Induction of SCEs and DNA fragmentation in bovine peripheral lymphocytes by *in vitro* exposure to tolyfluanid-based fungicide

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**Abstract**

The potential for genotoxic and cytotoxic effects of tolyfluanid-based fungicide (50% active agent) was evaluated using sister chromatid exchange (SCE) and proliferation indices (PI) in cultured bovine peripheral lymphocytes. For the detection of possible genetic damage, DNA fragmentation assay was also applied. Bovine lymphocytes cultured for 72 h were treated with the fungicide at the final concentrations of 1.75, 3.5, 8.75, and 17.5 μg/mL for the last 24 and 48 h of culture without S9 metabolic activation, and during the last 2 h of culture with S9 metabolic activation.

In the SCE assays no evidence for genotoxic activity of the fungicide was found in treatments of 24 h without and 2 h with S9. After the 24 h exposure to tolyfluanid, a weak decrease in the PI was observed. With the prolonged exposure time (48 h), dose dependence in the increase of SCE frequencies was observed. Moreover, after 48 h exposure slight fragmentation of DNA at the concentrations of 3.5 and 8.75 μg/mL was demonstrated. SCE quantification is the most widely used approach for the assessment of genotoxic/cytogenetic effects of chemical compounds. Positive results in the assay at 48 h exposure indicated a potential of the fungicide to increase frequency of chromosomal damage (replication injuries) that is the confirmation of early effect of exposure.

**Key words:** tolyfluanid-based fungicide, sister chromatid exchanges, DNA fragmentation.

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**Introduction**

Tolylfluanid is a member of the phenylsulfamide group of fungicides and it has been applied in agriculture for control of fungal diseases during thinning, pruning, and harvesting fruits and vegetables (Tielemans et al., 1999, Stajnbaher and Zupancic-Kralj, 2008). Fungicides such as tolyfluanid, captan, cyprodinil, tebuconazole, etc., tend to be more often detected in vegetables and fruits than are insecticides or herbicides (Looser et al., 2006, Basa Cesnik et al., 2006, Tichá et al., 2008, Knezevi et al., 2009). Tolylfluanid has also been used as an alternative antifouling compound (Bellas, 2006, Links et al., 2007, Takahashi 2009).

The acute toxicity of the fungicide was assessed to be low for mammalian cells and it was established as a readily absorbed and rapidly hydrolysed agent within 48 h (Pesticide Residues in Food – Joint FAO/WHO Meeting on Pesticide Residues, 2002). As regards genotoxic activity, tolyfluanid was reported as weakly clastogenic in Chinese hamster V79 cells in the presence of S9 metabolic activation. In mammalian chromosome aberration assay *in vitro* with and without S9 the cytotoxic effect of the fungicide was confirmed at concentrations of 1 to 10 μg/mL (-S9) and 5 to 10 μg/mL (+S9). On the basis of these findings, tolyfluanid was recognized by the United States Environmental Protection Agency (EPA, 2002) as a non-genotoxic agent both to bacteria and mammal cells. In further studies using Crg96 mutants of *Pseudomonas corrugate* (defective in the RecG gene encoding a RecG helicase); Decorosi et al. (2009) described an increased sensitivity of this mutant strain to the fungicides captan and tolyfluanid. Crg96 mutant was sensitive to numerous genotoxic compounds that are known to induce inhibition of DNA replication.

Several recent studies demonstrated the adverse effects of persistent exposure to pesticides including tolyfluanid and the related dichlofluanid on the integrity of immune (Pistl et al., 2003), nervous, endocrine (Rhind, 2002, Odermatt et al., 2006), and reproductive systems (Costa et al., 2006).

The results on possible cytogenetic and genotoxic effects of tolyfluanid in animal or human cells are insufficient or incomplete. Genotoxic potential of chemical agents represents a primary risk factor for long-term effects such as carcinogenic or reproductive disorders (Bolognesi, 2003). Pauluhn (2004) investigated acute lung edema induction in rats after exposure to naphthylene disocyanate aerosols and tolyfluanid. They could show that protein concentration in bronchoalveolar lavage fluid, a sensitive
early marker of lung edema was increased in rats exposed to tolylfluanid solid aerosol in size less than 3 μm.

The aim of our research was to investigate early effects of exposure to tolylfluanid-based fungicide in cultured bovine peripheral lymphocytes using sister chromatid exchanges (SCE) and proliferation indices (PI) to assess the cell cycle delay/cytotoxicity. Besides the cytogenetic markers, fragmentation of DNA as a prominent feature of apoptosis was investigated to evaluate the possible effect of tolylfluanid-based fungicide in the induction of apoptosis in peripheral lymphocytes of cattle. These parameters were analyzed because an indirect genotoxic mechanism (e.g., oxidative stress), or a mechanism involving significant cytotoxicity leading to compensatory increase in cell proliferation are considered to be a limiting step in tumorigenesis (Benford et al. 2010). Furthermore, apoptotic cell death is reflected by the degradation of genomic DNA by an endogenous endonuclease. (López Nigro and Carballo, 2008).

Material and Methods

The tolylfluanid formulation fungicide (1,1-dichloro-N-[[(dimethylamino) sulfonyl]-1-fluoro-N-(4-methylphenyl) methanesulfonamide (Bayer AG, Germany), containing 50% active agent, was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and added to culture media at the final concentrations of 1.75, 3.5, 8.75, and 17.5 μg/mL. The fungicide dose levels were chosen beforehand, taking into account the highest concentration for testing (17.5 μg/mL) causing a reduction in the mitotic index (MI > 50%).

Mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4 μM) and cyclophosphamide (CPA, Jenapharm, Ankerwerk, Rudolstadt, Germany, 4 μg/mL) were used as positive control agents in the assays with absence and presence of the metabolic activation (S9 mix), respectively.

Blood was obtained from two healthy young bulls (Slovak spotted cattle, 6 months old). For the SCE assays, experiments were made both in the presence and in the absence of the S9 mix. A freshly prepared S9 fraction (10% of the culture volume) from Aroclor 1254 (Supelco, Bellefonte, PA, USA) induced mice was prepared according to the method of Maron and Ames (1983) and applied to the SCE assays. DNA ladder assay was performed by means of the Apoptotic DNA Ladder Kit (Roche Diagnostics GmbH, Mannheim, Germany). Lymphocyte cultures without and with DMSO (0.1%) were used as negative controls. Apoptotic U937 cells of the kit (cell cultures treated with 4 μg/mL camptothecin for 3 h) served as a positive control. After incubation, the cells were collected by centrifugation at 500 g for 10 min. The supernatant was removed and the cell pellets were washed twice with PBS buffer (pH 7.2). DNA was isolated from a 200-300 μL sample as specified according to the manufacturer’s protocol. Briefly, after lysis of cultured cells in binding/lysis buffer (6 mM guanidine-HCl, 10 mM urea, 10 mM TRIS-HCl, 20% TritonX-100, pH 4.4), the lysate was applied to a filter tube and passed through centrifugation through the glass fiber fleece of this tube. Nucleic acid specifically binding to the surface of the glass fibres, was washed with 20 mM NaCl and 2 mM TRIS-HCl (pH 7.5) to clean the cellular impurities off the fleece and eluted using 10 mM TRIS elution buffer (pH 8.5) at 70 °C. The extracted DNA was analysed by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide (Merck, Darmstadt, Germany), examined under ultraviolet (UV) light, and photographed.
Statistical analysis

Analysis of variance (ANOVA) was used for statistical analysis followed by the Student's t-test for the comparison of SCE occurrence between treated and untreated groups. A χ² test was applied to evaluate cell cycle delay.

Results

Cytogenetic studies

Tables 1 to 3 show the data obtained from the cytogenetic experimental conditions: 24 h, 48 h, and 2 h exposure to tolylfluanid-based fungicide in cultured bovine lymphocytes without and with metabolic activation.

In the SCE study for 24 h, no dose dependence in the increases of sister chromatid exchanges was observed. A statistically significant elevation in chromosomal damage was seen after the fungicide exposure at the highest concentration (17.5 µg/mL, p < 0.01, Student's t-test) in both of the donors, however only an insufficient number of cells could be analyzed in the donor 1. A weak decrease (p < 0.05) in proliferation indices (PI) was observed at the fungicide concentration range 3.5-17.5 µg/mL. More expressive inhibition in the proliferation activity of tolylfluanid-based fungicide was seen in the donor 1 after the exposure to the highest concentration (p < 0.01, Table 1).

The prolonged time of exposure caused significant elevations in SCE frequencies with a dose dependence. However, a decrease in proliferation indices and the induction of cell cycle delays was not denoted (48 h, Table 2).

Similarly, in the SCE assay with 2 h exposure for metabolic activation no statistically significant increase in the SCE frequency was found. A weak decrease in proliferation indices was observed at the highest concentration of the fungicide (Table 3).

DNA fragmentation and cell death evaluation

At a 24 h exposure to tolylfluanid-based fungicide, no DNA fragments were recorded in bovine lymphocyte cultures at any concentration tested. On the contrary, a slight fragmentation of DNA was visible after the prolonged time of exposure to the fungicide. Nucleosomal laddering of cultivated lymphocyte was demonstrated at the concentrations of 3.5 and 8.75 µg/mL (Figure 1).

### Table 1 - Frequency of SCEs and proliferation indices in cultured bovine peripheral lymphocytes exposed to the tolylfluanid-based fungicide for 24 h.

| Dose                        | SCE / cell | PI    |
|-----------------------------|------------|-------|
| Control (DMSO)              | 6.98 ± 2.92| 1.76  |
| Tolylfluanid-based fungicide (µg/mL) 24 h |            |       |
| 1.7                         | 7.50 ± 3.45*| 1.66* |
| 3.5                         | 7.64 ± 3.21*| 1.61* |
| 8.75                        | 8.00 ± 2.28*| 1.59* |
| 17.5                        | 8.85 ± 2.22**| 1.53** |
| Positive control, 0.4 µM MMC| 9.74 ± 2.76***| 1.65* |

A total of 50 second-division metaphases of each group were analyzed for SCE, where possible. *p < 0.05), **(p < 0.01), *** (p < 0.001), ANOVA followed by Student’s t test was used for SCE and χ² test for PI evaluation a – no statistical significance, b, c – insufficient number of cells: 27, 28 analyzed second metaphases.

### Table 2 - Frequency of SCEs and proliferation indices in cultured bovine peripheral lymphocytes exposed to the tolylfluanid-based fungicide for 48 h.

| Dose                        | SCE / cell | PI    |
|-----------------------------|------------|-------|
| Control (DMSO)              | 6.40 ± 2.41| 1.76  |
| Tolylfluanid-based fungicide (µg/mL) 48 h |            |       |
| 1.7                         | 7.05 ± 2.64*| 1.76* |
| 3.5                         | 7.94 ± 3.41**| 1.73* |
| 8.75                        | 8.46 ± 2.76***| 1.73* |
| 17.5                        | 9.25 ± 3.29***| 1.68* |
| Positive control, 0.4 µM MMC| 23.2 ± 3.9***| 1.54**|

A total of 50 second-division metaphases of each group were analyzed for SCE. *(p < 0.05), ***(p < 0.01), ***(p < 0.001), ANOVA followed by Student’s t test was used for SCE and χ² test for PI evaluation a – no statistical significance.
We present herein the results of cytogenetic studies using SCE and PI as biomarkers, as well those on DNA fragmentation as a biochemical distinctive feature of apoptotic cell death.

The results of the SCE studies were dependent on the time of exposure to the fungicide. No increase in SCE induction was observed after a 24 h incubation. The treatment with the highest dose of the tested fungicide was demonstrated to cause an increase in SCE frequency in both donors, yet only incomplete second metaphases could be analysed in donor 1. Reduction in the PI percentage reflected a weak delay in cell proliferation.

A dose-related response in the induction of SCE was observed in cultures exposed to tolylfluanid-based fungicide for 48 h. Influence on proliferation kinetics of bovine lymphocytes was not found. In contrast, the SCE results for the 2 h exposure with metabolic activation (S9) were inadequate to confirm any genotoxic activity of the fungicide tested.

SCE quantification is the most widely used approach for the assessment of the genotoxic/cytogenetic effects of various chemical compounds, and is considered to be a measure of the dose as they also detect early effects of exposure (Albertini, 1998). Analysis of the SCE was adopted as an indicator of genotoxicity, although the mechanism of SCE induction is still unknown. Recent studies revealed that imbalance of the nucleotide pool can have severe consequences on DNA metabolism which is a critical point in SCE formation (Bolognesi, 2003).

Studies regarding the clastogenic/cytogenetic effects of tolylfluanid in farm animals are scant. Previously we performed experiments with bovine peripheral lymphocytes to detect the in vitro clastogenic effect of tolylfluanid formulation fungicide incubated for 24 h and 48 h. No clastogenic effect of the fungicide was observed using conventional chromosome aberration analysis (with Giemsa staining) or fluorescence in situ hybridisation (FISH) (Orosová et al., 2010).

A total of 50 second-division metaphases of each group were analyzed for SCE, where possible. * (p < 0.05), ** (p < 0.01), *** (p < 0.001), ANOVA followed by Student’s t test was used for SCE and χ2 test for PI evaluation a – no statistical significance, b, c – insufficient number of cells: a 27, b 28 analyzed second metaphases.

Table 3 - Frequency of SCEs and proliferation indices in cultured peripheral lymphocytes exposed to the tolylfluanid-based fungicide for 2 h with S9.

| Dose                     | SCE / cell | PI    |
|--------------------------|------------|-------|
| Donor 1                  |            |       |
| Control (DMSO)           | 7.20 ± 2.82| 1.75  |
| Tolyfluanid-based fungicide (µg/mL) 2 h+S9 |            |       |
| 1.7                      | 7.15 ± 2.94*| 1.80* |
| 3.5                      | 7.26 ± 3.12*| 1.80* |
| 8.75                     | 7.44 ± 3.54*| 1.76* |
| 17.5                     | 7.73 ± 2.99*| 1.64* |
| 4 µg/mL, cyclophosphamid | 11.96 ± 4.46***| 1.57**|
| Donor 2                  |            |       |
| Control (DMSO)           | 7.07 ± 2.86| 1.70  |
| Tolyfluanid-based fungicide (µg/mL) 2 h+S9 |            |       |
| 1.7                      | 7.13 ± 2.90*| 1.73* |
| 3.5                      | 7.22 ± 2.86*| 1.75* |
| 8.75                     | 7.34 ± 2.67*| 1.73* |
| 17.5                     | 7.63 ± 3.64**| 1.56* |
| 4 µg/mL, cyclophosphamid | 10.19 ± 5.24***| 1.47***|

Figure 1 - DNA gel electrophoresis of internucleosomal DNA fragmentation in 1.5% agarose gel after the exposure to tolylfluanid-based fungicide for 48 h. A slight fragmentation of DNA was demonstrated at the concentrations of 3.5 and 8.75 µg/mL. (1) Marker MW (2) DMSO (3) 1.75 µg/mL (4) 3.50 µg/mL (5) 8.75 µg/mL (6) 17.5 µg/mL (7) Negative control without treatment (8) Positive control (U937 cells).

Discussion

We present herein the results of cytogenetic studies using SCE and PI as biomarkers, as well those on DNA fragmentation as a biochemical distinctive feature of apoptotic cell death.

The results of the SCE studies were dependent on the time of exposure to the fungicide. No increase in SCE induction was observed after a 24 h incubation. The treatment with the highest dose of the tested fungicide was demonstrated to cause an increase in SCE frequency in both donors, yet only incomplete second metaphases could be analysed in donor 1. Reduction in the PI percentage reflected a weak delay in cell proliferation.

A dose-related response in the induction of SCE was observed in cultures exposed to tolylfluanid-based fungicide for 48 h. Influence on proliferation kinetics of bovine lymphocytes was not found. In contrast, the SCE results for the 2 h exposure with metabolic activation (S9) were inadequate to confirm any genotoxic activity of the fungicide tested.

SCE quantification is the most widely used approach for the assessment of the genotoxic/cytogenetic effects of various chemical compounds, and is considered to be a measure of the dose as they also detect early effects of exposure (Albertini, 1998). Analysis of the SCE was adopted as an indicator of genotoxicity, although the mechanism of SCE induction is still unknown. Recent studies revealed that imbalance of the nucleotide pool can have severe consequences on DNA metabolism which is a critical point in SCE formation (Bolognesi, 2003).

Few authors have documented toxicity of tolylfluanid. Bellas (2006) compared the toxicity of four biocides (chlorothalonil, dichlofluanid, tolylfluanid, and Irgarol) on early developmental stages of marine invertebrates. The highest toxicity to embryos and larvae of Mytilus edulis and sea-urchins was seen after the application of chlorothalonil and dichlofluanid. Pistl et al. (2003) demonstrated a significant cytotoxic effect of dichlofluanid at a concentration of 10^{-4} M in ovine peripheral leucocytes. The authors denoted a more expressive immunotoxic effect when cultures of ovine lymphocytes were treated with the fungicide after PHA proliferation stimulation. Similarly Holovská et al. (2007) described a significant suppression of the proliferative activity in RK13 cells after exposure to tolylfluanid at a concentration of 10^{-4} M.

Apoptosis (or programmed cell death) has been shown to be initiated by several factors, including exposure
to the pesticides (Kannan et al., 2000, Li et al., 2009). The effect of different groups of pesticides in apoptosis induced DNA fragmentation, has been investigated. Kaur et al. (2007) reported that low-level long-term organophosphate exposure resulted in oligonucleosomal DNA fragmentation as a hallmark of apoptosis. A derivative of the carbamate pesticide carbofuran, N-nitrosocarbofuran, induced apoptosis of Chinese hamster lung fibroblast cells (Yoon et al., 2001). Multiple in vitro exposures of mouse thymocytes to pesticide were examined by Olgun et al. (2004). As regards tolylfluanid formulation (with 50% active agent), Domaracky et al. (2007) reported cell death in mouse preimplantation embryos at relatively low doses. Nevertheless, the incidence of cell death has been mostly reported as necrosis.

No differences in DNA fragmentation among bovine culture samples on the agarose gel were observed after 24 h exposure. Nevertheless, a prolonged time of treatment with tolylfluanid-based fungicide at concentrations of 3.50 and 8.75 μg/mL resulted in a weak nucleosomal DNA ladder pattern. Apoptosis is thought to contribute to the elimination of the cells which might undergo irreversible transformation (Elhajouji et al., 1997). At the highest concentration of the fungicide, the cells are probably adapted to the changes and thus no DNA ladder formation occurs (Decordier et al., 2008). DNA fragmentation appears to be time-dependent and corresponds with the cell kinetics of our cytogenetic studies, where a delay of cell proliferation and cytotoxic/cytostatic effects of the fungicide could be denoted.

Positive results in the SCE assay for the 48 h treatment indicated that the fungicide has the potential to induce chromosomal damage (replication injuries). Based on our results in all of the experimental conditions, we suggest that tolylfluanid-based fungicide acts as a multi-site inhibitor (microtubule and proliferation inhibitors) and that in high concentrations could block cell division. There is evidence for an association of environmental chemical agents (including pesticides) with disturbing of glucocorticoid-regulated physiological processes. Xenobiotics that interfere with glucocorticoid-mediated functions are expected to disturb the cellular energy status by altering the expression of genes involved in carbohydrate, lipid, or protein metabolism. Glucocorticoid activation was also observed for tolylfluanid, hexachlorobenzene, and others, with IC50 values of about 1 mM promoting cell cycle arrest in the G1-phase (Johansson et al., 2005, Odermatt and Gumy, 2008). Taken together we infer that, environmental contamination with the fungicide can contribute to increase genetic risk for humans.

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**Internet Resources**

**EPA, Pesticide Fact Sheet**, http://www.epa.gov/opprd001/factsheets/tolylfluanid.pdf (February 25, 2010).

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