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Short Communication

Micronucleus assay in genotoxicity assessment

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Three different pesticide formulations were tested for micronuclei production and cell cycle arrest. Micronucleus test represents a suitable method for assessing chromosome damage because both chromosome breakage (clastogenicity) and chromosome loss (aneugenicity) can be measured simultaneously. In the experiments for 24 and/or 48 h exposure, no significant increase ($P < 0.05$) in MN frequency was found in comparison with negative control. On the contrary, the values of CBPI (cytochalasin blocked proliferative index) were significantly reduced in a dose dependent manner ($P < 0.05$, $P < 0.01$, $P < 0.001$) for both exposure time. Our results indicated an expressive cytotoxic effect of various pesticides in cultivated bovine peripheral lymphocytes.

Keywords: cell proliferation, cytotoxicity, genotoxicity, micronucleus, pesticide

1 Introduction

Pesticides are a significant source of environmental pollution due to their wide spectrum use in agriculture and forestry for controlling the spread of pests. Triazole fungicides (e.g. tebuconazole, prothioconazole, epoxiconazole etc.) are used as clinical drugs for the treatment and protection of cereals, soybeans and a variety of fruits. Their antifungal effect is based on the specific inhibition of CYP51, a sterol 14-α-demethylase that catalyzes the demethylation of lanosterol in the ergosterol biosynthesis pathway in fungi. Morpholine fungicides (e.g. fenpropimorph) act as inhibitors of the Δ8-Δ7 isomerase and Δ8, 14 sterol reductase, which results in the ergosterol biosynthesis impairment. Neonicotinoid insecticides (e.g. thiacloprid) provide effective control of a broad spectrum of sucking and chewing pests. They act as agonist of the post-synaptic nicotinic acetylcholine receptors, thus disturbing signal transmission in the insect's nervous system and potentially in mammals.

In this paper we present the effects of three different pesticide formulations on bovine peripheral lymphocytes *in vitro*, assessed by micronucleus test and cytochalasin-blocked proliferation index (CBPI) determination. Analysis of micronuclei (MN) frequencies serves as a biomarker of chromosomal damage for genotoxicity testing and biomonitoring studies (Decordier et al., 2009). This method allows detection of both clastogenic and aneugenic effects of chemical agents (Norppa and Falck, 2003).

2 Material and methods

2.1 Test samples, cell cultivation and chemicals

Blood was taken from 2 calves (Holstein crossbreed cattle, 6 months old, 150-200 kg) and was cultivated for *in vitro* MN assay according to Schwarzbacherová et al. (2015). Briefly, the whole blood was added to standard cultivation medium and cultivated for 72 h at 37 °C. Lymphocytes were treated with the pesticide for the last 24 and/or 48 h for induction of MNs. Two fungicides and an insecticide were used in the experiments. Prosaro 250 EC (125 g l^{-1} tebuconazole, 125 g l^{-1} prothioconazole), Tango Super (84 g l^{-1} epoxiconazole, 250 g l^{-1} fenpropimorph) and Calypso 480 SC (480 g l^{-1}) were added in to the cultures at concentrations: 1.5; 3; 7.5; 15; 30 µg ml^{-1} for Prosaro®, 0.5; 1.5; 3; 6; 15 µg ml^{-1} for Tango® and 30; 60; 120; 240; 480 µg ml^{-1} for Calypso®. Dimethylsulfoxide (DMSO; for Prosaro® and Tango®) at a final concentration of 1 % and water (for Calypso®) were used as dissolvent and negative controls.

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2.2 MN assay

To block cytokinesis, cytochalasin-B (Sigma Aldrich, USA) at the final concentration of 6 µg ml^{-1} was added to all cultures, 44 h after initiation of division. One thousand binucleated (BN) cells per donor and concentration were scored for micronuclei under the criteria for MN evaluation according to Fenech et al. (2003). At least 500 cells were scored for CBPI. The statistical analysis was performed using the chi square (χ2) test for MN induction and CBPI.

3 Results and discussion

The frequencies of MN and CBPI in bovine peripheral blood lymphocytes after exposure to the pesticides formulations for 24 and 48 h are presented in Fig. 1A, B and C. Some results have been published in works of Schwarzbacherová et al. (2015) and Galdíková et al. (2015). No statistically significant increases in the MN formation were found after exposure to all pesticides for both exposure times except the highest concentration of Calypso® (480 µg ml^{-1}; Fig. 1C). Similarly, negative results in MN frequencies were documented by numerous authors after exposure to several pesticides; e.g. after treatment of bovine peripheral lymphocytes with tebuconazole formulation (Orius®) (Šiviková et al. 2013); or human lymphocytes and rat bone marrow with imidacloprid formulation (Demsia et al., 2007), as well. It is assumed that MN frequencies can often be detected at lower levels than those seen for chromosome aberrations because not all fragments necessarily form a visible micronucleus (Savage, 1988).

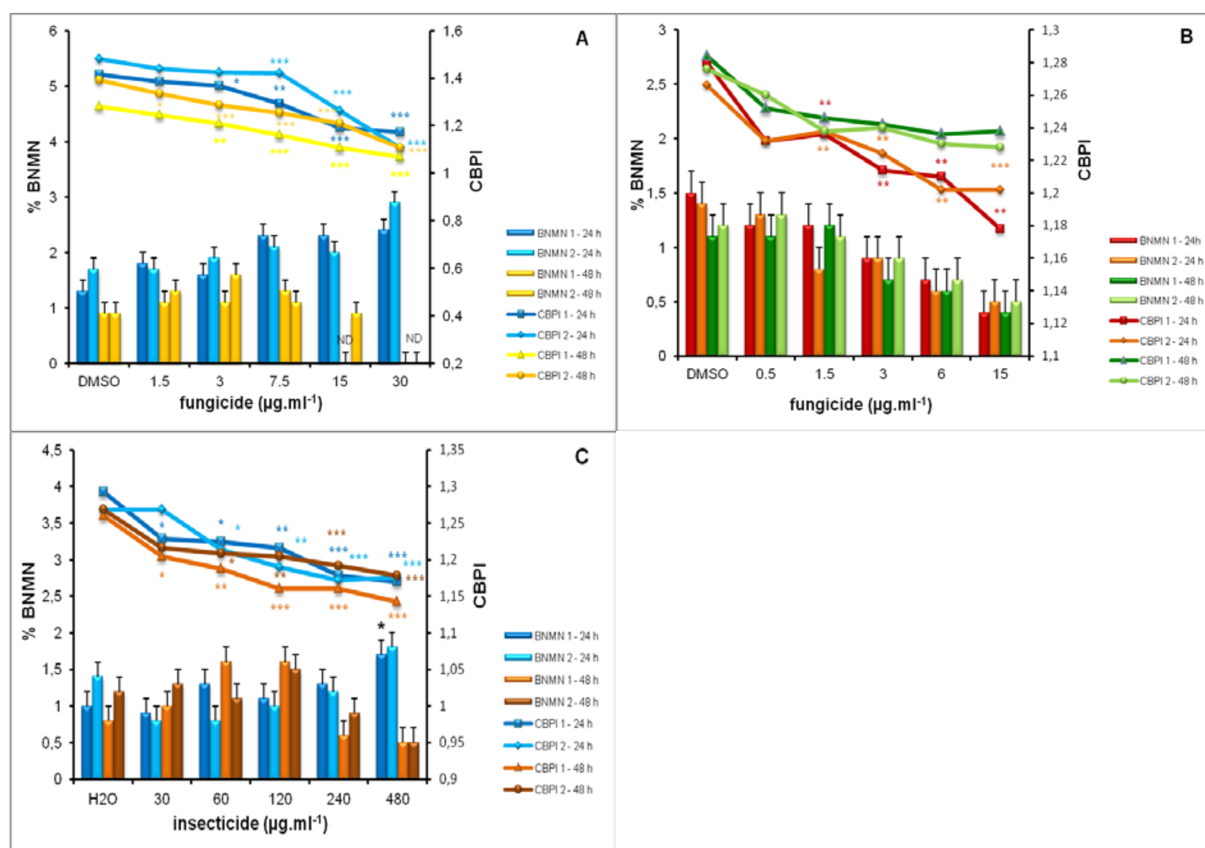

Figure 1 Induction of MN in bovine peripheral lymphocytes exposed to triazole fungicide Prosaro® (A), triazole-morpholine fungicide Tango® (B) and neonicotinoid insecticide Calypso® (C) for 24 and 48 h. BNMN, binucleated cell with MN; CBPI, cytokinesis block proliferation index; DMSO, dimethylsulfoxide, ND, not done, incomplete number of cells. Statistical significant data (χ2 test): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A reduction of CBPI was observed after exposure to all fungicides tested in a dose-dependent manner ($P < 0.05$; $P < 0.01$ and $P < 0.001$) for 24 h. The same results in the inhibition of proliferation was obtained after the prolonged time of exposure (48 h) except the results obtained with Tango® (Fig. 1B).

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Moreover, a total inhibition of proliferation activity was obtained after exposure of bovine lymphocytes to Prosaro® 250EC at the highest concentrations (15 and 30 µg ml^{-1}; Fig. 1A)

One explanation for the inhibition of cell proliferation could be the formation of reactive oxygen species (ROS) (Kocaman and Topaktas, 2007) as a result of oxidative stress. Another explanation for the antimitotic effect of tested fungicides could be in the inhibition of enzymes, which are more directly involved in spindle production, assembly and orientation (Hidalgo et al., 1989). Mitotic slippage as well as apoptosis and necrosis can change the number of cells that undergo mitosis, and thus lower frequency of binucleated cells could be analysed. Disorders of cell division and mitotic spindle apparatus could lead to aneuploidy through chromosome gain or loss. Animal aneuploidy is associated with reduced fertility/or increased incidence of developmental defects and birth defects. It is also one of the most important steps of carcinogenesis.

4 Conclusions

In conclusion, no genotoxic and clastogenic effect was obtained. Our results indicate that all tested pesticides are able to exert a clear cytotoxic effect in cultured bovine peripheral lymphocytes.

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