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Assessment of cytogenetic damage in bovine peripheral lymphocytes exposed to *in vitro* tebuconazole-based fungicide

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HIGHLIGHTS

- Bovine blood cultures were exposed to tebuconazole-based fungicide *in vitro*.
- Cytogenetic biomarkers were used to assess the genotoxic potential.
- Proliferation indices were analysed for confirmation of cytoxicity.
- Fluorescence *in situ* hybridisation was applied for determination of aneuploidies.

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ABSTRACT

The tebuconazole-based fungicide was tested *in vitro* for its potential genotoxic and cytotoxic effects on cultured bovine peripheral lymphocytes. Following 24 h and 48 h of incubation, several cytogenetic endpoints were investigated such as: Chromosome Aberrations (CAs); Sister Chromatid Exchanges (SCEs); Micronuclei (MN); Mitotic Index (MI); Proliferation Index (PI); and Cytokinesis Block Proliferation Index (CBPI). The cultured lymphocytes were exposed to the fungicide formulation at concentrations of 3, 6, 15, 30 and 60 μg mL^{-1}. Statistical significant increases were seen in the CA assays at concentrations ranging from 6 to 30 μg mL^{-1} for 24 h. The higher doses caused a decrease or total inhibition of chromosome damages in comparison to the last active dose, or the control values. The Fluorescence *in situ* Hybridisation (FISH) technique was also used for the study of stable/unstable structural chromosomal aberrations and numerical aberrations of aneuploidy/polyploidy at the concentrations of 6 and 15 μg mL^{-1}. Under conditions of our study, no reciprocal translocations were detected. The more frequent types of aberrations were trisomies and monosomies; both have been identified in association with either bovine chromosome 5 or 7. No statistical significant value was seen in the induced MN; but, the clear, evident reduction of the CBPI was observed. Significant elevations of SCE were observed after the applications of the fungicide formulation at doses from 15 to 60 μg mL^{-1} in each donor for 24 h. The highest concentrations also caused a statistical significant decrease in the PI. The treatment for 48 h failed to exhibit any genotoxic activity of the fungicide.

1. Introduction

Tebuconazole is a very effective fungicide used for the control of mildew and rust on wheat, barley, rice, fruits and vegetables. It belongs to the group of triazole fungicides, which mode of action is by the inhibition of the activity of lanosterol 14α-demethylase (CYP51), resulting in membrane disruption and subsequent inhibition of cell growth (Ghannoum and Rice, 1999; Tully et al., 2006). Besides the inhibition of fungal enzymes, conazoles also have a potential to interact with the mammalian cytochrome P450 system (Menegola et al., 2006; Goetz et al., 2007; Kjaerstad et al., 2010).

Several studies have demonstrated that tebuconazole has the capability to disrupt the endocrine balance of organisms. Endocrine disruption is related to a mechanism of toxicity resulting in numerous adverse health impacts in animals, such as: lowered fertility; spontaneous abortions; neurobehavioral disorders; impaired immune function; or cancer (McKinlay et al., 2008).

No alterations in immunological or reproductive functions were found after the administration of tebuconazole to Sprague–Dawley dams at concentrations of 6, 20 and 60 mg kg^{-1} by oral gavages daily, from gestation day 14 to the postnatal day. However, the fungicide produced neurobehavioral deficits and neuropathology in the rats (Moser et al., 2001).

The metabolic pathway of tebuconazole was studied in lactating goats (*Capra hircus*) after the administration of the fungicide orally at dose levels of 3 or 15 mg kg^{-1} d^{-1}. The results showed that

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tebuconazole was metabolized either via oxidation to hydroxyl tebuconazole with further conjugation with glucuronic acid to form hydroxy tebuconazole glucuronide detected in the liver, kidney, fat, muscle and milk or via the conjugation of the parent compound with glucuronic acid to form tebuconazole glucuronide (in the liver and kidney only) (Pest Management Regulatory Agency, 2006).

Tebuconazole was found to be readily absorbed and excreted within 72 h of its administration (Pesticides Residues in Food, 2010). The acute toxicity was assessed to be low by the oral or dermal route, and mild by the inhalation route of exposure.

The mutagenic effects of tebuconazole were not confirmed in several *in vitro* bacterial mutation experiments using *Salmonella typhimurium* (TA 98, 100, 1535, 1537, 1538 strains) or *Escherichia coli* WP strains and *in vivo* cytogenetic mammalian assays (EPA, 1999). Because of no convincing evidence for direct DNA damage *in vivo* or *in vitro*, the fungicide was concluded as unlikely to be genotoxic. In contrast to these outcomes, tebuconazole (trade name Folicur) was established to be toxic to aquatic organisms (Sancho et al., 2010). Taking into account the possibility of absorbing dissolved pesticides, fish seem to be very sensitive organisms for investigating the possibility of detrimental health impacts. The authors documented that zebrafish (*Danio rerio*) exposed to a sublethal dose of tebuconazole, at the concentration of 230 μg L^{-1} for 7 and 14 d, exhibited alterations in lipid and carbohydrate metabolism and in several enzymatic activities, as well.

Furthermore, Toni et al. (2011) studied changes in the metabolism of carp (*Cyprinus carpio*) following their exposure to tebuconazole in both natural (rice field) and laboratory conditions. The authors demonstrated the increase in protein carbonyl content observed in fish liver that was a result of the inhibition of cytochrome P450-mediated steroid metabolism. Their results indicated that hydroxyl radicals ($OH^{\cdot}$) that have been generated in the specific process resulting in oxidative stress, were responsible for the formation of carbonyl groups in proteins and the subsequent alteration in their function. In other work, Ferreira et al. (2011) demonstrated hepatic cell injuries induced by sublethal concentrations of tebuconazole in *Rhamdia quelen* (Teleostei).

The teratogenic effect in rat embryos was described by Di Renzo et al. (2011) after the treatment of pregnant rats with tebuconazole at concentrations ranging from 62.5 to 250 M. After the exposure of *Xenopus laevis* embryo to the same fungicide concentrations, ectomesenchymal abnormalities were identified. Transcriptional analysis of liver tissue from genomic studies of triazole agents suggested that these compounds induce constitutive androstane (CAR) and pregnant X receptors (PXRs) (Goetz and Dix, 2009; Nesnow et al., 2009, 2011). Taxvig et al. (2007) reported that the predominant effect on rats exposured *in utero* to tebuconazole resulted in a feminisation of the male offspring and conversely, virilising effects in female foetuses. Based on the effects observed in a species from developmental studies, the possible health risks for an unborn child may be assumed (EFSA, 2008).

With regard to the possible oxidative stresses induced by tebuconazole leading to DNA damage, the aim of our study was to evaluate chromosomal changes and lymphocyte proliferation kinetics after the exposure of bovine peripheral lymphocytes to tebuconazole-based fungicide (trade name Orius 25 EW). Considering the human and animal exposures to the fungicide, the results of cytogenetic studies using cytogenetic endpoints such as: Chromosomal Aberrations (CAs); Micronuclei (MN); Sister Chromatid Exchanges (SCEs); Mitotic Index (MI); and Proliferation Indices (PIs and CBPI), could provide valuable data on the genotoxic and immunotoxic potential of the agent. Besides the conventional cytogenetic analysis, the Fluorescence *in situ* Hybridisation (FISH) technique was studied also in order to determine the stable and/or unstable structural chromosomal aberrations, and aneuploidy/polyploidy which may be caused by tebuconazole.

2. Material and methods

The tebuconazole-based fungicide, trade name Orius 25 EW (α-terc.butyl-α-{4-chlorophenylethyl}-1H-1,2,4-triazolyl-ethanol, 25% of active agent) was obtained from the Research & Plant Station, Slovak Republic (holder of registration – Makhteshim Agan Holding B.V., Rotterdam, Netherlands).

The agent was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and applied into culture flasks at concentrations of 3, 6, 15, 30 and 60 μg mL^{-1}. For the MN assays, a dose of 1.5 μg mL^{-1} was also used for treatments. The fungicide dose levels were chosen according to the cytotoxicity of the fungicide that had been identified at a concentration of more than 60 μg mL^{-1}. The final DMSO concentration in the treated and control cultures was 0.1%.

Ethylmethanesulfonate (EMS, Sigma, St. Louis, MO, USA, 250 μg mL^{-1}), mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4 μM) were used as positive control agents.

Experiments were carried out with two healthy bull donors (Slovak spotted cattle, 6 months old).

2.1. Cell cultivation

Whole blood cultures (0.5 mL) were cultivated for 72 h at 38 °C in 5 mL of RPMI 1640 medium supplemented with L-glutamine, 15 μM HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum, antibiotics (penicillin 250 U mL^{-1} and streptomycin 250 μg mL^{-1}), and phytohaemagglutinin (PHA, 180 μg mL^{-1}, Welcome, Dartford, England).

2.2. CA and SCE assays

Chromosome preparations were obtained by the standard cytogenetic method. Treatment times in all experimental conditions were performed for the last 24 h of incubation (tebuconazole-based fungicide was added to the experimental cultures at 48 h after the initial divisions) and at 48 h (tebuconazole-based fungicide was added to the experimental cultures at 24 h after the initial divisions). For the CA and SCE analyses, colchicine (Merck, Darmstadt, Germany) was added to all control and experimental flasks at a concentration of 5 μg mL^{-1} for 2 h before harvest. The cells were collected by centrifugation, hypotonized in 0.075 M KCl for 20 min and fixed in Carnoy solution (methanol/acetic acid 3:1 – v:v). The slides were prepared by an air-dried method and stained with 2% Giemsa solution.

For the SCE assay and the PI (cell cycle kinetics), bromodeoxyuridine (8 μg mL^{-1}, BrdUrd, Sigma, St. Louis, MO, USA) was added to all cultures 24 h after the initial divisions. Slides were stained with the FPG (Fluorescence Plus Giemsa) technique to differentiate sister chromatids and cell cycles as described previously (Šiviková and Dianovský, 2006).

One hundred metaphases were analysed for the detection of chromatid and isochromatid breaks (CB, IB) and chromatid and isochromatid exchanges (CE, IE). Two thousand cells per donor and at the different concentrations were scored in order to calculate the mitotic index.

Fifty differentially stained metaphases per donor and at the different concentrations were examined for SCE, and 100 metaphases were analysed for the determination of M_1, M_2 and M_{3+} mitotic divisions according to Lamberti et al. (1983).

2.3. MN assay

In the cytokinesis block micronucleus assay, cytochalasin B (Cyt. B, Sigma, St. Louis, MO, USA) at a concentration of 6 μg mL^{-1}