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ANALYSIS OF SISTER CHROMATID EXCHANGES AND PROLIFERATION OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES EXPOSED TO EPOXICONAZOLE

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ABSTRACT

The potential genotoxic/cytotoxic effect of epoxiconazole was evaluated by means of sister chromatid exchanges (SCE) following the 24 and 48 h *in vitro* exposure of human peripheral blood lymphocytes to epoxiconazole at concentrations of: 5, 10, 25, 50 and 100 µg.ml^{-1}. Dimethyl sulphoxide (DMSO), used as an epoxiconazole solvent, was used as a negative control and mitomycine (MMC) as a positive control. After the 24-hour exposure, we failed to observe a significant increase in SCE frequencies in comparison with the negative control, however, the concentrations of 10—100 µg.ml^{-1} caused a significant decrease in the proliferation index (PI; $P < 0.001$). Also, the 48-hour exposure produced no significant alterations in the SCE frequencies in comparison with the control. At epoxiconazole concentrations ranging from 10 to 50 µg.ml^{-1} we recorded a moderate to strong, dose-dependent inhibition of PI ($P < 0.05$; $P < 0.01$; $P < 0.001$), while at the highest dose (100 µg.ml^{-1}) the reduction in PI compared to the control was less pronounced ($P < 0.05$). The reduction in PI at the concentration range of 10—100 µg.ml^{-1} depended on the number of cells in the M_1, M_2 and M_3 phases of the cell cycle per total number of 100 evaluated metaphases. Our results indicated a significant cytotoxic or cytostatic effect on human peripheral blood lymphocytes.

Key words: epoxiconazole; proliferation index; sister chromatid exchanges

INTRODUCTION

Azole antimycotics are widely used not only in agriculture but also in human and veterinary medicine against a broad spectrum of fungal infections [6]. The mechanism of effect of azole compounds is based on an inhibition of the enzyme lanosterole 14α-demetylase (CYP51), responsible for conversion of lanosterole. Because of this inhibition, ergosterole, the essential component of membranes of yeasts and fungi, is not produced [3]. In agriculture, epoxiconazole has appeared effective in the prevention and treatment of fungal infections of plants caused by an as-

comycete fungus *Mycosphaerella fijiensisa* and *M. musicola* (black and yellow sigatoka) from the family *Mycosphaerellaceae*, occurring on banana leaves, and rust forming fungus *Hamileia vastatrix* (*Pucciniaceae*) parasitizing leaves of plants from the genus *Coffea* [2]. A characteristic feature of epoxiconzole is its low biodegradability in the environment that may result in its accumulation in the soil and, subsequently, contamination of water [7]. Water fauna is therefore considerably used as suitable objects in the evaluation of toxic and teratogenic effects of various azole compounds. For example, Zhu et al. [15] exposed *Gobiocypris rarus* embryos to five triazole compounds (myclobutanil, fluconazole, flusilazole, triflumizole and epoxiconazole) at various concentrations and confirmed their teratogenic effects. Because azole compounds are capable of inhibiting biosynthesis of androgens and oestrogens, one may assume that chronic exposure to these compounds could result in a number of disturbances in humans and other animals, for example reproductive disorders, foetal malformations, feminisation, neuro-behavioural and tumorous diseases [12, 16].

With regard to the potential exposure of humans and animals to azole compounds we focused on the investigation of the potential *in vitro* genotoxic and/or cytotoxic effects of epoxiconazole by means of analysis of SCE and PI in human peripheral blood lymphocytes.

MATERIALS AND METHODS

Materials and the tested compounds

Epoxiconazole (CAS registration number 133855-98-8, purity 99 %, Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulphoxide (DMSO, Sigma, St. Louis, MO, USA) and added to culture media at concentrations of: 5, 10, 25, 50 and 100 $\mu g.ml^{-1}$. DMSO served as a negative control and its final concentration in the experimental and control media was 0.1 %. Mitomycine (MMC) (Sigma, St. Louis, MO, USA, 0.4 $\mu g.ml^{-1}$) was used as a positive control.

In the experiment we used lymphocytes from the peripheral blood of a healthy, 20 years old woman.

Cultivation of lymphocytes

The culture medium contained 4 ml RPMI 1640 (Sigma Chemical Co., St. Louis), L-glutamine and 15 µmol HEPES (GE Healthcare Hycone Lab, Utah, USA), 1 ml BoFeS (bovine foetal serum; Sigma Chemical Co., St. Louis, MO, USA), phytohaemagglutinine (PHA; 20 $\mu g.ml^{-1}$, Welcome, Dartford, England), mixture of antibiotics and antimycotics (100 U. ml^{-1} penicillin, 0,1 $mg.ml^{-1}$ streptomycin a 0,25 $mg.ml^{-1}$ amphotericin, Sigma Chemical Co., St. Louis, MO, USA) and 0.2 ml of blood. The cultivation took place in test tubes in a thermostat set to 37 ± 1 °C and lasted 72 hours. Fifty µl aliquots of epoxiconazole solution in DMSO of concentrations specified above were added to the cultures 24 and 48 hours before termination of cultivation. After 24 hours of cultivation, we added bromdeoxyuridine (BrdU; 8.0 $\mu g.ml^{-1}$, Sigma Chemical Co., St. Louis, MO, USA) to all culture flasks (control and experimental) in order to detect SCE and differentiate the cell cycles. For detection of SCE and differentiation of cell cycles in metaphases we used the FPG (Fluorescence Plus Giemsa) method.

Evaluation of results and statistical methods

For each concentration and time of exposure we counted SCE in 30 metaphases selected at random (M_2 cells, i. e. cells in the second cell cycle). At the same time, we calculated and recorded the numbers of cells in the first, second and third cycle from among the total number of 100 evaluated cells. PI was calculated by the following formula:

$$PI = (M_1 + 2M_2 + 3M_3)/N$$

Where: M_1 is the number of metaphases in the first (uniformly stained chromosomes) cell cycle; M_2 in the second (the so-called harlequin chromosomes) and M_3 in the third cycle (Fig. 1) and N is the total number of metaphases, altogether in 100 cells.

The statistical evaluation of the occurrence of SCE in the experimental groups in comparison to the control was carried out by ANOVA and subsequently by the Student's t-test; the proliferation rate indices were evaluated by the χ^2 test. The significance level was set to $P < 0.05$.

RESULTS

Detection of SCE in human lymphocytes exposed to epoxiconazole

The results of the detection of the sister chromatid exchanges following the exposure to epoxiconazole are shown in Fig. 2 and the relationship between the PI and epoxiconazole concentration is depicted in Fig. 3.

After 24-hour exposure, we failed to observe a signifi-

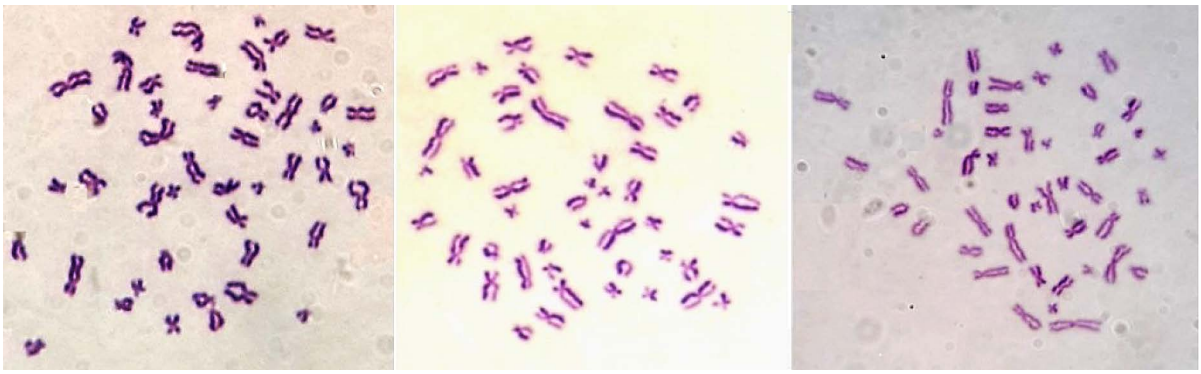

Fig. 1. M_1, M_2 and M_3 cell cycle phases

On the left: M_1 phase, uniformly stained, dark chromosomes (46, XX). In the centre: M_2 phase with BrdU incorporated in both DNA strands of one chromatid — during two cell cycles (light chromatid), second chromatid coloured dark — BrdU is incorporated in one of two DNA strands, the so-called harlequin chromosomes that enable evaluation of SCE. On the right: M_3 phase, chromosomes are light (with incorporated BrdU in both DNA strands of both chromatids) or of harlequin type

cant increase in SCE frequency in comparison with the negative control (DMSO), however, starting with the concentration of 10 μg.ml^{-1}, up to the concentration of 100 μg.ml^{-1}, we recorded a significant decrease in the proliferation rate index (PI; $P < 0.001$; χ^2 test).

Similarly, after the 48-hour exposure, there were no significant changes in the SCE frequency in comparison with the control. Starting from the epoxiconazole concentration of 10 μg.ml^{-1} up to 50 μg.ml^{-1} we recorded a dose-dependent inhibition of PI ($P < 0.05$; $P < 0.01$; $P < 0.001$; χ^2 test), but at the highest dose (100 μg.ml^{-1}) the PI reduction in comparison with the control was less pronounced ($P < 0.05$; χ^2 test).

The differences in the number of the observed M_1, M_2 and M_3 metaphases produced by individual concentrations are illustrated in Fig. 4 and Fig. 5. Fig. 6 shows metaphase M_2 with sister chromatid exchanges marked with arrows.

The curves show proportions of metaphases in the first, second and third cell cycles in dependence on concentration of epoxiconazole. DMSO served as a negative control and MMC as a positive control. We evaluated 100 mitoses for each concentration.

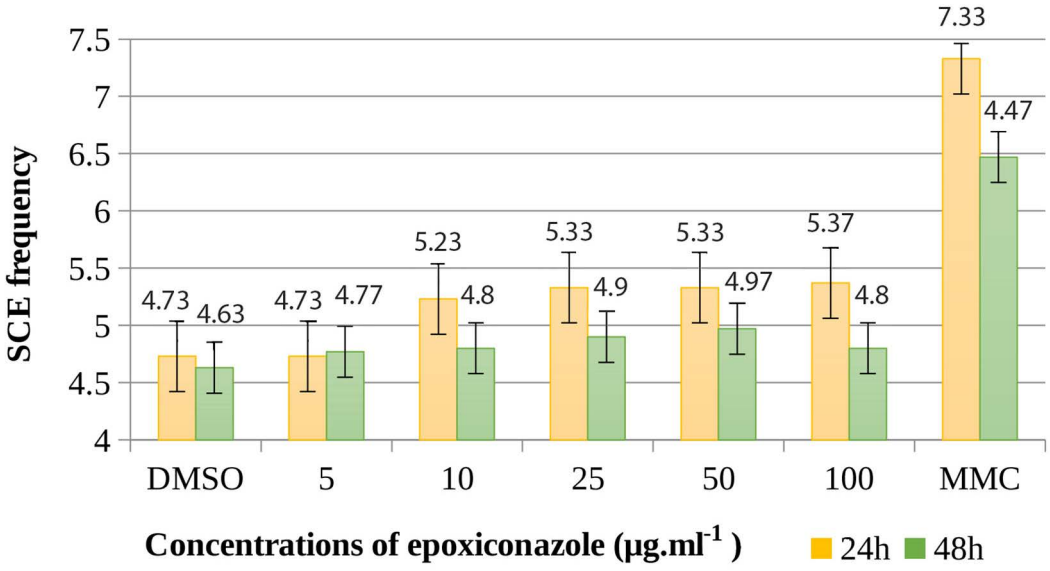

Fig. 2. Relationship between SCE frequency and concentration of epoxiconazole after 24h and 48h exposure of human peripheral blood lymphocytes

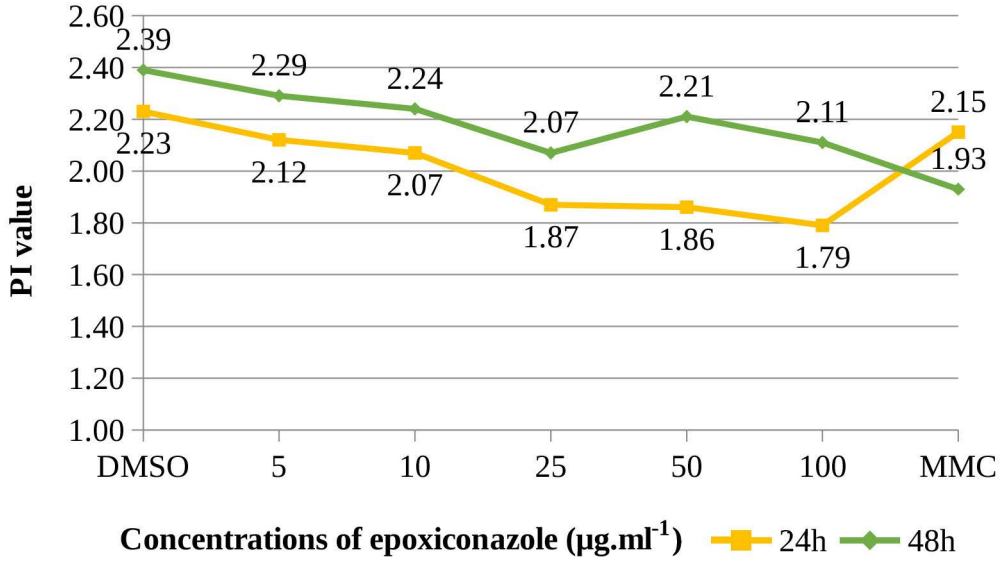

Fig. 3. Relationship between PI value and concentration of epoxiconazole after 24h and 48h exposure of human peripheral blood lymphocytes

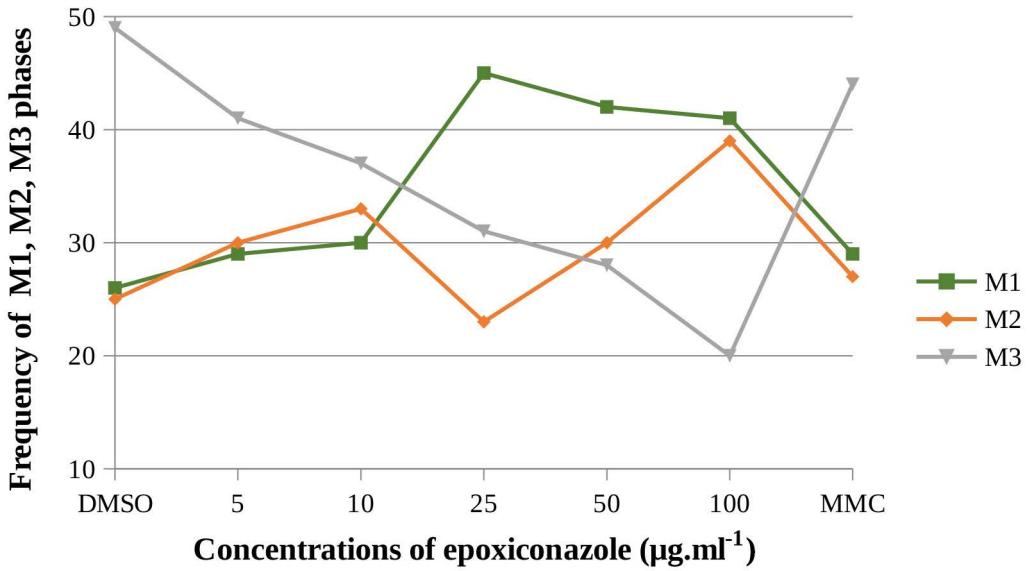

Fig. 4. Relationship between frequency of M_1, M_2 and M_3 phases of the cell cycle and the concentration of epoxiconazole after 24 h exposure

DISCUSSION

The evaluation of sister chromatid exchanges (SCE) in the human peripheral blood lymphocytes is one of the complex of cytogenetic methods required by Organisation for Economic Cooperation and Development (OECD) for the evaluation of the potential mutagenic/carcinogenic effects of chemicals. Despite its advantages, there are two main practical issues associated with its use. First, after the exposure to genotoxic chemicals, the SCE frequencies are evaluated only in cells that have reached the metaphase stage. The cells are able to reach this stage after exposure to non-cytotoxic doses of chemicals. The cells with the cell cycle arrested in the G_2 phase are not included in the analysis. Because the undamaged cells, the cell cycle of which was not affected, pass through metaphase, the conventional SCE analysis cannot sufficiently detect the genotoxic/mutagenic potential of various chemical

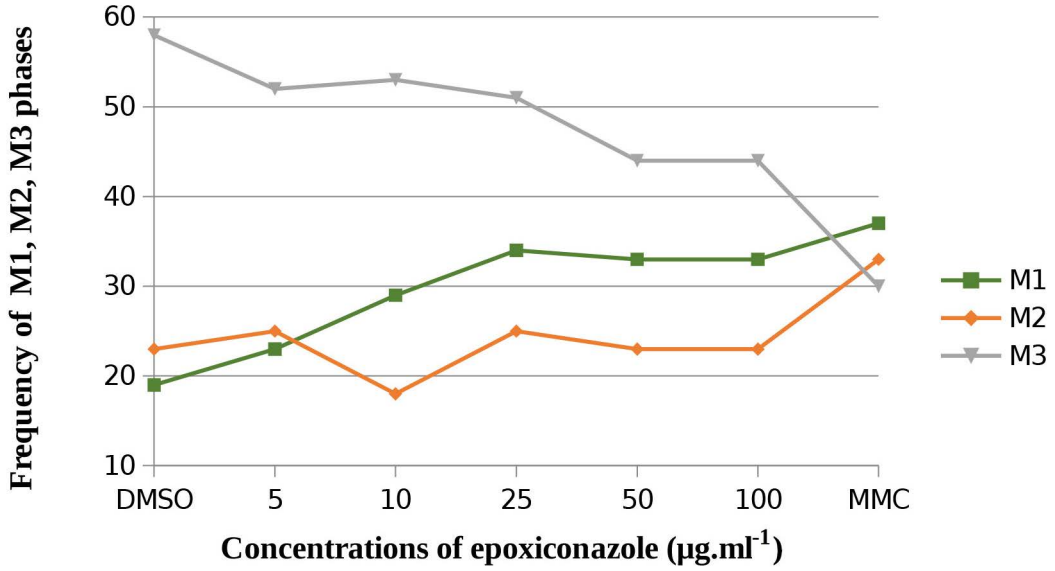

Fig. 5. Relationship between frequency of M_1, M_2 and M_3 phases of the cell cycle and the concentration of epoxiconazole after 48 h exposure

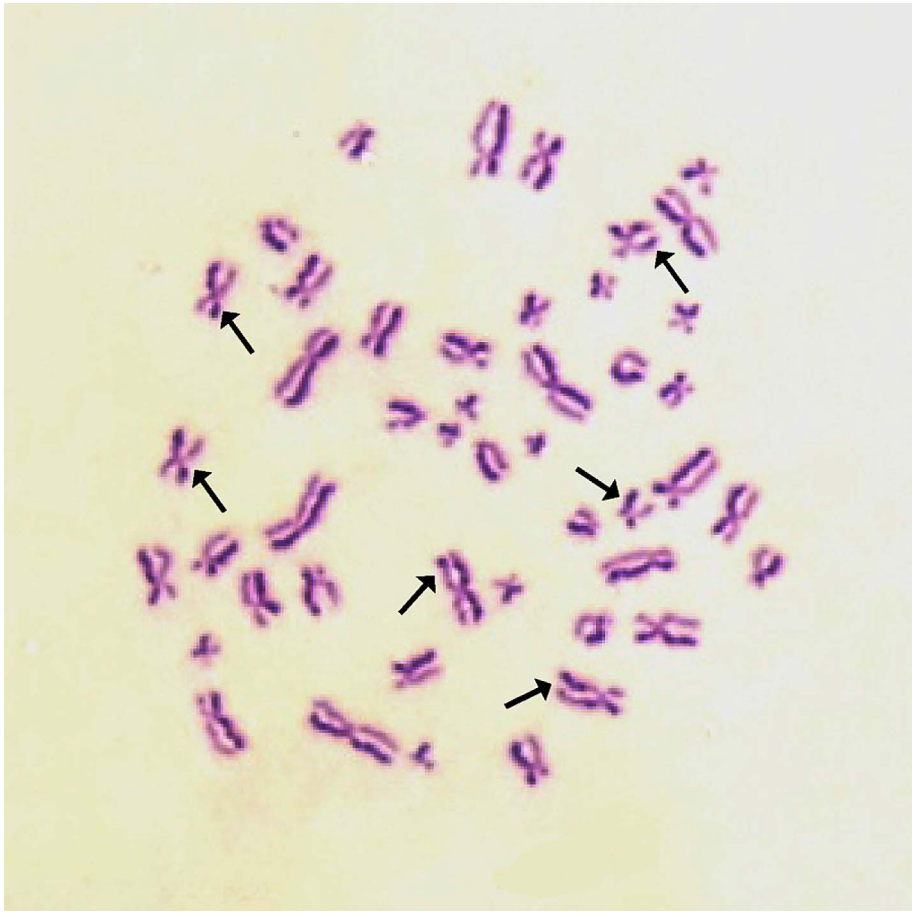

Fig. 6. M_2 phase of the cell cycle
Human metaphase — woman (46, XX); second cell cycle (M_2 phase) — harlequin chromosomes; sister chromatid exchanges are marked with arrows

compounds. Second, the influence of the concentration of BrdU incorporated in DNA can be sometimes higher than the effect associated with the exposure to genotoxic carcinogens [13].

Our experiment, that involved analysis of SCE after 24 and 48 h exposure to epoxiconazole, showed no genotoxic effect of pure azole antimycotic on human peripheral lymphocytes (Fig. 2).

Amara et al. [1] described a synergistic effect of epoxiconazole and pyrethroid insecticides and herbicides on aquatic crustaceans and phytoplankton and their studies allowed them to present an assumption that azole antimycotics are toxic to aquatic fauna — crustaceans, fish and some algae. Similarly, biomonitoring studies that involved farmers professionally exposed to pesticides (cyproconazole, epoxiconazole, tebuconazole, hexaconazole and other) conducted by Lebailly et al. [5] documented a significant damage to DNA detected by alkaline single-cell gel electrophoresis (SCGE — single-strand breaks, alkaline labile DNA sites and double-strand breaks). Ross et al. [8, 9] conducted a study on murine liver cells and observed that triadimefon at a dose of 1800 ppm and propiconazole at a dose of 2500 ppm induced increased frequencies of mutations during a 4-day exposure. Additional authors, for example, Zhu et al. [15] demonstrated teratogenic effects on embryos of *Gobiocypris rarus* fish.

In agreement with our results obtained in the study on human lymphocytes, no genotoxic and/or clastogenic effect of epoxiconazole was confirmed in cultivated bovine peripheral lymphocytes [11]. However, the proliferation rate was affected significantly after 24 and 48 h of exposure to epoxiconazole with cytotoxic or cytostatic effects. In a similar experiment conducted by Schwarzbacherová et al. [10], the authors obtained negative results by evaluation of clastogenic and aneugenic effects of commercial triazole fungicide containing tebuconazole and protioconazole on bovine peripheral lymphocytes *in vitro*. In their evaluation they used a micronuclei and comet assays. The observed inhibition of cytokinesis-block proliferation index (CBPI) indicated a significant cytostatic/cytotoxic effect of the investigated fungicide.

Our experiments showed a pronounced effect on proliferation at both exposures of human lymphocytes to epoxiconazole (basic and prolonged). In dependence on concentration, we recorded a reduction in PI in the concentration range of 10—100 $\mu g.ml^{-1}$ after 24 h exposure and in the range of 10—50 $\mu g.ml^{-1}$ after 48 h exposure to epoxiconazole. On the basis of the reduced number of M_1 metaphases and persistence of cells in M_2 and M_3 cell cycles (Fig. 5) we assumed that after 48 h exposure to epoxiconazole, the cell division was arrested in the G_1/S phase of the cell cycle, therefore the cells could not proceed to the replication phase. This indicates the cytotoxic/cytostatic effect of epoxiconazole on the peripheral lymphocytes (Fig. 5).

A similar effect of azole compounds on cell proliferation was documented also by other authors. For example, Yilmaz et al. [14] described a genotoxic effect of a preparation Conan 5FL, containing hexaconazole (50 $g.l^{-1}$), on murine bone marrow cells *in vivo* and human lymphocytes *in vitro* and a significant, dose-dependent decrease in mitotic index (17.5, 30 and 70 $\mu g.ml^{-1}$).

Our results agree also with those obtained by Herrera–Martínez et al. [4], who evaluated the effects of ketoconazole (imidazole antimycotic) on cell lines BON-1 (human serotonin-producing neuroendocrine pancreas tumour, derived from lymph node metastases) and DMS-79 (small-cell lung carcinoma). A significant suppression of cell growth by ketoconazole in BON-1 cells was dose and time dependent. The authors recorded a maximum inhibitory effect (41—95 %) at the concentration of 10 µM after 3 to 7 days ($P < 0.0001$). Ketoconazole also induced stopping of the cell cycle in the G_1 phase, accompanied by reduction of S and G_2 phases and significantly induced apoptosis ($P < 0.001$). In the less susceptible DMS-79 cells, the authors observed the highest inhibitory effect (44—94 %) at concentration of 50 µM after 3 to 7 days. Ketoconazole at the concentration of 10 µM suppressed the secretion of adrenocorticotropic hormone.

The results of our experiments allowed us to conclude that the concentrations used of epoxiconazole did not induce damage to the DNA or chromosomes but significantly affected the proliferation as manifested by the cytotoxic and cytostatic effects.

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REFERENCES

1. **Amara, A., Quiniou, F., Durand, G., EL Bour, M., Boudabous, A., Hourmant, A., 2013:** Toxicity of epoxiconazole to the marine diatom Chaetoceros calcitrans: Influence of growth conditions and algal development stage. *Water Air Soil Pollut.*, 224, 1—9.
2. **EPA (Office of Prevention, Pesticides and Toxic Substances), 2006:** *Pesticide Fact Sheet: Epoxiconazole*, 21 pp.
3. **Heise, T., Schmidt, F., Knebel, C., Rieke, S., Haider, W., Pfeil, R., et al., 2015:** Hepatotoxic effects of (tri)azole fungicides in a broad dose range. *Arch. Toxicol.,* 89, 2105—2117.
4. **Herrera-Martínez, A. D., Feelders, R., Castano, J., Dogan, F., Koetsvelt, P., Hofland, L., 2017:** Effects of ketoconazole on ACTH-producing and non ACTH-producing neuroendocrine tumour cells. *Endocrine abstracts*, 49, 182.
5. **Lebailly, P., Gladys, M., Herin, F., Lecluse, Y., Sales, B., Boutet-Robinet, E., 2015:** DNA damage in B and T lymphocytes of farmers during one pesticide spraying season. *Int. Arch. Occup. Environ. Health*, 88, 963—972.
6. **Pérez-Rivera, A. A., Hu, T., Aardema, M. J., Nash, J. F., 2009:** Evaluation of the genotoxicity of the imidazole antifungal climbazole: Comparison to published results for other azole compounds. *Mutat. Res.*, 672, 27—39.
7. **Roelofs, M., Temming, A. R., Piersma, A. H., Van Den Berg, M., Van Dursen, M. B. M., 2014:** Conazole fungicides inhibit Leydig cell testosterone secretion and androgen receptor activation *in vitro*. *Toxicol. Rep.*, 1, 271—283.
8. **Ross, J., Moore, T., Leavitt, S. A., 2009:** *In vivo* mutagenicity of conazole fungicides correlates with tumorigenicity. *Mutagenesis*, 24, 149—152.
9. **Ross, J., Leavitt, S. A., Schmid, J. E., Nelson, G. B., 2012:** Quantitative changes in endogenous DNA adducts correlate with conazole *in vivo* mutagenicity and tumorigenicity. *Mutagenesis*, 27, 541—549.
10. **Schwarzbacherová, V., Šiviková, K., Drážovská, M., Dianovský, J., 2015:** Evaluation of DNA damage and cytotoxicity induced by triazole fungicide in cultured bovine lymphocytes. *Caryologia*, 68, 233—238.
11. **Šiviková, K., Holečková, B., Schwarzbacherová, V., Galdíková, M., Dianovský, J. 2018:** Potential chromosome damage, cell-cycle kinetics/and apoptosis induced by epoxiconazole in bovine peripheral lymphocytes *in vitro*. *Chemosphere*, 193, 82—88.
12. **Taxvig, C., Hass, U., Axelstad, M., Dalgaard, M., Boberg, J., Andeasen, H. L., et al., 2007:** Endocrine-disrupting activities *in vivo* of the fungicides tebuconazole and epoxiconazole. *Toxicol. Sci. Off. J. Soc. Toxicol.*, 100, 464—473.
13. **Terzoudi, G. I., Malik, S. I., Pantelias, G. E., Margaritis, K., Manola, K., Makropoulos, W., 2003:** A new cytogenetic approach for the evaluation of mutagenic potential of chemicals that induce cell cycle arrest in the G_2 phase. *Mutagenesis,* 18, 539—543.
14. **Yilmaz, S., Aksoy, H., Unal, F., Celik, M., Yuzbaloglu, D., 2008:** Genotoxic action of fungicide Conan 5FL (hexaconazole) on mammalian cells *in vivo* and *in vitro*. *Russian J. Genet.*, 44, 273—278.
15. **Zhu, B., Liu, L., Gong, Y. X., Ling, F., Wang, G. X., 2014:** Triazole-induced toxicity in developing rare minnow (*Gobiocypris rarus*) embryos. *Environ. Sci. Pollut. Res. Int.,* 21, 13625—13635.
16. **Lv, X., Pan, L., Wang, J., Lu, L., Yan, W., Zhu, Y., et al., 2017:** Effects of triazole fungicides on androgenic disruption and CYP3A4 enzyme activity. *Environ. Pollut.*, 222, 504—512.

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