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USE OF SISTER CHROMATID EXCHANGES AND COMET ASSAY IN GENETIC RISK ASSESMENT

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ABSRACT

Sister chromatid exchanges (SCEs) and comet assay (SCGE) are considered as sensitive bioindicators for detection of genotoxic activity of chemicals agents. Both methods, SCEs and SCGE were used for evaluation of genotoxic/cytotoxic effects after treatment of cultured bovine peripheral lymphocytes with neonicotinoid insecticide Calypso 480 SC (active ingredient: thiacloprid). Frequency of SCEs, proliferation indices (PI) and % DNA in tail were evaluated. For 24h, a weak statistical significance increase of SCEs was observed at the concentration of 240 and 480 $\mu g.ml^{-1}$ in donor 2. Reduction of PI was obtained only at the highest concentration. For 48h statistical significant elevation in the SCE frequency was found at concentrations ranging from 120 to 480 $\mu g.ml^{-1}$ in each donor. Reduction of the PI was recorded at these concentrations too, but most significantly at highest dose (480 $\mu g.ml^{-1}$) in both donors. A statistical significance in the increase of DNA strand breaks was seen at the concentration ranged from 60 to 480 $\mu g.ml^{-1}$ for 1h with the same compound by comet assay.

Keywords: sister chromatid exchanges, comet assay, genotoxicity, cytotoxicity, thiacloprid

INTRODUCTION

SCEs belong to the most frequently employed cytogenetic biomarkers. Despite the lack of specificity in the detection of mutagenic activity of chemical agents they remain very attractive in short term assays **(Tucker** and **Preston, 1996)** *in vivo* and also *in vitro* **(Tucker et al., 1993)**. Most studies that involve SCEs analysis have been carried out in humans and rodents **(Arruga et al., 1992)**. As the basic component of the feed of ruminants is of plant origin, they represent the first consumers exposed to environmental mutagens. Thus biomonitoring studies using farm animals could be very useful and sensitive indicator to evaluate the genotoxic effect chemical substances with predictive value for human health risks **(Parada** and **Jaszczak, 1993)**.

SCEs represent the cytological manifestation of interchanges between DNA replication products at apparently homologous loci **(Tucker et al., 1993)**. The mechanism of SCE formation is still not known, though they appear to be a consequence of errors of DNA replication, possibly at the replication fork itself **(Painter, 1980)**. There is no direct association between SCE induction and an adverse health outcome. Thus, the analysis of SCEs has limited value in risk assessment; however, they are except as a biomarker of exposure **(Tucker** and **Preston, 1996)**.

The comet assay or single cell gel electrophoresis (SCGE) is a simple and frequently used method for measuring DNA strand breaks in eukaryotic cells. The assay attracts adherents except its simplicity also by its sensitivity, versatility, speed and economy. This assay has become one of standard methods for assessing DNA damage, with applications in genotoxicity testing, ecogenotoxicology, human biomonitoring as well as fundamental research in DNA damage and repair **(Collins, 2004)**. At present, two basic modifications of the comet assay are used – alkaline SCGE, and neutral SCGE. Both methods were developed by

Östling and **Johanson (1984)**. Alkaline SCGE is applied for detection single strand breaks; neutral SCGE is applied for detection double strand breaks **(Dvořák and Matejovičová, 2008)**.

The principle of alkaline SCGE is that cells embedded in agarose on a microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA. Loops containing a break lose their supercoiling and become free to extend toward the anode therefore they escape from the nucleoid comet head to form a tail. Finally, electrophoresis at high pH result in structures resembling comets, which can be observed by fluorescence microscopy **(Collins, 2004; Horváthová et al., 2004)**.

In this study, SCE analysis and SCGE were used for assessing of genotoxic/cytotoxic effects of commercial neonicotinoid insecticide Calypso 480 SC (active ingredient: thiacloprid) in bovine peripheral lymphocyte. As additional endpoints, the proliferation indices (PI) were evaluated to detect cytotoxic activity of the insecticide. Neonicotinoids represent a new class of synthetic insecticides that are widely used to control sucking insects. They act as agonist at the insect nicotinic acetylcholine receptor **(Tomizawa and Casida, 2003)**. Genotoxic effects are considered as most serious of the possible potential side effects of agricultural agents **(Anwar, 1997)**. The genotoxic effect of the thiacloprid insecticide was reported by **Kocaman et al. (in press)** in human peripheral blood lymphocytes using chromosome aberrations, sister chromatid exchanges and micronuclei. In another study Comet assay was applied to detect the genotoxic and cytotoxic effects of neonicotinoid insecticides in human peripheral lymphocytes *in vitro* **Calderón-Segura et al. (in press)**.

MATERIAL AND METHODS

The experiments were performed with peripheral blood obtained from two healthy bull donors (Slovak spotted cattle, 6 months old). Calypso 480 SC (with active component thiacloprid) was used as toxic substance. The agent was dissolved in purified water and applied into culture flasks at concentration of 30, 60, 120, 240 and 480 $\mu g.ml^{-1}$. For the SCEs analysis, mitomycin C (MMC, Sigma, St. Louis, MO, USA; 0.4 $\mu g.ml^{-1}$) and for the comet assay, hydrogen peroxide (H_2O_2, Mikrochem, SR; 300 µM) were used as positive control agents.

Sister chromatid exchanges test

Whole blood (0.5 ml) was cultivated for 72 h at 38°C in 5 ml of RPMI 1640 medium supplemented with L-glutamine and HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum, antibiotics (streptomycin 250 $\mu g.ml^{-1}$ and penicillin 250 $U.ml^{-1}$) and phytohaemagglutinine (PHA, Welcome, Dartford, England; 180 $\mu g.ml^{-1}$).

Chromosome preparations were obtained by the standard cytogenetic method. Bromodeoxyuridine (BrdU, Sigma, St. Louis, MO, USA; 8 $\mu g.ml^{-1}$) was supplemented to all groups 24 h after the initial divisions. Experimental lymphocyte cultures were exposed to the insecticide Calypso 480 SC for the last 24 h and 48 h. 2 h before harvest, colchicine (Merck, Darmstadt, Germany; 5 $\mu g.ml^{-1}$) was added to control and also to experimental cultures. The slides were prepared by an air-dried method and stained with the fluorescence plus Giemsa (FPG) technique to differentiate sister chromatids and cell cycles.

Comet assay

Whole blood was diluted with PBS buffer (1:1) and subsequently suspension was layered at separation medium (Histopaque). After centrifugation at 1000 rpm for 15 min according to **Singh** and **Stephens (1997)** lymphocyte were harvested and suspended with PBS buffer in total volume of 1 ml.

Bovine peripheral lymphocytes (0,3 ml) were incubated with insecticide Calypso 480 SC at each concentration in 4,7 ml of 1640 RPMI medium supplemented with L-glutamine and

HEPES (Sigma) including 15% fetal calf serum, 2% phytohemagglutinin (PHA, Welcome, Dartford, England; 180 μg.ml^{-1}) and antibiotics (streptomycin 250 μg.ml^{-1} and penicillin 250 U.ml^{-1}) at 37°C for 1 h according to **Chen et al. (2008)**. After treatments, the cells were centrifuged at 1800 rpm for 3 min and then were mixed with low melting point agarose for the alkaline comet assay.

Each concentration was represented by three microscope slides or gels. Plain glass microscope slides were pre-coated by dipping in a solution of 1% normal melting point agarose (NMP, Serva, Germany) in distilled water and drying at room temperature. This pre-coating ensures adhesion of agarose gels that has been used subsequently. 100 μl 1% NMP diluted in PBS buffer and 85 μl cells suspension mixed in 0.75 % low melting point agarose by Serva were placed on microscope slide. Each layer was left to set at 4°C and cover-slips were removed. Microscope slides were placed into lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, plus 1% Triton X-100) for 1 h at 4°C. Slides were then placed in horizontal gel electrophoresis tank and immersed in 0.3 M NaOH, 1 mM Na_2EDTA for 40 min, before electrophoresis at 25 V (300 mA) for 30 min at 4°C. After electrophoresis, slides were neutralized by washing three times for 5 min with 0.4 M Tris-HCl, pH 7.5. Finely slides were stained with 20 μl ethidium bromide (5 μg/ml) and covered with cover-slip. Comets were analyzed by NIKON Labophot 2A fluorescence microscope, equipped with single band pass filter Texas Red.

For SCEs frequencies, fifty differentially stained well-spread metaphases (Figure 1a) per donor and at the different concentrations were examined and 100 metaphases were analyzed for determination of M_1, M_2 and M_3 mitotic divisions and subsequent calculation of the proliferation index according to **Lamberti et al. (1983)**.

A total of 100 comets on each slide were classified visually into five categories (Figure 1b, 1c); based on the DNA fluorescence intensity in proportion comet tail and head, and an overall score for each gel of between 0 (all undamaged) and 400 (all maximally damaged) was calculated.

Statistical analysis of results was performed using simple analysis of variance (ANOVA), and then the Student's t test was applied to evaluate SCE occurrence and DNA breaks between treated and untreated groups. Chi square test (χ^2) was applied for estimation of the cell cycle delay too.

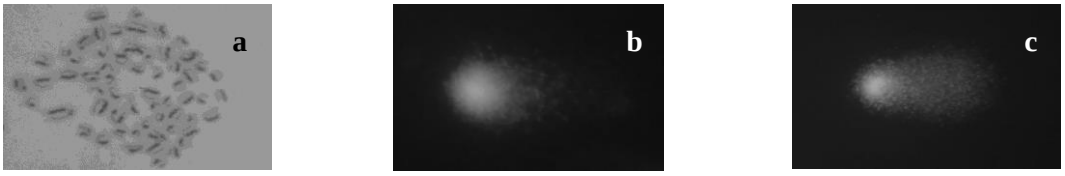

Figure 1

a – metaphases in M_2 mitotic division; b, c – different categories of comets

RESULTS and DISCUSSION

The results obtained from the SCE studies and lymphocyte proliferation kinetics after exposure to thiacloprid-based insecticide for 24 and 48 h are summarized in Figure 2 and 3.

For 24h no significant elevation in the SCE frequency was obtained in donor 1. A weak statistical significance in the increase of SCEs was observed in donor 2 after exposure to the insecticide at the concentration of 240 and 480 μg.ml^{-1}. The highest concentration also reflected in a reduction of the PI ($p < 0.05$, ANOVA, Student's *t* test, and χ^2 test, respectively, Fig. 2).

In the experiments for 48h a statistical significance in the increase of frequency SCEs was found after the treatment with the neonicotinoid insecticide Calypso 480 SC at

concentrations ranging from 120 to 480 µg.ml^{-1} in each donor ($p < 0.05$, $p < 0.01$, respectively, by ANOVA and Student's *t* test, Figure 3). These results were reflected in the reduction of the PI most significantly at highest dose (480 µg.ml^{-1}, $p < 0.01$, χ^2 test) in both donors (Fig. 3)

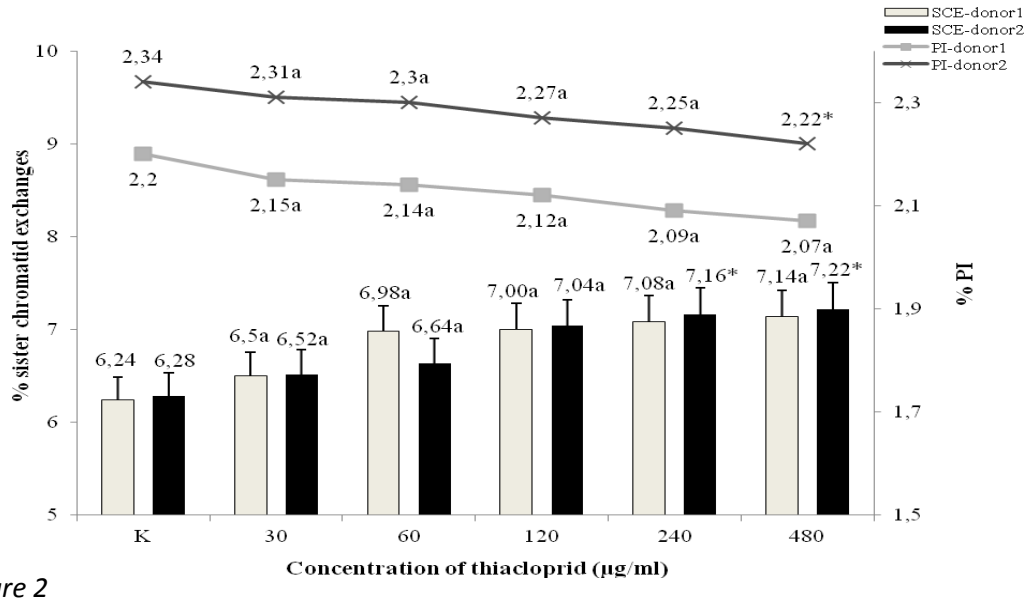

Figure 2

Frequency of SCEs and proliferation indices in cultured peripheral lymphocytes exposed to the insecticide thiacloprid for 24h

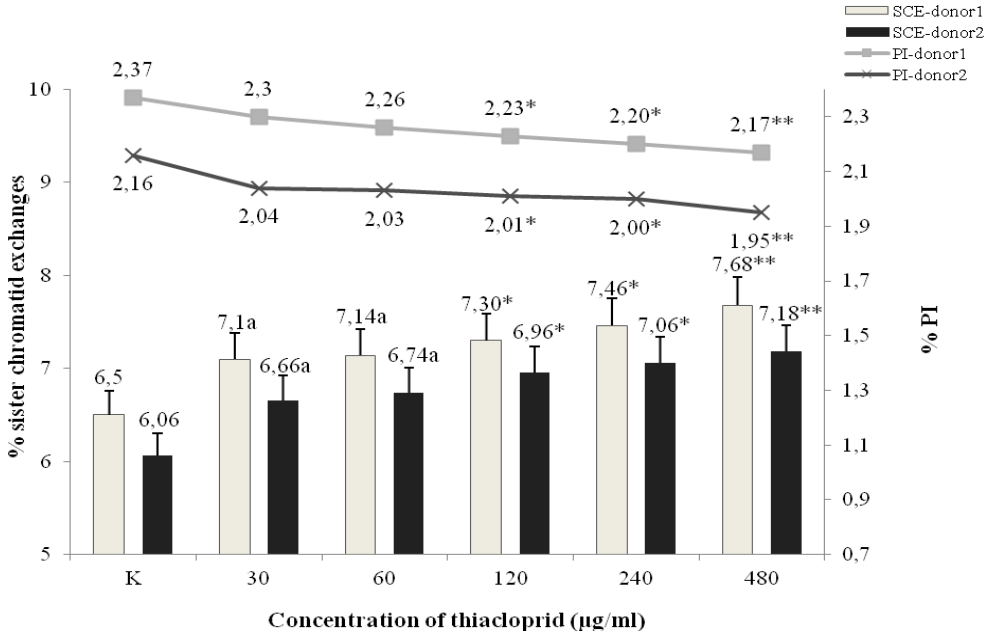

Figure 3

Frequency of SCEs and proliferation indices in cultured peripheral lymphocytes exposed to the insecticide thiacloprid for 48h

A total of 50 second - division metaphases of each group were analysed for SCE
* (P<0.05), ** (P<0.01), *** (P<0.001) - statistical significant data, ANOVA, Student`s t test was used for SCE and χ^2 test for the PI a - no statistical significance.

The results of DNA strand breaks after treatment with various concentrations of the insecticide tested in bovine peripheral lymphocytes are shown in Figure 4. After treatment of the bovine peripheral lymphocytes with the insecticide for 1h an increase in DNA damage was found. A dose dependence in elevations of DNA strand breaks was seen at the concentrations ranged from 60 $\mu g.ml^{-1}$ ($p < 0.05$) to 480 $\mu g.ml^{-1}$ ($p < 0.01$).

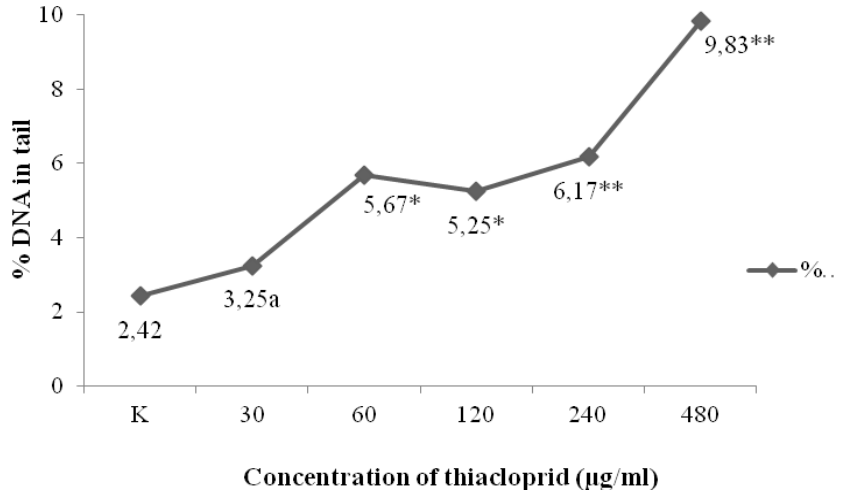

Figure 4

DNA damage in bovine peripheral lymphocytes exposed to different doses of insecticide thiacloprid for 1h

SCEs are widely used in cytogenetic studies and they are considered as very sensitive bioindicators for the assessment genotoxic agents **(Šiviková and Dianovský, 2006)**. The ratio of induced SCEs is different for each compound, suggesting that each type of lesion formed is processed by the cell to form either an SCE, an aberration, or a mutation (Carrano and Natarajan, 1988). The reduction of PI represents an additional check on the chemical agent potency that can be interpreted as cell cycle delays and/or in terms of induced cytotoxicity. A more expressive cytotoxic effect was found with the prolonged time sampling of insecticide incubation. With respect to found cell proliferation delay, treatment for 48h incubation was provided to confirm evidence of insecticide genotoxicity.

The alkaline comet assay is an excellent and sensitive test for the detection of DNA damage induced by chemicals agents, such as pesticides **(Calderón-Segura, in press)**. According to **Tice et al. (2000),** comet assay, in comparison with other cytogenetic tests, such as sister chromatid exchanges, micronuclei formation and chromosomal aberration assays, is the most rapid and sensitive method to evaluate the genotoxic agents. Also **Feng et al. (2005)** indicates that the comet assay was more sensitive than SCEs test. On the other hand **Hartmann et al. (1995)** reported that comet assay was 100 times less sensitive than the SCEs test. Our study indicated genotoxic/and cytotoxic effect of insecticide Calypso 480 SC in bovine peripheral lymphocytes *in vitro*.

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