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Genotoxicity testing of bovine lymphocytes exposed to epoxiconazole using alkaline and neutral comet assay

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Abstract. Epoxiconazole belongs in the class of azoles which have been developed to protect crops from fungal diseases. The mechanism of action of these fungicides is to inhibit the specific cytochrome P450 enzyme (CYP), CYP51 (lanosterol 14α-demethylase) which contributes to ergosterol biosynthesis. Since ruminants and cattle are exposed to contaminants during grazing, they are a suitable experimental model for genotoxicity testing. In our experiment, epoxiconazole (EPX) (active agent, 99% of purity) was tested *in vitro* for its potential genotoxic and cytotoxic effects on bovine lymphocytes, isolated from whole peripheral blood. We exposed the lymphocytes to EPX at concentrations of 2.5, 5, 10, 25, 50 and 100 μg/mL by two different ways: immediately after isolation of lymphocytes during 2 h in RPMI 1640 medium (without phytohaemagglutinin, PHA) as well as on the last 2 h of 48-h culture (with PHA). In a second case, we chose 48 h culture because the lymphocytes usually start DNA replication 24 h after the start of the cultures; therefore, we incubated the cells longer to obtain dividing (proliferating) cells. The levels of DNA damage were measured using alkaline and neutral comet assays. The results of alkaline comet assay showed the significantly increased percentage of DNA breaks in both lymphocytes in medium without PHA (2 h of exposure; non-proliferating cells) and lymphocytes cultured during 48 h in medium with PHA (exposure for the last 2 h of cultivation; proliferating cells). Similarly, neutral comet assay showed dose-dependent elevation of the DNA migration induced in both non-proliferating and proliferating lymphocytes treated with EPX when compared with negative controls. Our results suggest that epoxiconazole fungicide is capable of causing damage to the genetic material of the bovine cells.

Keywords: epoxiconazole, genotoxicity, cattle, comet assay.

INTRODUCTION

Pesticides are a significant source of environmental pollution due to their wide-ranging application in agriculture and forestry. Exposure to these pollutants can have both acute and chronic effects on target and non-target

organisms (Berenzen et al. 2005). Long-term exposure and chronic poisoning with pesticides can trigger genotoxic and epigenetic processes through various pathways, interactions and doses resulting from the intensive use of pesticides and can cumulatively lead to genetic change in humans, covertly and without clinical evidence (Bull et al. 2006). As later indicated by Kaur and Kaur (2018), occupational exposure to pesticides in agricultural workers has been associated with an increased incidence of various diseases such as cancer, Parkinson's disease, Alzheimer's disease, reproductive disorders, and birth defects.

Conazoles are a class of azole-based fungicides which are widely used as pesticides in the cultivation of crops despite their suspected endocrine disrupting properties (Roelofs et al. 2014) but also as human and veterinary pharmaceuticals for the treatment of oropharyngeal, vaginal as well as systemic candida and mycosis infections (Kjaerstad et al. 2010). These fungicides act by inhibiting a specific cytochrome P450 (CYP) enzyme, CYP51 (lanosterol 14α-demethylase), which mediates a critical step in the biosynthesis of ergosterol, a steroid required for the synthesis of the fungal cell wall (Zarn et al. 2003). For this reason they are called demethylation inhibitor (DMI) or ergosterol-biosynthesis-inhibiting (EBI) fungicides. Besides their effects on fungal CYP51, triazole-based conazoles have the potential to interact with the mammalian cytochrome P450 (CYP) system, e.g. via inhibition of aromatase (CYP19) which can lead to numerous toxicological effects (Chambers et al. 2014). As reported by Roelofs et al. (2013) conazoles also cause catalytic inhibition of the CYP17 enzyme, responsible for the conversion of pregnenolone and progesterone to androgen precursors. Exposure to these compounds from multiple environmental matrices can cause many negative effects including carcinogenicity hepatotoxicity, reproductive and developmental toxicities (Goetz and Dix 2009; Hester et al. 2012; Heise et al. 2015; Mu et al. 2016; Heise et al. 2018). In spite of the large production and extensive usage of many conazoles, accurate data on human exposure levels are scarce. Besides occupational and pharmaceutical exposure, individuals can also be exposed to conazoles through environmental, food, resident or bystander exposure. This is confirmed by the increasing concentrations of conazole pesticides found in surface and waste waters (Kahle et al. 2008).

Epoxiconazole (EPX) belongs in the triazole class of pesticides and is used worldwide as a fungicide for plant protection. It is known to combat various target fungal diseases in cereals, rice, sugar beets, bananas, coffee, and soybeans (Passeport et al. 2011). This DMI fungicide was effectively used for the control of Fusarium head blight of wheat in China (Chen et al. 2012). In Europe and Australia, the epoxiconazole is part of several commercially successful one-compound fungicide formulations (Epic, Opus) or two-compound formulations composed from combinations of epoxiconazole with different pesticide (Splice, Swing Gold, Tango Super, Venture etc.). Glyphosate, DDTs and the broad-spectrum fungicides boscalid, epoxiconazole and tebuconazole were the most frequently found in agricultural soil samples of 11 member states of the European Union (EU) (Silva et al. 2019). These findings confirmed the previous study of Hvězdová et al. (2018), where conazoles showed the second most frequent occurrence among currently used pesticides (CUPs) in Central European arable soils. In the Czech Republic, Vašíčková et al. (2019) determined that epoxiconazole was one of the main contributors to the overall pesticide mixture toxicity: the measured levels and its frequent presence in soils represented a risk for the agroecosystems. This contribution might be a result of low biodegradability and photochemical stability of the EPX molecule that makes it very persistent in soil and aquatic sediment (Passeport et al. 2011) and allows entering multiple environmental media through spray drift or surface runoff (Potter et al. 2014).

Bovine farm animals are exposed to chemical agents through grazing, so they are the first in which adverse effects of pesticides might occur (Drážovská et al. 2016). For this reason, in this study, we would like to present new data from an experiment where DNA damage was investigated after exposure of bovine peripheral lymphocytes to epoxiconazole. Both alkaline and neutral comet assays were used as the methods of choice for detection of single-strand and double-strand DNA breaks.

MATERIALS AND METHODS

Blood samples were collected by means of jugular venipuncture from two healthy bulls (Slovak indigenous cattle, 6 month old). The animals were kept in healthy conditions, not treated with any drugs and fed with clean feed. The study was conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare. Lymphocytes isolated from whole blood were used for the comet assays.

Epoxiconazole (CAS registry number 133855-98-8, 99% purity, Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and introduced into culture flasks at concentrations of 2.5, 5, 10, 25, 50 and 100 μg/mL. The fungicide doses were chosen according to study of Šiviková et al. (2018), where the fungicide cytotoxicity level was identified at a

concentration of more than 100 µg/mL. The final DMSO concentration was 0.1% in both the treated and untreated (negative control) cells. Hydrogen peroxide (H_2O_2, Mikrochem, SR, 250 µM) was used as a positive control agent.

Cell cultivation and treatment

For comet assay, lymphocytes were immediately isolated from bovine whole blood using the Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA) separation medium. Isolated lymphocytes were treated with epoxiconazole for 2 h in two different ways: immediately after isolation (non-proliferating lymphocytes) and for the last 2 h of 48 h cultivation (i.e. pre-cultivation of lymphocytes before 2 h treatment to obtain proliferating lymphocytes). Medium for non-proliferating lymphocytes consisted from 4 ml RPMI 1640 medium supplemented with L-glutamine and 15 µM HEPES, 1 ml bovine foetal serum (BOFES) and 40 µl antibiotic/antimycotic mixture (100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin) (Sigma-Aldrich, St. Louis, MO, USA). Immediately after isolation lymphocytes were added to the medium and exposed to the test fungicide for two hours (2 h) (i.e. concurrently with their addition to the medium) according the procedure of Calderón-Segura et al. (2012).

In the experiment with proliferating lymphocytes phytohaemagglutinin (PHA-L, 20 µg/mL, PAN Biotech, Germany) was added to the above-described culture medium. The isolated lymphocytes were subsequently incubated at 37°C for 48 h and exposed to epoxiconazole for the last 2 h of cultivation.

The cells of positive controls were treated with H_2O_2 (250 mM) for 5 minutes (Horváthová et al. 2006).

Cytotoxicity

After exposure completed, the cells were washed twice with phosphate-buffered saline (Dulbecco A, pH 7.4) and resuspended to a final volume 1mL with PBS. Cytotoxic effects on the bovine peripheral lymphocytes were evaluated using the trypan blue dye exclusion staining (0.4% trypan blue), where the number of viable (shiny) and dead (blue) cells were scored (viability test).

Alkaline comet assay

The alkaline comet assay procedure was the same for both non-proliferating and proliferating lymphocytes. Each concentration tested was represented on special microscope comet slides (CometSlides™ 2-Well, TREVIGEN**,** Gaithersburg, Maryland, US) treated to promote agarose adherence, in this case ready-to-use low melting point agarose (LMPA). The cells were mixed with 0.75% LMPA in PBS. The cell suspension was pipetted onto the agarose layer, fitted with a cover slip and left to set at 4°C. After removal of the cover slips, the microscope slides were immersed in cold lysing solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, plus 1% Triton X-100) for 1 h at 4°C. The slides were then transferred to a horizontal gel electrophoresis tank with electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH>13) for 40 min unwinding at 4 °C, and then electrophoresis was conducted at 25V and 300mA for 30 min. The slides were neutralized two times for 10 min with 0.4 M Tris-HCl (pH=7.4), stained with ethidium bromide (5 µg/mL) on both sides, and fitted with cover slips. All of these steps were carried out in the dark and cold (4°C) to prevent the occurrence of additional DNA damage (Collins 2002).

Neutral comet assay

The slides were lysed in cold lysing solution (2.5 M NaCl, 0.1M disodium ethylene diaminetetraacetic acid (EDTA disodium salt), 10 mM Tris-HCl, pH=9.5, 1% N-lauroylsarcosine sodium salt, 1% TritonX-100) for 1h at 4°C. Then the slides were moved to an electrophoretic tank with TBE buffer in which the "unwinding" was performed for 1 hour, followed by electrophoresis (20V) for 40 min. After electrophoresis, the slides were neutralized in blossom with neutralizing solution (0.4 M Tris, pH=7.4) for 2 x 10 min. After drying, the glasses were stained with ethidium bromide (5 µg/mL) (Gyori et al. 2014).

DNA damage evaluation

Comets were analysed with a Nikon ECLIPSE N*i*-U fluorescence microscope, equipped with a Texas Red single band pass filter. A total of 100 nucleoids per slide (three slides for each concentration - 300 nucleoids) were scored visually and five classes of damage were recorded, from 0 (undamaged) to 4 (maximally damaged) according to DNA fluorescence intensity in proportion comparing the comet tail and head. The scores 0-4 were attributed according to visual analysis of nucleoids. The overall score for each slide was therefore between 0-400 (Collins 2002). The percentage of damaged cells and the extent of % DNA damage in the comet tail were calculated.

Statistical analysis

Statistical analysis was performed using simple analysis of variance (ANOVA, Student's t test), which was used to evaluate % DNA breaks comparing treated and untreated groups (controls).

RESULTS

The results of our analysis of DNA damage using both alkaline and neutral comet assays in non-proliferating (2 h exposure to fungicide) and proliferating (48h cultivation and exposure to fungicide for the last 2h) lymphocytes from bovine peripheral blood after exposure to epoxiconazole at concentrations of 2.5; 5; 10; 25; 50 and 100 μg/mL, are summarized in Fig. 1*a, b* and Fig. 2*a, b*. The percentage viability of non-proliferating and proliferating lymphocytes from bovine peripheral blood following exposure to epoxiconazole is shown in Fig. 3*a, b* (alkaline comet assay) and Fig. 4*a, b* (neutral comet assay).

Regarding the results of alkaline comet assay after 2h exposure of non-proliferating lymphocytes to epoxiconazole, increases in DNA damage with statistical significance were found starting from concentration 5 μg/mL in donor 1 (5 μg/mL * $p<0.05$; 10, 25, 50 and 100 μg/mL ** $p<0.01$; ANOVA and Student's t test; Fig. 1*a*) as well as donor 2 (5 μg/mL * $p<0.05$; 10, 25, 50 and 100 μg/mL ** $p<0.01$; ANOVA and Student's t test; Fig. 1*a*).

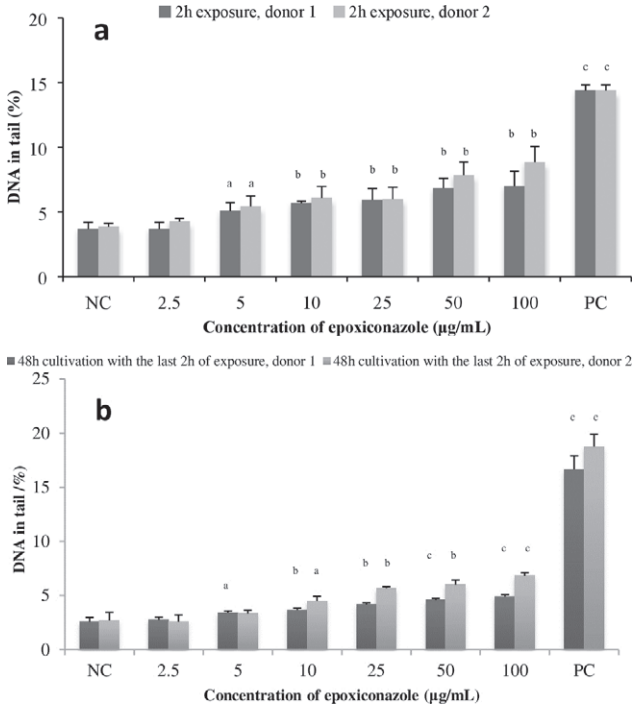

Figure 1. Percentages of DNA in tail estimated by means of alkaline comet assay in bovine peripheral blood lymphocytes (non-proliferating) treated with epoxiconazole for 2 h (a) and in bovine peripheral blood lymphocytes (proliferating 48 h) treated with epoxiconazole for the last 2 h (b). NC (negative control): DMSO; PC (positive control): H_2O_2 (250μM); a: $p<0.05$; b: $p<0.01$; c: $p<0.001$; mean ± SD.

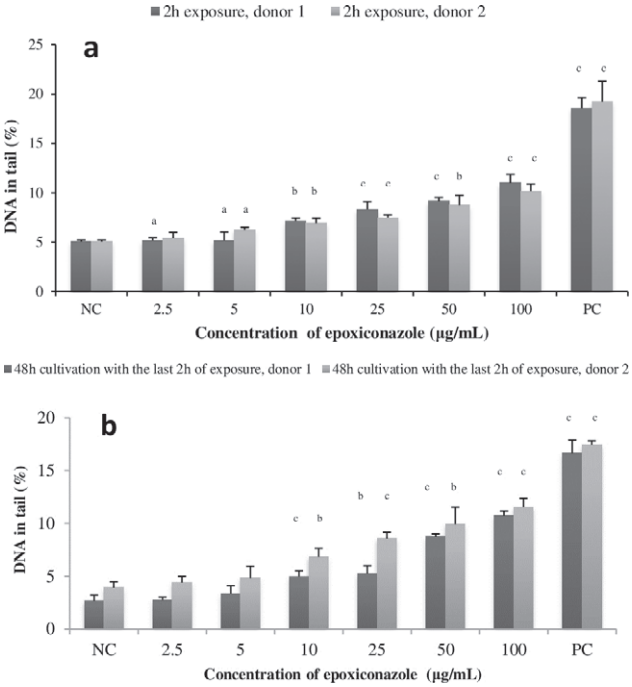

Figure 2. Percentages of DNA in tail estimated by means of neutral comet assay in bovine peripheral blood lymphocytes (non-proliferating) treated with epoxiconazole for 2 h (a) and in bovine peripheral blood lymphocytes (proliferating 48 h) treated with epoxiconazole for the last 2 h. NC (negative control): DMSO; PC (positive control): H_2O_2 (250μM); a: $p<0.05$; b: $p<0.01$; c: $p<0.001$; mean ± SD.

After 48h cultivation and exposure to epoxiconazole for the last 2 h, DNA damage was observed in proliferating lymphocytes with statistical significance in donor 1 (5 μg/mL * $p<0.05$; 10, 25 μg/mL ** $p<0.01$; 50 and 100 μg/mL *** $p<0.001$; ANOVA and Student's t test; Fig. 1*b*) as well as donor 2 (10 μg/mL * $p<0.05$; 25, 50 μg/mL ** $p<0.01$; 100 μg/mL *** $p<0.001$; ANOVA and Student's t test; Fig. 1*b*).

The viability of non-proliferating lymphocytes was greater than 95% in both donors (Fig. 3*a*), and for proliferating lymphocytes it was greater than 94.7% in both donors, too (Fig. 3*b*).

Statistically significant increases in DNA damage with double-stranded breaks in proliferating and non-proliferating lymphocytes were detected using neutral comet assay after exposure to epoxiconazole (Fig. 2*a, b*) at the same concentrations as for alkaline comet assay. Lymphocyte viability is shown in Fig. 4 *a, b*.

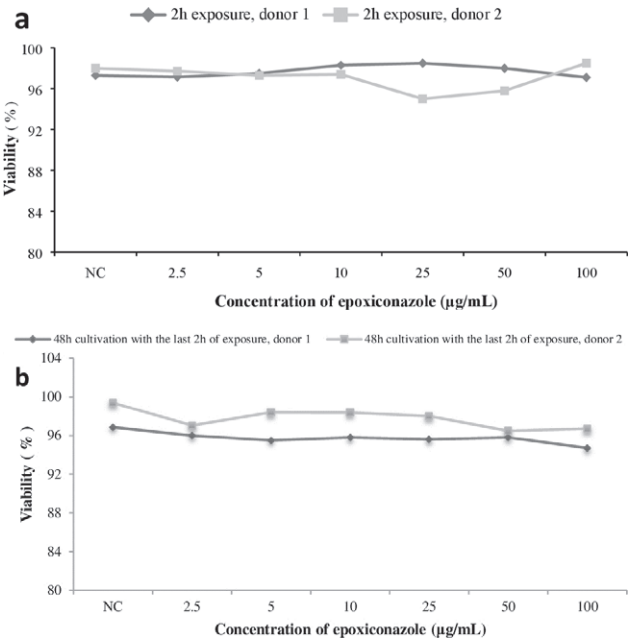

Figure 3. Viability of bovine peripheral blood lymphocytes used in alkaline comet assay. Cells were treated with fungicide epoxiconazole for 2h (a) and for the last 2h of 48h cultivation (b). NC (negative control): DMSO.

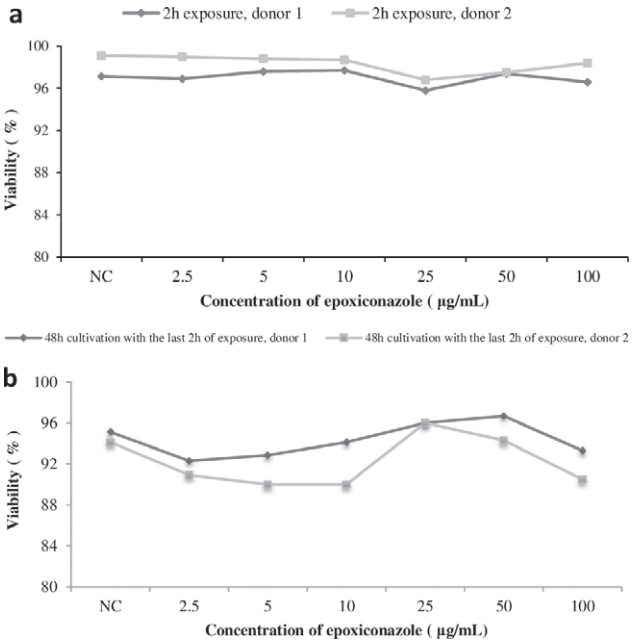

Figure 4. Viability of bovine peripheral blood lymphocytes used in neutral comet assay. Cells were treated with fungicide epoxiconazole for 2h (a) and for the last 2h of 48h cultivation (b). NC (negative control): DMSO.

DNA damage results detected using neutral comet analysis after exposure of non-proliferating lymphocytes to epoxiconazole indicate statistically significant DNA damage in donor 1 from the lowest concentration (2.5 and 5 µg/mL *p<0.05; 10 µg/mL ** p<0.01; 25, 50 and 100 µg/mL *** p<0.001; ANOVA and Student's t test; Fig. 2*a*) and in donor 2 from concentration 5 µg/mL (5 µg/mL * p <0.05, 10, 50 µg/mL ** p <0.01; 25 and 100 µg/mL *** p<0.001; ANOVA and Student's t test; Fig. 2*a*).

Proliferating lymphocytes showed statistical significance in donor 1 from starting from concentration 10 µg/mL (25 µg/mL ** p<0.01; 10, 50 and 100 µg/mL *** p<0.001; ANOVA and Student's t test; Fig. 2*b*) and in donor 2 starting from concentration 10 µg/mL (10, 50 µg/mL ** p<0.01; 25 and 100 µg/mL *** p<0.001; ANOVA and Student's t test; Fig. 2*b*).

Lymphocyte viability found in both donors after epoxiconazole exposure was higher than 95.8% (Fig. 4*a*) in non-proliferating lymphocytes and higher than 90% in proliferating ones (Fig. 4*b*).

DISCUSSION

Comet assay (single-cell gel electrophoresis) is one of the most popular methods employed for the evaluation of DNA damage and repair in eukaryotic cells (Singh 2016; Lu et al. 2017; Moller 2018) This method is used to study processes dealing with DNA damage in various fields, such as environmental toxicology, biological process monitoring, radiation biology, nutritional studies and cancer studies (Olive 2009; Wasson et al. 2008). This test has a wide spread in genotoxicity testing mainly due to advantages such as simplicity of the test, low cost and high sensitivity (Hartmann et al. 2003; Tice et al. 2000). The comet test is a universal and sensitive method measuring single-stranded and / or double-stranded DNA breaks as well as photodimers (Collins et al. 2008). There are two basic variants for determining DNA damage using comet analysis under alkaline or neutral conditions (Östling 1984; Singh 1988). Visual classification of nucleoids and calculation of percentage DNA at the tail is commonly presented up today (Collins et al. 2002; García et al. 2004; Bruschweiler et al. 2016; Hamdi et al. 2018) as an alternative to image analysis.

In the present study, the possible genotoxic and cytotoxic effects of epoxiconazole fungicide were assessed in bovine lymphocytes using alkaline and neutral variants of the comet assay. Treatment was performed on non-proliferating and proliferating lymphocytes to evaluate whether the status of cells has an impact on the DNA damage level. Therefore, we evalu-

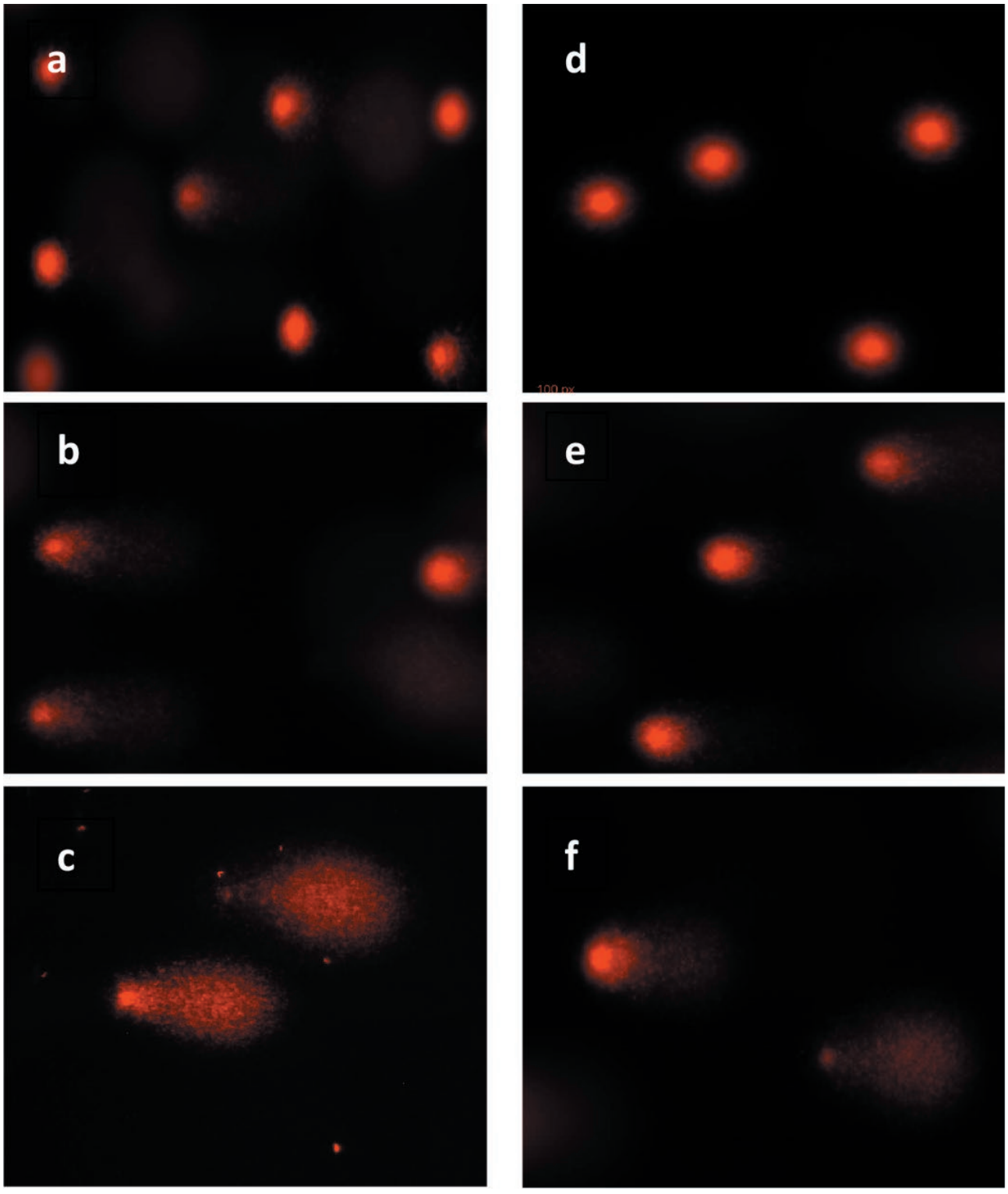

Figure 5. DNAdamage was investigated after exposure of bovine peripheral lymphocytes to epoxiconazole. The cells were treated with the fungicide for 2 h (non-proliferating lymphocytes) and for the last 2 h of the 48-hour culture (proliferating lymphocytes). Positive control was H_2O_2 (5 min). The results of the alkaline comet assay are shown in the first column (picture a, b, c) and the neutral comet assay in the second column (d, e, f). Negative control: a, d. Selected concentration 50 µg/mL: b, e. Positive control: c, f.

ated two different experiments. The first one was with non-dividing (non-proliferating) lymphocytes exposed to EPX immediately after isolation for 2 hours, as indicated by Calderón-Segura et al. (2012). The second with lymphocytes stimulated to divide by phytohaemagglutinin (PHA) during 48 hours taking account the results of Bausinger and Speit (2014) who revealed that DNA synthesis starts in T lymphocytes (similarly like in peripheral blood mononuclear cells, PBMC) around 24 h after stimulation with PHA. Therefore we chose 48-hour (24 h plus 24 h) cultivation allowing lymphocyte proliferation in the medium at least during one cell cycle. We treated the cultured lymphocytes with EPX the last 2 h for the examination of the epoxiconazole ability to induce DNA damage in proliferating cells. Using alkaline comet assay we showed that epoxiconazole induced statistically significant DNA damage in both non-dividing (without PHA) and dividing (with PHA stimulation) lymphocytes of cattle. EPX induced DNA migration in PHA-stimulated cultured lymphocytes in the same range of concentrations like in non-stimulated ones but to a different extent; less DNA migration was observed in PHA-stimulated cells. It is likely that these results correspond with different capability of proliferating cells to repair DNA damage. On the contrast to alkaline comet assay, neutral comet assay showed that DNA breaks were induced in different ranges of concentrations in proliferating (dividing) lymphocytes (from 10 μg/mL) when compared with non-proliferating cells (from 2.5 μg/mL) (Fig. 2*a, b*). One of explanation might be that the lower concentration did not induce DNA damage or that neutral comet assay detects mostly double-strand breaks (Lu et al. 2017), which were probably more effectively repaired in proliferating lymphocytes than in non-proliferating ones.

The results of the comet assay can be affected by the exposure time which is one of a crucial factor. Long incubation periods may not be appropriate for the comet assay because DNA lesions may be repaired during the time that mutagens are inactivated, leading to false negative results (Sekihashi et al. 2003). According to Tice et al. (2000), an appropriate exposure time for chemical *in vitro* genotoxicity assessment should be around 3 to 6 hours; other papers refer 1 h, 2 h, 4 h or 24 h exposure times (Lebaily et al. 1997; Calderón-Segura et al. 2012; Želježić et al. 2016).

It is known that cattle can accumulate foreign substances not only in the liver but also in the muscle (García-Repetto et al. 1997), milk (Pokorná et al. 1996) and fat (Ferré et al. 2018) thereby increasing the genetic risk to humans through the food chain. Guitart et al. (2010) reported that as a result of the application of fungicides in agricultural production, livestock poisoning may occur, the clinical manifestations of which are only rarely addressed. Exposure of livestock to genotoxic substances may also induce mutations, lead to metabolic disorders, immunosuppression and decreased fertility. Cattle are exposed to chemicals during grazing, so adverse effects may occur primarily in them. Besides, some of the chemical agents have a long-term cumulative effect and can contribute to cancer through chronic exposure.

Genotoxicity assessment is an essential component of the safety analysis of all types of substances, ranging from pharmaceuticals, industrial chemicals, pesticides, biocides, food additives, cosmetics ingredients, to veterinary drugs, relevant in the context of international legislation aiming at the protection of human and animal health (ECVAM 2013). As reported by Bolognesi and Morasso (2000) pesticides have been considered potential chemical mutagens. The genotoxicity of pesticides is generally considered to be the most serious of the possible side effects of their usage. The formation of highly reactive substances during oxidation processes, coupled with the ability to interact with DNA, leads to a series of measurable changes, for example point mutations, chromosomal rearrangements, DNA adducts, DNA strand fragments and increased number of micronuclei (Medina et al. 2007). There are several studies testing the genotoxicity of pesticides and mycotoxins on bovine lymphocytes (Lioi et al. 2004; Holečková et al. 2013; Schwarzbacherová et al. 2017; Ferré et al. 2020). Šiviková et al. (2018) tested epoxiconazole *in vitro* in cultured bovine peripheral lymphocytes using chromosome aberrations, sister chromatid exchanges and micronucleus test. She found that epoxiconazole was not related to genotoxic and / or clastogenic / aneugenic effects, but had the ability to significantly affect cell-cycle kinetics and induce apoptosis.

Our results show that epoxiconazole can induce significant levels of DNA damage in bovine lymphocytes, as revealed in both alkaline and neutral variants of the comet assay. On the other hand, no statistically significant DNA damage was detected by Drážovská et al. (2016), who investigated DNA damage using alkaline comet assay after 2 h exposure of bovine lymphocytes to Tango® Super fungicide (epoxiconazole/fenpropimorph). Potential genotoxic/cytotoxic effects of the epoxiconazole/fenpropimorph-based fungicide were also investigated by cytogenetic assays: chromosomal aberrations, sister chromatid exchanges, micronuclei and fluorescence *in situ* hybridization. The final results indicated that the tested fungicide was capable of evoking cytotoxic effect / cell-cycle delay in peripheral cattle lymphocytes. On the contrary Schwarzbacherová et al. (2017)

reported stimulation od DNA-double strand breaks after 4 h exposure to epoxiconazole / fenpropimorph-based fungicide (Tango Super) using neutral comet assay. When compared with pure EPX, the results of both studies mentioned above were probably affected by the presence of fenpropimorph and inert ingredients in the tested pesticide formulation, as well as by different exposure times and variants of comet assay.

Similarly to our observations, significantly increased percentages of comets and tail lengths were obtained after epoxiconazole treatment in the human colon carcinoma cell line (HCT116) (Hamdi et al. 2018). Epoxiconazole was able to induce a range of cell damage in HCT116 cells by generating ROS, which in turn induces mitochondrial DNA dysfunction and fragmentation leading to cell death, as confirmed by the attenuated death of cells treated with the antioxidant N-acetylcysteine (NAC) prior to treatment with epoxiconazole. In the later study with F98 glioma cells the same author (Hamdi et al. 2019) showed that EPX induced cytotoxic effects, cell cycle arrest, cytoskeleton disruption, DNA damage and apoptosis via caspases dependent signalling. In addition Akram et al. (2019) confirmed that epoxiconazole was the potent inhibitor of 11-bhydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) in hamster and human adrenal H295R cells; these enzymes catalyse the formation of cortisol and aldosterone in the adrenal cortex therefore in this study epoxiconazole seems to be an endocrine disruptor. Similarly, Taxvig (2007) concluded that disruption of a crucial enzyme such as CYP17, which is involved in steroid synthesis hormone, is one of the main endocrine-disrupting mechanisms of azole fungicides like tebuconazole and epoxiconazole.

In our experiment, bovine lymphocytes were tested under *in vitro* conditions to obtain significant results, preceded by the testing of several methods and procedures to create optimal experimental conditions. Treatment of cells with epoxiconazole was followed by determination of cell viability for each test concentration using the trypan blue exclusion method, where the percentage of viability represents the number of viable cells compared to the total number of cells surviving after treatment. The cell viability was greater than 90% at all concentrations tested. Tice et al. (2000) recommended that *in vitro* treatment with chemicals should not reduce cell viability by more than 30%, and extended this similarly to *in vivo* experiments. Other researcher maintain that the allowed cell viability after exposure should be at least 70-75% (Želježić et al. 2018), or above 85 % (Evans et al. 2016), and some also report up to 95% (Lebailly et al. 2015) upon performing comet analysis.

In general, the question of determining the sensitivity of both alkaline and neutral comet analysis is frequently discussed, so the comparison of sensitivity of individual methods is interesting from the practical point of view (Afanasieva and Sivolob 2018; Azqueta and Collins 2013; Peycheva et al. 2009; Collins et al. 2008). The alkaline variant demonstrates increased sensitivity in the investigation of agents causing DNA strand breaks or inducing alkaline labile lesions of DNA. Currently, the first choice is to detect low levels of DNA damage, either in lymphocyte samples or in genotoxicity testing *in vitro* and *in vivo*. According to other authors, the neutral variant is more sensitive than the alkaline one. For instance, Afanasieva et al. (2009) indicate that the neutral variant is the more sensitive method for assessing small numbers of DNA breaks.

CONCLUSION

Our data acquired using alkaline and neutral comet assays suggest that epoxiconazole induces significant DNA damage in both non-proliferating and proliferating bovine lymphocytes.

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