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# EVALUATION OF THE GENOTOXIC EFFECT OF THE COMMERCIAL FUNGICIDE TANGO® SUPER ON BOVINE LYMPHOCYTES

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## ABSTRACT

**This study investigated the potential genotoxic effects of the fungicide Tango® Super using methods of conventional cytogenetic analysis, fluorescence *in situ* hybridization (FISH) and detection of DNA fragmentation in bovine lymphocytes. After exposure of two donor cell cultures to several concentrations of fungicide (0.5, 3.0 and 15.0 mg.ml$^{-1}$ for conventional cytogenetic analysis; 0.5 and 3.0 mg.ml$^{-1}$ for FISH) we detected the insignificant occurrence of chromosome and chromatid breakages. In both donors we observed a significant decrease in mitotic index (MI) percentage with increasing concentrations of fungicide ($P < 0.01$; $P < 0.001$), which indicated a cytotoxic effect of the preparation. Electrophoretic analysis of DNA fragmentation in lymphocytes exposed to increasing concentrations (0.5; 1.5; 3.0; 6.0 and 15.0 mg.ml$^{-1}$) of this preparation showed its ability to induce formation of fragments, which is a characteristic manifestation of the last stage of apoptosis.**

**Key words: chromosomal analysis; DNA fragmentation; fluorescence *in situ* hybridization; lymphocyte culture; systemic fungicide**

## INTRODUCTION

Pesticides intended for the protection of plants against pests form the largest group of toxic chemical substances introduced into the environment. In addition to the load on the environment, these substances can also raise risk to health of humans and other animals [4, 5, 6].

Tango® Super is a spray fungicide with two active ingredients, fenpropimorph and epoxiconazole, with systemic and contact effects. It is used in the form of an emulsion to control leaf and spikelet diseases of cereals. This commercial preparation is known for various undesirable effects on humans and other animals. However, the genotoxic effects of the fungicide Tango® Super and its influence on farm animal's genetic material have not been investigated sufficiently.

Fenpropimorph belongs to the group of morpholins and inhibits the biosynthesis of sterols, compounds essen-

tial to fungi [1]. It has been assumed that this compound is not carcinogenic to man but experiments have shown that it induces malformations in rats (cheilognathopalatoschisis) and rabbits (skeletal retardations) [8]. Epoxiconazole belongs to the group of triazoles and affects budding of spores, growth of infectious mycelium and its branching. Studies revealed its effect on laboratory rats resulting in a significant increase in cholesterol and high levels of its residues in the liver, kidneys and testes [8].

The objective of our study was to evaluate the potential genotoxic effects of the fungicide Tango® Super by means of conventional cytogenetic analysis, fluorescence *in situ* hybridization (FISH) and detection of DNA fragmentation in bovine lymphocytes.

## MATERIALS AND METHODS

Our study was conducted in order to test the commercial fungicide Tango® Super (BASF SE, Germany), containing two active ingredients, fenpropimorph (cis-2,6-dimethyl-4-{2-methyl-3-[4-(2-methyl-2-propanyl)phenyl]propyl} morpholine; 250 $g.l^{-1}$) and epoxiconazole (2RS,3SR)-1-[3-(2-chlorophenyl)-2,3-epoxy-2-(4-fluorophenyl)-propyl]-1H-1,2,4-triazole; 84 $g.l^{-1}$).

Heparinized blood collected from two young healthy bulls was cultivated in 5 ml of the cultivation medium RPMI 1640 (Sigma, St. Louis, MO, USA), enriched with 15 % bovine foetal serum (BOFES, Sigma, Chemical Co. St. Louis, MO, USA), growth factors, antibiotics and an antimycotic (penicillin 100 $U.ml^{-1}$ a streptomycin 100 $\mu g.ml^{-1}$, amphotericin B 0.25 $\mu g.ml^{-1}$), and phytohaemagglutinin (PHA, 180 $\mu g.ml^{-1}$, Welcome, Dartford, England). Twenty four hours before the termination of the cultivation we added the fungicide Tango® Super to the cell culture of lymphocytes in concentrations of 0.5, 3.0 and 15.0 $mg.ml^{-1}$. Fifty minutes before the end of cultivation, we added the inhibitor colchicine at a dose of 5 $\mu g.ml^{-1}$ (Merck, Darmstadt, Germany). Ethyl methane sulphonate (EMS, Sigma, St. Louis, MO, USA) in the concentration of 250 $\mu g.ml^{-1}$ was used as a positive control and dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) in concentration of 0.1 % as a negative one.

The preparations intended for conventional cytogenetic analysis were stained with a 3 % solution of Giemsa stain diluted with phosphate buffer pH 7. A light microscope (Nikon, ECLIPSE E200) was used to evaluate the chromosome and chromatid breakages in 100 well-distributed chromosomes in metaphase cells. We evaluated also the mitotic index (MI), a ratio between the cells in metaphase and 1 000 cells that did not undergo division.

The statistical evaluation of the MI reduction and induction of chromosomal aberrations by the pesticide was carried out by c2 method.

For the fluorescence *in situ* hybridization, 15 min before the termination of pre-hybridization, the preparation was placed into a denaturation solution (70 % formamide/2xSSC), dehydrated in alcohol sequence (70 %, 80 %, 96 % ethanol) and then a hybridization mixture prepared by mixing hybridization buffer MM with salmon DNA, DNA isolated from calf thymus and whole chromosome probes (BTA5 a BTA1) was pipetted onto the preparation. The preparation was then stained with 10 µ DAPI (4´,6´-diamidino-2-fenolindol) and stable chromosomal aberrations of the type of reciprocal and non-reciprocal translocations were evaluated under a fluorescence microscope (NIKON LABOPHOT 1A/2, fluorescence filter FITC/TRITC).

For electrophoresis, DNA was isolated by means of the Apoptotic DNA Ladder kit (Roche Diagnostics GmbH, Mannheim, Germany). The DNA was analysed by electrophoresis in 1 % agarose solution (90 min., 75 V, buffer 1x TAE; TRIS-acetate-EDTA), stained with Gel RedTM (Biotium) and the results were documented by means of the Gel documentation system D1-HD (Major Science).

## RESULTS

After 24 h exposure of lymphocyte cultures to various concentrations of fungicide Tango® Super (0.5, 3.0 and 15.0 $mg.ml^{-1}$), the conventional cytogenetic analysis showed the presence of unstable aberrations involving chromatid and chromosome breakages. The increased frequency of chromatid breakages was observed in both donors but the increase in chromosome breakages was detected only in one donor. Separately we investigated formation of chromatid and chromosomal gaps the frequency of which increased in both donors starting from the 3.0 $mg.ml^{-1}$ concentration of fungicide. Although the increase in frequency of breakages was insignificant, evaluation of the mitotic index (MI) showed a significant decrease in mitotic index percentage with increasing concentration of the fungicide in both

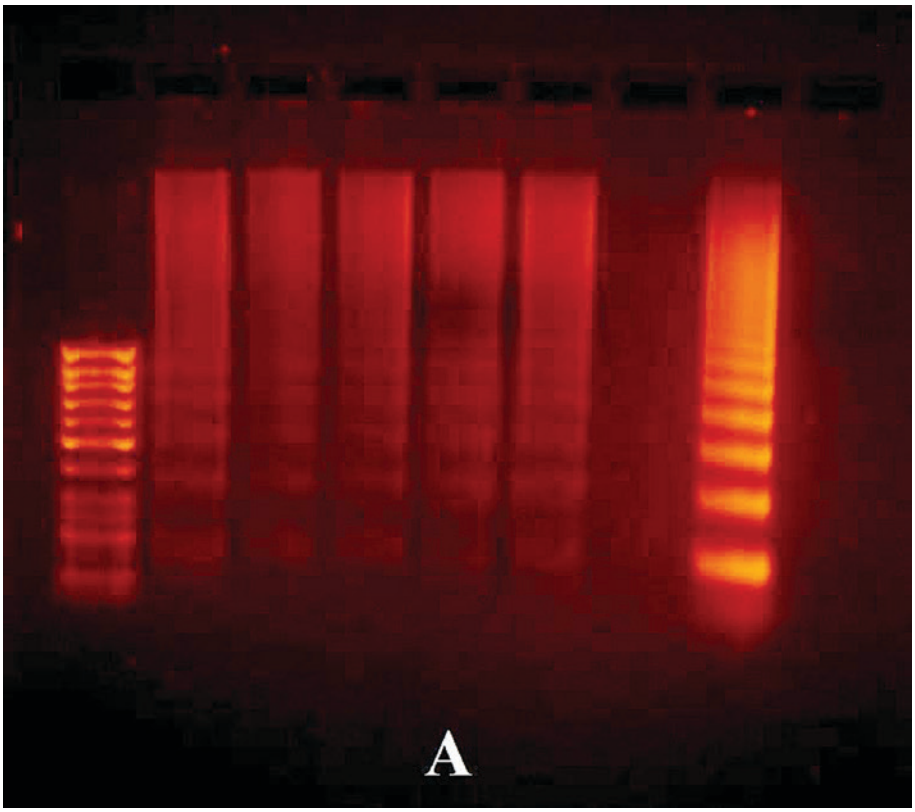
**Table 1. Unstable chromosome aberrations and gaps in cultivated bovine peripheral lymphocytes after 24h exposure to fungicide Tango® Super. Results were obtained by conventional cytogenetic analysis.**

| Dose | Number of metaphases | Gaps | | Breakages | | Exchanges | | % B (± SD) | % G + B (± SD) | % MI |
|---|---|---|---|---|---|---|---|---|---|---|
| | | G I. | G II. | CB | IB | CE | IE | | | |
| **DONOR 1** | | | | | | | | | | |
| **Negative control (DMSO)** | 100 | 1 | 0 | 1 | 2 | 0 | 0 | 2.0 ± 0.14 | 4.0 ± 0.2 | 3.5 |
| **Tango® Super concentration** | | | | | | | | | | |
| **0.5 µg.ml$^{-1}$** | 100 | 1 | 1 | 3 | 1 | 0 | 0 | 4.0 ± 0.20a | 6.0 ± 0.24a | 2.9 |
| **3.0 µg.ml$^{-1}$** | 100 | 4 | 3 | 2 | 1 | 0 | 0 | 3.0 ± 0.17a | 10.0 ± 0.30a | 1.5** |
| **15.0 µg.ml$^{-1}$** | 100 | 3 | 5 | 4 | 1 | 0 | 0 | 5.0 ± 0.22 a | 13.0 ± 0.34* | 1.1*** |
| **Positive control (EMS 250 µg.ml$^{-1}$)** | 100 | 6 | 2 | 15 | 4 | 1 | 0 | 21 ± 0.40*** | 29 ± 0.45*** | 1.2*** |
| **DONOR 2** | | | | | | | | | | |
| **Negative control (DMSO)** | 100 | 3 | 0 | 1 | 0 | 0 | 0 | 1.0 ± 0.1 | 4.0 ± 0.2 | 3.3 |
| **Tango® Super concentration** | | | | | | | | | | |
| **0.5 µg.ml$^{-1}$** | 100 | 2 | 1 | 1 | 2 | 0 | 0 | 3.0 ± 0.17a | 6.0 ± 0.24a | 2.6 |
| **3.0 µg.ml$^{-1}$** | 100 | 5 | 1 | 1 | 2 | 0 | 0 | 3.0 ± 0.17a | 9.0 ± 0.29a | 1.6* |
| **15.0 µg.ml$^{-1}$** | 100 | 4 | 4 | 2 | 1 | 0 | 0 | 3.0 ± 0.17a | 11.0 ± 0.31a | 1.2** |
| **Positive control (EMS 250 µg.ml$^{-1}$)** | 100 | 5 | 2 | 14 | 5 | 0 | 0 | 19.0 ± 0.39*** | 26.0 ± 0.43*** | 1.3*** |

a — insignificant; *, **, *** – significant at the levels P < 0.05, P < 0.01, P < 0.001, respectively; G1, G2 — chromatid and chromosome gaps; CB, IB — chromatid and chromosome breakage; CE, IE — chromatid and chromosome exchange; MI — mitotic index

**Table 2. Frequency of aberrations in cultivated bovine peripheral lymphocytes after 24 h exposure to the fungicide Tango® Super. Results were obtained by fluorescence *in situ* hybridization**

| | Number of metaphases | Fragmentation BTA5 | Fragmentation BTA1 | Separation in centromere BTA5 | Separation in centromere BTA1 | Monosomy BTA5 | Monosomy BTA1 |
|---|---|---|---|---|---|---|---|
| **DONOR 1** | | | | | | | |
| **Control (DMSO)** | 250 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Tango® Super concentration** | | | | | | | |
| **0.5 µg.ml$^{-1}$** | 250 | 1 | 1 | 0 | 0 | 0 | 0 |
| **3.0 µg.ml$^{-1}$** | 250 | 1 | 0 | 0 | 0 | 0 | 1 |
| **DONOR 2** | | | | | | | |
| **Control (DMSO)** | 250 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Tango® Super concentration** | | | | | | | |
| **0.5 µg.ml$^{-1}$** | 250 | 1 | 0 | 0 | 0 | 0 | 0 |
| **3.0 µg.ml$^{-1}$** | 250 | 0 | 0 | 2 | 0 | 0 | 0 |



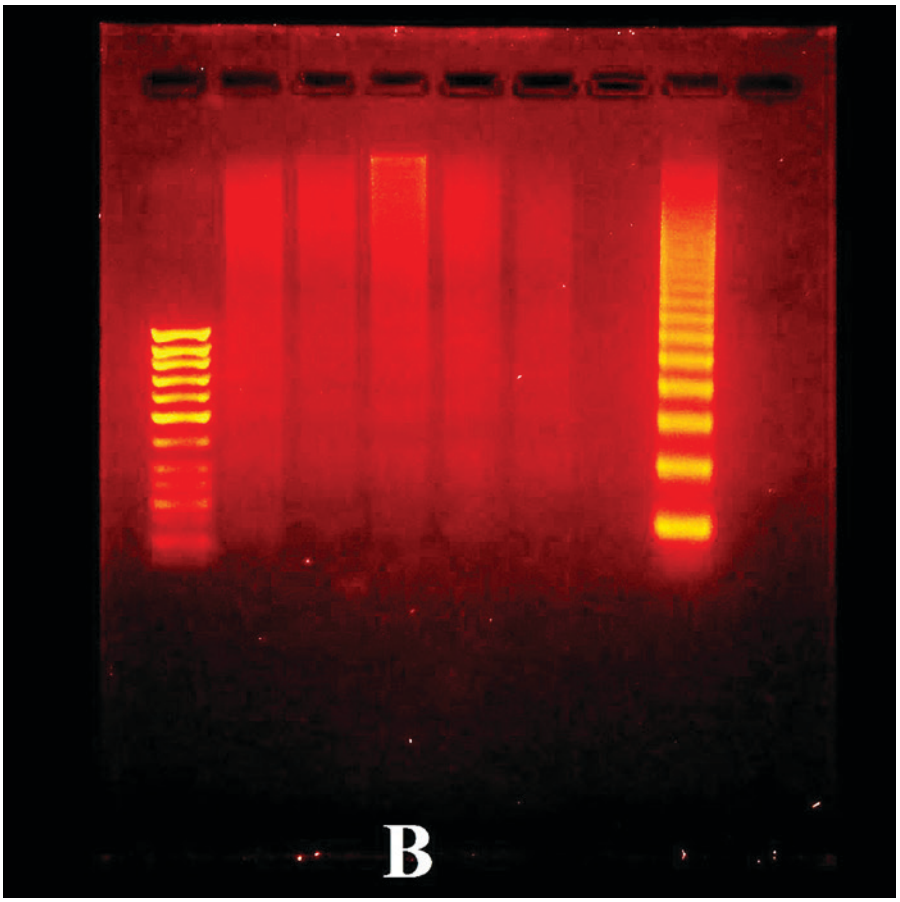




**Fig. 1. Fragmentation of DNA in cultivated bovine lymphocytes exposed to the tested fungicide**
Patways from the left: standard of molecular weight 100 bp, Tango® Super 0.5; 1.5; 3.0; 6.0; 15.0 mg.ml$^{-1}$; negative control DMSO; positive control — U937 cells treated by camptothecin 4 mg.ml$^{-1}$; negative control — water; A — donor 1, B — donor 2

donors ($P < 0.01$; $P < 0.001$). The changes observed in the mitotic index can be considered a manifestation of cytotoxic properties of the preparation. The frequency of chromosomal aberrations and % MI in cultivated lymphocytes following the 24 h exposure to fungicide Tango® Super are presented in Table 1.

In the second part of our study, we investigated the effect of Tango® Super (0.5 a 15.0 $mg.ml^{-1}$) by means of fluorescence *in situ* hybridization, using whole-chromosome probes BTA1 (green fluorescence stain) and BTA5 (red fluorescence stain) for the detection. In the lymphocytes from the first bull, both concentrations of the fungicide caused the production of fragments which formed separate groups. Their development was considered proof that the preparation causes breakages. BTA1 monosomy was observed after exposure to the concentration of 3.0 $mg.ml^{-1}$. In lymphocytes from the second bull, the concentration of 0.5 $mg.ml^{-1}$ produced one fragment and 3.0 $mg.ml^{-1}$ induced two separations in the centromere which were categorised again as a specific aberration. The frequency of aberrations after 24 h exposure to the fungicide Tango® Super is presented in Table 2.

In the third part of our study we used electrophoretic analysis of DNA fragmentation in agarose gel and found out that all tested concentrations of the fungicide (0.5; 1.5; 3.0; 6.0; 15.0 $mg.ml^{-1}$) were able to induce fragments (Fig. 1).

## DISCUSSION

Thee genotoxic effects of the fungicide Tango® Super on the health of humans and other animals have not yet been studied sufficiently. Conventional cytogenetic analysis used in our study failed to confirm the genotoxic effect of the tested fungicide as no significant dose-dependent increase in chromosomal aberrations was observed in comparison with the negative control (DMSO). Our results are supported by the findings of Galdíková et al. [3] who failed to observe a significant clastogenic effect of this preparation on bovine lymphocytes. Schwarzbacherová et al. [9] conducted experiments with Tango® Super and reported that it induced apoptosis already at a concentration of 1.5 $mg.ml^{-1}$, with the highest proportion of apoptotic cells occurring between concentrations 3.0 and 6.0 $mg.ml^{-1}$. Similar to the above study, our results obtained by electrophoretic separation of characteristic DNA fragments allowed us to assume that the fungicidal preparation is capable of inducing apoptosis. The formation of fragments (DNA laddering) is a typical feature of degradation of DNA by DNases that are activated by caspases in the key phase of apoptosis [2]. The test of DNA fragmentation is considered a simple and rapid method of evaluation of cell apoptosis [7].

## CONCLUSIONS

When evaluating the potential genotoxic effect of various types of fungicides, it is important to investigate their lowest concentrations as their long-term use may result in development of tumour diseases. The conventional cytogenetic method used in our study indicated a significant dose-dependent decrease in the percentage of the mitotic index and the presence of unstable aberrations. By means of the FISH method, we evaluated the formation of DNA fragments, separation in the centromere, and monosomy. Electrophoretic analysis allowed us to detect DNA fragmentation induced by all concentrations, probably due to apoptosis.

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## REFERENCES

1. **Bjornlund, L., Ekelund, F., Christensen, S., Jacobsen, C. S., Kroh, P. H., Johnsen, K. , 2000:** Interaction between saprotrophic fungi, bacteria and protozoa on decomposing wheat roots in soil influenced by the fungicide fenpropimorph (Corbelâ): a field study. *Soil Biol. Biochem.*, 32, 967—975.
2. **Elmore, S., 2007:** Apoptosis: A review of programmed cell death. *Toxicol. Pathol.*, 35, 495—516.
3. **Galdíková, M. et al., 2014:** Chromosomal aberrations induced by fungicide Tango® Super in bovine peripheral lymphocytes. *Priemyselná toxikológia (Industrial Toxicology)* (In Slovak). Slovak Technical University, Bratislava, 33—37.
4. **Garaj-Vrhovac, V., Zeljezic, D., 2002:** Assessment of genome damage in a population of Croatian workers employed in pesticide production by chromosomal aberration analysis,

Micronucleus assay and Comet assay. *J. Appl. Toxicol.*, 22, 249—255.

5. **Matušova, M., Ďurčanska, J., 2010:** Evaluation of official control of pesticide residues in foodstuffs in 2009 (In Slovak). *Slovenský veterinársky časopis* (Slovak Veterinary Journal), 35, 186—191.
6. **Pistl, J., Kovalkovičová, N., Legáth, J., Mikula, I., Holovská, V., 2004:** *Immunotoxicology in Veterinary Medicine* (In Slovak). 1st edn., University of Veterinary Medicine, Košice, 154 pp.
7. **Saadat, Y. R., Saeidi, N., Zununi Vahed, S., Barzegari, A., Barar, J., 2015:** An update to DNA ladder assay for apoptosis detection. *Bioimpacts*, 5, 25—28.
8. **Schmidt, F., Marx-Stoelting, P., Haider, W., Heise, T., Kneuer, C., Ladwig, M. et al., 2016:** Combination effects of azole fungicides in male rats in a broad dose range. *Toxicology*, 355—356, 54—63.
9. **Schwarzbacherova, V., Wnuk, M., Lewinska, A., Potocki, L., Zebrowski, J., Koziorowski, M., 2017:** Evaluation of cytotoxic and genotoxic activity of fungicide formulation Tango® Super in bovine lymphocytes. *Environ. Pollut.*, 220, 255—263.

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