of CBZ with other substances/pesticides. Searching by hand for published data was also conducted through the reference lists of identified articles. Moreover, the most recent report from the International Program of Chemical Safety (IPCS, 1993), which describes studies from the toxicological dossiers submitted to the international authorities in order to request the registration of this pesticide, was used. The final literature search was conducted on November, 2013.

The toxicological studies retrieved were evaluated and tables were constructed summarizing reproductive and developmental endpoints as well as summarizing studies that aimed to investigate endocrine effects of CBZ.

2.2. Uterotrophic and Hershberger tests

2.2.1. Drugs

Estradiol valerate and tamoxifen citrate were obtained from Pharma Nostra Comercial (Rio de Janeiro, Brazil) and dissolved in corn oil and distilled water, respectively. Testosterone propionate and flutamide were obtained from Fragon (São Paulo, Brazil) and dissolved in corn oil. Carbendazim (97%), from Sigma–Aldrich (EUA), was dissolved in propylene glycol.

2.2.2. Animals

Male and female Wistar rats from the colony of State University of Londrina were mated. Litters with 8–12 pups were used and, if litters had more than 12 pups, culling was conducted. The day of birth was considered postnatal day (PND) 0 and on PND 18, female pups (uterotrophic assay) were weaned whereas male pups (Hershberger test) were weaned on PND 21. Littermates were not used in the same experimental group. Animals, separated by the experimental group, were housed in collective polypropylene cages ($29 \times 18 \times 13$ cm) with wood shavings bedding. They were kept in a controlled environment with temperature at 21 ± 2 °C; 12 h light/dark cycle (lights on at 6:00 AM) and had free access to regular lab chow (Nuvilab™, Quimtia SA, Brazil) and tap water.

The animals used in this study were maintained in accordance with Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and the experimental protocol was approved by the institutional ethics committee (CEUA UEL 149/2013).

2.2.3. Uterotrophic assay

The study design followed the guideline OPPTS 890.1600 from EPA (EPA, 2009a). On PND 18 rats were weighed and assigned to each experimental group. Weight variation among animals in the first day of treatment was 12%.

In order to evaluate the possible estrogenicity of CBZ, rats were divided in the following groups:

- Vehicle ($n = 6$): rats were treated with propylene glycol (vehicle of CBZ).
- Estradiol ($n = 5$): rats were treated with 0.3 mg/kg estradiol.
- CBZ 200 ($n = 5$): rats were treated with 200 mg/kg CBZ.
- CBZ 400 ($n = 7$): rats were treated with 400 mg/kg CBZ.
- CBZ 800 ($n = 6$): rats were treated with 800 mg/kg CBZ.

For the evaluation of the antiestrogenicity of CBZ, rats were divided in the following groups:

- Estradiol ($n = 5$): rats were treated with 0.3 mg/kg estradiol.
- Estradiol + tamoxifen ($n = 5$): rats were treated with 0.3 mg/kg estradiol in addition to 10 mg/kg tamoxifen.
- CBZ 200 + estradiol ($n = 6$): rats were treated with 200 mg/kg CBZ in addition to 0.3 mg/kg estradiol.
- CBZ 400 + estradiol ($n = 6$): rats were treated with 400 mg/kg CBZ in addition to 0.3 mg/kg estradiol.
- CBZ 800 + estradiol ($n = 6$): rats were treated with 800 mg/kg CBZ in addition to 0.3 mg/kg estradiol.

The doses of all chemicals were chosen based on manuscripts published in the open literature and on the recommendations from the EPA guideline. The chemicals were administered by gavage for 3 consecutive days (from 1:00 to 1:30 PM) after a 3-h food restriction. During the 3-day treatment period, animals were observed daily for mortality, morbidity, and general signs of toxicity, such as changes in behavior (e.g., agitation, lethargy hyperactivity), neurological changes (e.g., convulsions, tremors, muscle rigidity and hyperreflexia), and autonomic signs (e.g., lacrimation, piloerection, pupil size, and unusual respiratory patterns). Twenty-four hours after the final dose (i.e., PND 21), animals were weighed, examined for vaginal opening and anesthetized with sodium pentobarbital (40 mg/kg, ip). Uterus was removed, trimmed free of fat, weighed (wet weight), blotted to remove fluid and weighed again (blotted weight).

2.2.4. Hershberger test

The study design followed the guideline OPPTS 890.1400 from EPA (EPA, 2009b).

On PND 42 rats were castrated under ether anesthesia by placing an incision in the scrotum and removing both testes and epididymis with ligation of blood vessels and seminal ducts. On PND 49, rats were randomly assigned to the experimental groups. Weight variation among animals in the first day of treatment was 10%.

In order to evaluate the possible androgenicity of CBZ, rats were divided in the following groups:

- Vehicle ($n = 6$): rats were treated with propylene glycol (solvent of CBZ).

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