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Deleterious effects of benomyl and carbendazim on human placental trophoblast cells

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ABSTRACT

Benomyl and carbendazim are benzimidazole fungicides that are used throughout the world against a wide range of fungal diseases of agricultural products. There is as yet little information regarding the toxicity of benzimidazole fungicides to human placenta. In this study, we utilized human placental trophoblast cell line HTR-8/SVneo (HTR-8) to access the toxic effects of benomyl and carbendazim. Our data showed that these two fungicides decreased cell viability and the percentages of cells in G0/G1 phase, as well as induced apoptosis of HTR-8 cells. The invasion and migration of HTR-8 cells were significantly inhibited by benomyl and carbendazim. We further found that benomyl and carbendazim altered the expression of protease systems (MMPs/TIPMs and uPA/PAI-1) and adhesion molecules (integrin α5 and β1) in HTR-8 cells. Our present study firstly shows the deleterious effects of benomyl and carbendazim on placental cells and suggests a potential risk of benzimidazole fungicides to human reproduction.

1. Introduction

Benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbarnate] and its major active metabolite carbendazim (methyl 2-benzimidazolecarbamate) are benzimidazole fungicides that have been widely used in agriculture since 1970s. Benomyl and carbendazim suppress fungal growth by disrupting tubulin polymerization [1]. Although accumulating toxicological evidence of these fungicides led the US Environmental Protection Agency to cancel their registrations for a decade [2], benomyl and carbendazim are still particularly applied in Europe and extensively used in some developing countries such as China. Benomyl rapidly degrades to its metabolite carbendazim and it has a half-life of a few hours in the environmental compartments (such as soil, water, plants) [3]. However, the half-life of carbendazim varies from several days to a few months in water and soil [4]. Carbendazim was reported as one of the most commonly detected pesticides in fruits [5]. The residual half-life of carbendazim in whole fruits was approximately 7.0 and 6.5 days respectively from pre-harvest and post-harvest treatment [6]. The residues of carbendazim were found at levels up to 1 mg/L (5.5 μM) in fruits in China and Italy [7,8]. Due to the occurrences of benomyl and carbendazim in environment and the potential exposure to them through diet, there continues to be ongoing concern over the risks to human health of these fungicides.

A plenty of evidences in laboratory mammals support the deleterious effects of benomyl and carbendazim on male reproduction. Administration of benomyl to male rats and mice caused reproductive damage by decreased testicular and epididymal weights and a reduction in epididymal sperm counts and fertility [9,10]. Carbendazim induced spermatogenic failure by altering Sertoli cell morphology and functions, as well as increased Leydig cellular oxidative stress and apoptosis in germ cells of testes in rats [11–13]. Several studies have revealed toxic effects of benomyl and carbendazim on female reproduction and fetal development. Exposure of pseudopregnant rats to benomyl inhibited the growth of deciduoma, which represented the maternal portion of the placenta [14]. Benomyl had teratogenic effects on cultured rat embryos by decreasing the morphological score, somite number and optic development [15]. A recent study showed that exposure of pregnant mice to carbendazim induced maternal and developmental toxicity and produced an increase in postimplantation loss

Abbreviations: MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of MMPs; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor-1.

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[16]. Although the toxic consequences on maternal and fetal tissues have been investigated, information regarding toxicity of benomyl and carbendazim to placenta is scarce.

Placenta is an organ that affords physiological exchange of nutrients, hormones and gases between mother and fetus. Its development is critical for pregnancy success and fetal health. The trophoblasts are the main functional cells of placenta and play pivotal roles in placenta establishment. During placental development, trophoblast cells proliferate, migrate and invade the uterine endometrium and arterioles, thereby ensuring a continuous blood supply to developing fetus. The processes of trophoblast invasion and migration involve the coordinated interactions of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor (PAI-1), as well as adhesive molecules such as integrins [17]. It has been reported that a lot of environmental pollutants might disrupt trophoblast function and transplacental exposure to these chemicals had adverse effects on the developing fetus [18]. Therefore, human placental trophoblast cell has been used as an *in vitro* model to screen the potential reproductive and developmental toxicants [19–21]. In the present study, we utilized a human trophoblast cell line HTR-8/SVneo (HTR-8) to evaluate the effects of benomyl and carbendazim on cell viability, cell cycle, apoptosis, cell invasion and migration, as well as the expression of invasion and migration-related genes.

2. Materials and methods

2.1. Materials

Benomyl, carbendazim and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used in cell exposure experiments were dissolved in DMSO and control cells were treated with the same concentration of DMSO (0.1%, v/v).

2.2. Cell culture and exposure

HTR-8 cells, a human first trimester extravillous trophoblast cell line, were grown in DMEM medium (Hyclone, Logan, USA) containing 10% fetal bovine serum (FBS) (Hyclone) and incubated at 37 °C in humidified atmosphere under 5% CO_2 in air. Cells were seeded in 12-well culture plates and maintained in growth medium. Next day, cells were exposed to fresh serum-free media containing various concentrations of benomyl or carbendazim for 24 h. At different time points, cells were collected for RNA extraction.

2.3. MTS cell viability assay

HTR-8 cells were seeded in 96-well culture dish at a density of 5000 cells per well and exposed to benomyl or carbendazim at concentrations of 1, 2.5 or 5 μM for 24 h. As described previously, CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, USA) was used to assess cell viability [22–24]. In brief, at end of culture, 20 μL of reagent was added to each well and incubated for an additional 2 h. The optical density (OD) was determined at 490 nm in the Infinite M200 PRO microplate reader (Tecan Group, Switzerland). The amount of OD is directly proportional to the number of viable cells per well [25,26].

2.4. Flow cytometric analysis of cell cycle

The cell cycle distribution was analyzed by flow cytometry (Becton Dickinson) as described previously [27]. Briefly, HTR-8 cells were seeded in 6 cm Petri dish at the concentration of 2×10^5 cells per well and treated with benomyl or carbendazim at doses of 1, 2.5 or 5 μM for 24 h. Then, cells were harvested, fixed in 75% ice-cold ethanol and stored at −20 °C overnight. Ethanol-fixed cells were washed with phosphate-buffered saline (PBS) and treated with 50 μg/mL ribonuclease A and incubated at 37 °C for 30 min. Afterward, cells were treated with 50 μg/mL of propidium iodide (PI; Invitrogen, USA) for 15 min in the dark on ice. Cell cycle analysis was performed with excitation at 488 nm in a flow cytometer (FACS, Becton-Dickinson, USA) at Zhejiang University. Three independent experiments were performed for each treatment and at least 20,000 cells were analyzed for each sample.

2.5. Flow cytometric analysis of apoptosis

The detection of early and late apoptotic cells was conducted using a commercial Annexin V-FITC kit (BD Biosciences, Massachusetts, USA) according to the manufacturer's instructions. HTR-8 cells were exposed to benomyl or carbendazim at dose of 1, 2.5 or 5 μM for 24 h. After washing with PBS, cells were digested with trypsin and then collected by centrifugation at $1200 \times g$ for 2 min. As described previously, cells were briefly resuspended in Annexin V-binding buffer and incubated with FITC-labeled Annexin V and PI for 15 min at room temperature in the dark [28–30]. Afterward, the samples were immediately analyzed by flow cytometry. Early apoptotic cells (intact cell membranes) were defined as PI-negative cells with annexin V staining. Late apoptotic/necrotic cells (non-intact cell membranes) were defined as PI-positive cells with annexin V staining [31,32].

2.6. Transwell cell invasion assay

Cell invasion assay was determined as previously described [33]. Briefly, transwell inserts with 8-μm pore size membranes (Corning, Now York, USA) were coated with growth factor reduced Matrigel (BD Biosciences, Massachusetts, USA) at a concentration of 200 μg/ml and placed in 24-well plate. HTR-8 cells were plated in the upper chambers at density of 2×10^4 cells per well in 200 μL serum-free DMEM. The lower chamber of the transwell was filled with DMEM supplemented with 10% FBS. Cells were exposed to benomyl or carbendazim at concentrations of 1, 2.5, or 5 μM for 24 h. Noninvaded cells on the upper surface of membrane were completely removed by gentle swabbing. The invaded cells remaining on the lower surface of membrane were fixed with 95% ethanol for 20 min and stained with hematoxylin for 3 min and then stained with eosin for 1 min. Invaded cells were viewed under optical microscopy at 40× magnification and counted in at least fifteen fields. The fields were randomly chosen from the top, bottom, left, right, and center position of each membrane for three times respectively. The invasion index was calculated as the percentage of invaded cells compared to the control group.

2.7. Wound-healing assay

Wound-healing assay was performed as previous described [34]. Briefly, HTR-8 cells were seeded in 12-well plates and allowed to form confluent monolayers. Cell monolayers were scratched using a 10-μL pipette tip to create a wound and washed once with PBS. Then cells were exposed to fresh DMEM medium containing benomyl or carbendazim at doses of 1, 2.5, or 5 μM for 24 h. Wound width was monitored in each well under optical microscopy at 10× magnification and photographed immediately (0 h) and at 24 h after wounding. The migration index was calculated as the percentage of wound width compared to the control group.