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Effects of mancozeb, metalaxyl and tebuconazole on steroid production by bovine luteal cells in vitro

Nurgul Atmaca[a], Sevket Arikan[a], Dinc Essiz[b], Hakan Kalender[c], Ozkan Simsek[a], Fatih Sultan Bilmen[a], Ruhi Kabakci[a,*]

[a] Kirikkale University, Faculty of Veterinary Medicine, Department of Physiology, Yahsihan, 71450, Kirikkale, Turkey
[b] Kirikkale University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Yahsihan, 71450, Kirikkale, Turkey
[c] Kirikkale University, Faculty of Veterinary Medicine, Department of Obstetric and Reproductive Disease, Yahsihan, 71450, Kirikkale, Turkey

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ABSTRACT

Mancozeb, metalaxyl and tebucanazole are widely used pesticides in agriculture and industry to treat plant pathogenic fungi. Livestock may be exposed to such substances by consuming contaminated plants. The present study was designed to evaluate the effects of these three fungicides on bovine luteal cell steroidogenesis. Luteal slices from mid-cycle corpus luteum were dissociated into single cell suspension in aerated (O_2) culture media (DMEM/F12) by enzymatic digestion. The cells were incubated in newborn calf serum (10%) for 18 h and then with serum-free media containing mancozeb (0.01 μM, 0.1 μM, 1 μM), tebuconazole (1 μM, 10 μM, 100 μM) or metalaxyl (100 μM, 500 μM, 2500 μM) for additional 96 h. The medium was replaced on day 1 and 3; and the retrieved medium was stored at −20 °C until progesterone assay. Treatment of cells with three different fungicides induced dose dependent variable decrease in steroid synthesis during incubation periods. Incubation of cells with 1 μM mancozeb exhibited a 33% decline in steroid synthesis on day 3 and 48% decline on day 5 compared with controls. Treatment of cells with 100 μM tebuconazole and 500 μM metalaxyl resulted in a 65% and 31% decrease, respectively, in progesterone accumulation on day 5 of incubation. Fungicide induced suppressive effects on luteal steroidogenesis were as metalaxyl < tebuconazole < mancozeb. Results of the present study suggest that designated concentrations of all three fungicides studied might have varying degrees of adverse effects on luteal steroidogenesis.

1. Introduction

Fungicides are the chemicals used widely to protect various fruits and indoor plants against fungal diseases. Nevertheless, fungicides may be hazardous to human and animals and exist in the environment for long time (Komarek et al., 2010). Mancozeb, which is one of the fungicides widely used today, is a metal ethylene-bis-dithiocarbamate used to protect various fruits, vegetables and field crops against fungal diseases (Gullino et al., 2010). It has been reported that mancozeb is a multipotent carcinogenic agent with potential risk of tumor formation in various organs (Belpoggi et al., 2002) and a genotoxic chemical (Calviello et al., 2006).

Studies revealed that mancozeb causes fungicide toxicity in somatic ovarian cells, blocks ovulation by inhibiting LH secretion (Cecconi et al., 2007), and has unfavorable effects on the functions and morphology of granulosa cells in rats (Paro et al., 2012). It has been reported that mancozeb can produce toxic effect at such low concentrations as ≤1 μg/ml and impairs reproductive performance in females (Paro et al., 2012). It was determined that diestrus phase is prolonged, proestrus, estrus and metestrus phases are shortened and the number of estrus cycles are decreased (Baligar and Kaliwal, 2001), the number of atretic follicles is increased and the number of healthy follicles is decreased (Mahadevaswami et al., 2000) in the rats exposed to mancozeb.

Metalaxyl is an acylalanine fungicide with systemic function and prevents fungal growth by inhibiting nucleic acid synthesis. Besides, it inhibits protein synthesis in fungi by influencing ribosomal RNA synthesis (Davidse et al., 1983). Metalaxyl has been classified by WHO as moderately hazardous (WHO, 2009). Although it was reported that metalaxyl dose-dependently stimulates chromosome aberration in human peripheral lymphocyte culture (Hrelia et al., 1996) and leads to histopathological alterations in the mice liver (Lamfon, 2011), maternal or embryonic toxicity has not been reported in rats (EPA, 1994; Farag et al., 2012).

* Corresponding author.
E-mail address: ruhikabakci@kku.edu.tr (R. Kabakci).

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Tebuconazole is an organic compound containing nitrogen and carbon bonds and belongs to the group of triazole fungicides. Azole fungicides are widely used in the fields of both agriculture and human and veterinary medicines for the treatment of local and systemic fungal diseases (Musiol and Kowalczyk, 2012). The mechanism of fungicidal activity consists of decreasing ergosterol biosynthesis and impairing structure of fungal membrane by inhibiting α-lanosterol demethylase (Kwok and Loeffler, 1993; Lamb et al., 1998). It was reported that tebuconazole has endocrine-disrupting potential (Taxvig et al., 2008). In addition, Kjaerstad et al. (2010) demonstrated androgen receptor antagonist activity of tebuconazole in the Chinese hamster ovarian cell culture. A recent study has reported that tebuconazole reduces cell viability and induces apoptosis in human placental trophoblast cells (Zhou et al., 2016).

Progesterone is secreted by both corpus luteum and placenta for maintaining pregnancy. Cholesterol is converted to pregnenolone by CYP11A1 (P-450scc) in mitochondria, and finally to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3B1) in the smooth endoplasmic reticulum (Tomac et al., 2011). Fungicide induced inhibition in progesterone synthesis and HSD3B1activity has been reported in human placental cell line Jeg-3 (Rieke et al., 2014). The fall in progesterone synthesis was attributed to diminished HSD3B1 and CYP11A1 activities.

Several studies have also demonstrated that fungicides can also generate oxidative stress in steriodogenic cells. Rajeswary et al. (2007) reported that treatment of rats with fungicide (carbendazim) induced significant increase in lipid peroxidation (LPO) and reactive oxygen species (ROS) in leydig cells. They also reported significant decrease in superoxide dismutase (SOD) and catalase (CAT) activities. Additionally, an increase in fungicide-induced intracellular ROS production was also reported in a more recent study (Roelofs et al., 2014) in which isolated rat leydig cells were treated with flusilazole, hexoconazole, prochloraz and tebuconazole. Lundqvist et al. (2016) reported that incubation of cells with 15, 20 and 50 μM prochloraz induce oxidative stress in cultured human adrenocortical carcinoma NCI-H295R cells.

Every year, livestock are accidentally exposed to fungicides applied to the crops, potato, or other agricultural products. Studies on the toxic effects of fungicides have been usually conducted in laboratory animals such as mouse, rat and rabbit (Komarek et al., 2010). The present study aimed to demonstrate the effects of various doses of widely used fungicides mancozeb, metalaxyl and tebuconazole, which have been determined previously to have unfavorable effects on both male and female laboratory animals, on progesterone synthesis, which is a marker for luteal cell function in the corpus luteum of bovine.

2. Materials and methods

2.1. Luteal tissue

Ovaries containing midcycle corpus luteum were collected from a local abattoir immediately after slaughter. Midcycle corpora lutea were identified according to criteria described by Ireland et al. (1980). Ovaries were transported on ice to the laboratory within 40 min. The corpora lutea were dissected free from the stroma of the ovaries under aseptic conditions.

2.2. Cell dissociation

Luteal tissues were minced into small pieces in a plastic petri dish. The minced tissues were washed with culture media (Dulbecco's Modified Eagle's and HAM'S F-12) through a cell strainer (BD Biosciences, San Jose, CA, USA) to remove blood cells. The cells from minced tissues were dissociated by collagenase digestion as described previously (Arikan and Yigit, 2001) with minor modifications. The cell isolation was performed by 4 successive 1 h incubations in aerated (O_2, 2 min) culture media (DMEM/F12) containing collagenase (0.03%, Type V), DNase (0.005%), bovine serum albumin (BSA, 0.5%) and antibiotic/antimicotic solution (1%) in an erlenmeyer flask on a shaking water bath (Julabo, Labortechnik GmbH, Seelbach, Germany, 90 cycles/min, 37 °C). Undigested tissue fragments were removed by filtering cells through a cell strainer supported by a glass funnel into a falcon tube (50 ml). The filtrate was then centrifuged (400 × *g*) to wash off the cell debris and chemicals, used in cell dissociation, for 5 min. All chemicals used in the cell dissociation were purchased from Sigma Chemical Company (Sigma-Aldrich, Co., Munich, Germany).

2.3. Identification of cells having steroidogenic activity

Luteal tissue is heterogeneous and consists of both steroidogenic and non-steroidogenic (fibroblast, immune cells, smooth muscle cells, etc.) cells. 70% of the cells of midluteal phase corpora lutea consist of nonsteroidogenic cells (O'Shea et al., 1989). Therefore, identification of cells having steroidogenic activity is crucial for excluding cells having no steroidogenic activity during cell counting. Biosynthesis of luteal progesterone depends on availability of 3β-hydroxysteroid dehydrogenase (3β-HSD) in cells. Thus, the cells having steroidogenic activity can easily be described by staining cells for 3β-HSD activity. Cells were stained for 3β-HSD as described previously (Arikan and Yigit, 2001). In brief, the cells were fixed in paraformaldehyde (1%) for 20 min at 37 °C. After removing paraformaldehyde cells were incubated in staining solution [phosphate buffered saline (PBS) containing 0.1% BSA, 1.5 mM NAD, 0.25 mM nitro blue tetrazolium and 0.2 mM 5β-androstene-3β-ol-17 one (prepared from 8 mM stock solution in ethanol) in the dark at 37 °C for 4 h.

2.4. Cell incubation

Luteal cells were cultured in 6-well cell culture plates (Corning Life Sciences, Cat no: 3516) in CO_2 incubator (BINDER GmbH, Germany) in the culture medium (DMEM/F12), containing newborn calf serum (10%) and antibiotic–antimycotic solution (1%) as described previously (Arikan and Rodway, 2000). In brief, the cells were incubated without treatment for 18 h and then with serum-free media containing 1% ITS premix (1 mg/ml insulin, 0.55 mg/ml transferrin, and 0.5 μg/ml sodium selenite) plus the specific fungicide treatments [0.01 mM, 0,1 mM, and 1 mM mancozeb dissolved in ethanol; 100 μM, 500 μM, and 2500 μM metalaxyl dissolved in ethanol; or 1 μM, 10 μM, and 100 μM tebuconazole dissolved in dimethyl sulfoxide (DMSO)] for additional 4 days. All treatment groups and controls were treated with 22(R)-hydroxycholesterol (22(R)-HC, 10 μg/ml), precursor for luteal biosynthesis of progesterone (Arikan and Yigit, 2009). The medium was replaced on day 1 and 3; and the retrieved medium was stored at −20 °C until progesterone assay by radioimmunoassay (RIA). Each fungicide dose was repeated 4 times.

2.5. Monitoring cell growth in culture

Additional to experimental and control groups, extra control cells were also cultured in 3 separate culture dishes for monitoring cell growth during first 3 days of the culturing period. The cells grown on the bottom surface of the tissue plates were stained for 3β-HSD activity on day 1–3 of the incubation as describe previously (Arikan and Yigit, 2009). The stained cells were examined on an inverted microscope (Olympus, Tokyo, Japan) to evaluate cell attachment, cell growth and cell-to-cell contact.

2.6. Progesterone measurement

Steroid concentrations in the media collected on day 3 and 5 of the incubation were measured by a commercial RIA kit (Beckman Coulter, Inc. Czech Republic) following the manufacturer's instruction. The intra- and interassay coefficients of variations were 4.1% and 7.2%,