

ASSESSMENT OF TRYPTOPHOL GENOTOXICITY IN FOUR CELL LINES *IN VITRO*: A PILOT STUDY WITH ALKALINE COMET ASSAY

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Tryptophol is an aromatic alcohol and secondary metabolite of the opportunistic fungus *Candida albicans*. Although its toxicity profile at cell level has been poorly investigated, recent data point to cytotoxic, cytostatic, and genotoxic effects in lymphocytes and the induction of apoptosis in leukaemic blood monocytes. In this pilot study we evaluated the genotoxicity of tryptophol *in vitro* on four permanent cell lines of animal and human origin: ovary cells, alveolar epithelium, liver cells, and blood monocytes using the alkaline comet assay. We selected cells that might be principal targets of tryptophol and other low-molecular geno(toxins) secreted by *Candida albicans* during host invasion. Our results suggest that tryptophol applied *in vitro* at 2 mmol L⁻¹ for 24 h damages DNA in HepG2, A549 and THP-1 cells, obviously due to bioactivation and/or decomposition of the parent compound, which results in the formation of more genotoxic compound(s) and production of reactive species that additionally damage DNA. On the other hand, notably lower levels of primary DNA damage were recorded in CHO cells, which lack metabolic activity. Future studies with tryptophol should look further into mechanisms involved in its toxic action and should focus on other cell types prone to infection with *Candida* spp. such as vaginal epithelial cells or keratinocytes of human origin.

KEY WORDS: *aromatic alcohol, Candida albicans, DNA damage, permanent cell lines, secondary metabolite*

Microorganisms produce various secondary metabolites, low-molecular-weight compounds that are not essential for their normal development, but play an important role in cell defence and survivability and also mediate cell-to-cell communication. The last, also termed as “quorum sensing” is a well-known cell-signalling mechanism in bacteria (1). *Candida albicans* is the first simple eukaryotic microorganism described to possess the quorum-sensing ability (2). Its main quorum-sensing molecules are aromatic alcohols tyrosol, farnesol, tryptophol, and 2-phenylethanol (2-4).

Although *C. albicans* is one of the most important human pathogens, the mechanisms of infection with this opportunistic fungus are still not fully elucidated. The members of genus *Candida* normally exist as a commensal microflora on the skin, mucosal surfaces, and in the gastrointestinal tract of healthy humans and animals (5). In some conditions, however, they become harmful and invade their host. Recent findings suggest that the virulence of *C. albicans* is linked to

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its ability for phenotypic switching from yeast-form to filamentous growth (6). Oval yeast cells are more prevalent in commensal interactions, while in opportunistic infections filamentous forms prevail. Such infections range from localised mycoses (superficial and deep-seated ones) to serious systemic diseases that may lead to death (mainly in immunocompromised hosts) (5, 7).

The morphological transition of *C. albicans* is influenced by numerous stimuli such as increased pH and temperature, low availability of nutrients, low cell density, and the presence of certain compounds that promote or block filamentation (7-9). Aromatic alcohol tyrosol, for instance, stimulates morphological transition of *C. albicans* from yeasts to hyphae (3), while farnesol, farnesoic acid, tryptophol, and 2-phenylethanol mostly repress hyphal development (2, 4, 9-11).

The role of tryptophol (3-indole-ethanol) in the quorum sensing of *C. albicans* was established in 2000's (7). This aromatic alcohol (Figure 1) is formed as the end product of tryptophan catabolism in certain

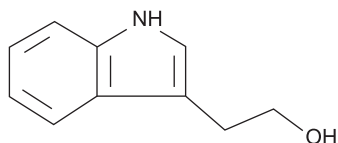


Figure 1 Structural formula of tryptophol

bacteria, yeasts, and fungi (7, 12-14). In higher plants, tryptophol is an endogenous constituent and growth regulator (15). It is also a product of tryptophan metabolism by *Trypanosoma brucei* and is involved in the pathophysiology of sleeping sickness caused by these blood parasites (16, 17). Tryptophol has also been found in the bloodstream of patients with chronic trypanosomiasis (18).

The toxicity profile of tryptophol at the cell level is not complete. Tanaka et al. (19) observed mutagenic effects of 1-nitrosotryptophol and 6-nitrosotryptophol in Ames' *Salmonella typhimurium* tester strains. Others have found that it induces apoptosis in the human leukemic cell line U937 (20-22). In the dose range (0.25 to 2.00) mmol L⁻¹ tryptophol induced cytotoxic and cytostatic effects and caused DNA breaks in human lymphocytes (23), but was not mutagenic to Ames' *Salmonella* TA 98 and TA 100 (24).

Current evidence on tryptophol toxicity is obviously not sufficient for human risk assessment. Considering that infection with *C. albicans* - confirmed

as tryptophol producer (7, 14) - may be localised but can also become a systemic disease, various organs, tissues, and cell types in the host invaded by *C. albicans* are potential targets of tryptophol toxicity. It is also reasonable to anticipate that different cells would express different DNA sensitivity to tryptophol, but we have not got this information as yet. Assuming different exposure scenarios, in this pilot study we tried to establish the genotoxicity of tryptophol on four cell lines of animal and human origin: ovary cells, alveolar epithelium, liver cells, and blood monocytes. The level of primary DNA damage was evaluated using the alkaline comet assay. Regardless of the simple design, we expect that results of this study might help to better understand how important tryptophol is for the pathogenic action of *Candida* spp.

MATERIAL AND METHODS

Cell lines

We used commercially available cell lines (strain number in brackets): A549 (human Caucasian lung carcinoma, ECACC 86012804), CHO (Chinese hamster ovary cells, ECACC 85050302), HepG2 (human Caucasian hepatocyte carcinoma, ECACC 85011430), and THP-1 (human monocyte leukemia, ECACC 88081201) that were purchased from the European collection of cell lines (ECACC, United Kingdom).

Chemicals

Tryptophol (3-indole-ethanol, CAS No. 526-55-6; MW 161.20) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

If not otherwise specified, other chemicals and reagents were also purchased from the same supplier.

Culture conditions and toxicity testing

All cells were cultured in Dulbecco's modified Eagle's Medium supplemented with foetal calf serum (10 % V/V), penicillin (100 U mL⁻¹), streptomycin (100 µg mL⁻¹), and amphotericin B (0.25 µg mL⁻¹) at 37 °C in atmosphere humidified with 5 % CO₂.

One day prior to experiments, the cells were sub-cultured in 96-well microtitre plates. Each well contained from 50,000 to 75,000 cells (in the case of THP-1). Cells were treated with tryptophol in the

concentration of 2.00 mmol L⁻¹. As before use, tryptophol was dissolved in DMSO, this solvent was also added to control cultures. The final concentration of DMSO in the medium did not exceed 1 %. Treated cell cultures and controls were incubated at 37 °C for 24 h. Tested concentration of tryptophol and the exposure period were based on results obtained in previous dose-range studies (20, 23).

Each test was repeated four times and data were pooled.

The alkaline single-cell gel electrophoresis (comet assay)

Immediately after treatment, aliquots of each cell suspension (V=40 µL) were collected with sterile pipette and used for the preparation of agarose

Table 1 Alkaline comet assay findings for A549, HepG2, THP-1, and CHO cells treated in vitro with tryptophol in the concentration of 2 mmol L⁻¹ for 24 h and for corresponding controls

Alkaline comet assay	A549		HepG2		THP-1		CHO	
	Tryptophol	Control	Tryptophol	Control	Tryptophol	Control	Tryptophol	Control
Tail length / µm	17.08±0.35*	15.08±0.15	18.80±0.25*	16.11±0.13	15.80±0.38*	13.69±0.11	14.40±0.17	14.32±0.16
Tail intensity / DNA%	1.27±0.25*,#	0.49±0.06	0.95±0.09*,#	0.33±0.04	1.05±0.34	0.49±0.06	0.48±0.08*	0.26±0.04
Tail moment	0.22±0.05*	0.07±0.01	0.17±0.02*	0.05±0.01	0.19±0.07	0.06±0.01	0.07±0.01*	0.03±0.004

Data are expressed as mean ± S.E. of 200 comets measured for each sample. Statistical evaluation was performed using the Mann-Whitney U test. Values significantly higher than corresponding control (P<0.05) are marked with an asterisk (*). Intra-group differences were studied only for tail intensity using the one-way ANOVA with post hoc Schéffe test. # denotes values significantly higher (P<0.05) than for CHO cells

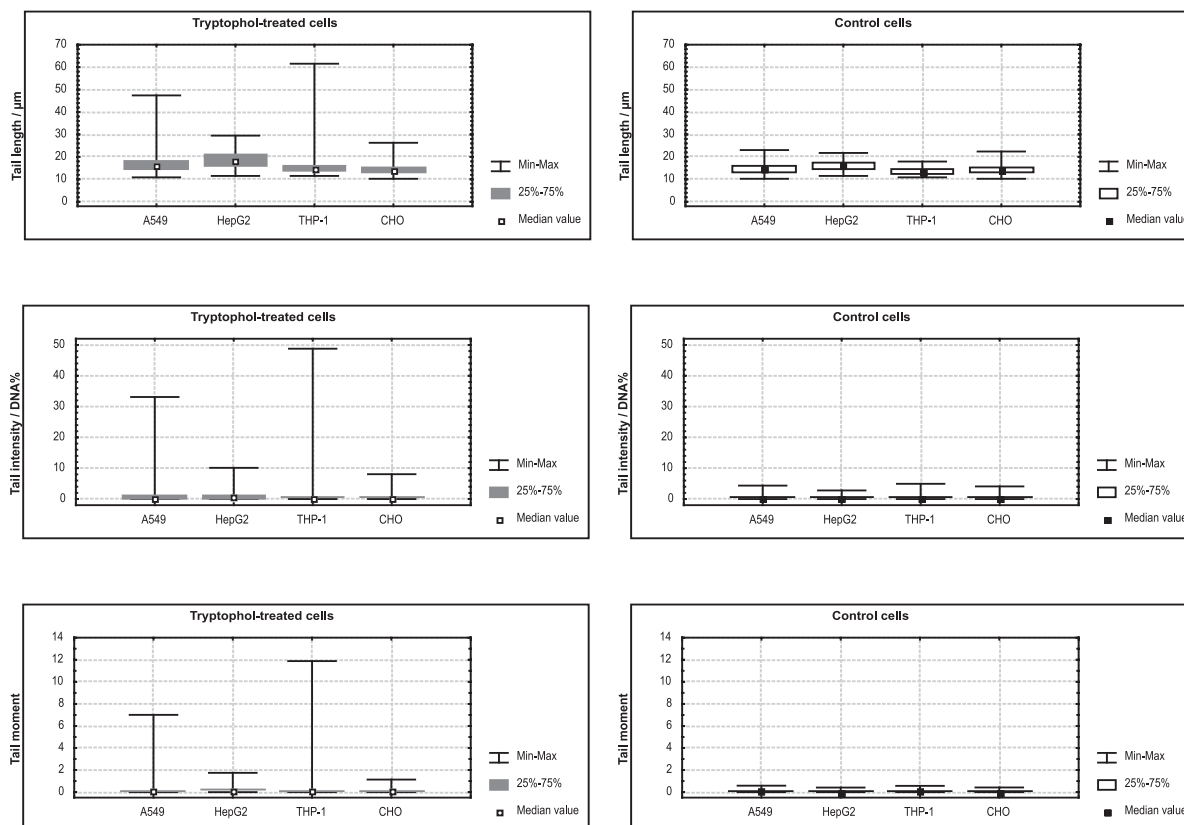


Figure 2 Alkaline comet assay findings for A549, HepG2, THP-1, and CHO cells treated in vitro with tryptophol in the concentration of 2 mmol L⁻¹ for 24 h and for corresponding controls

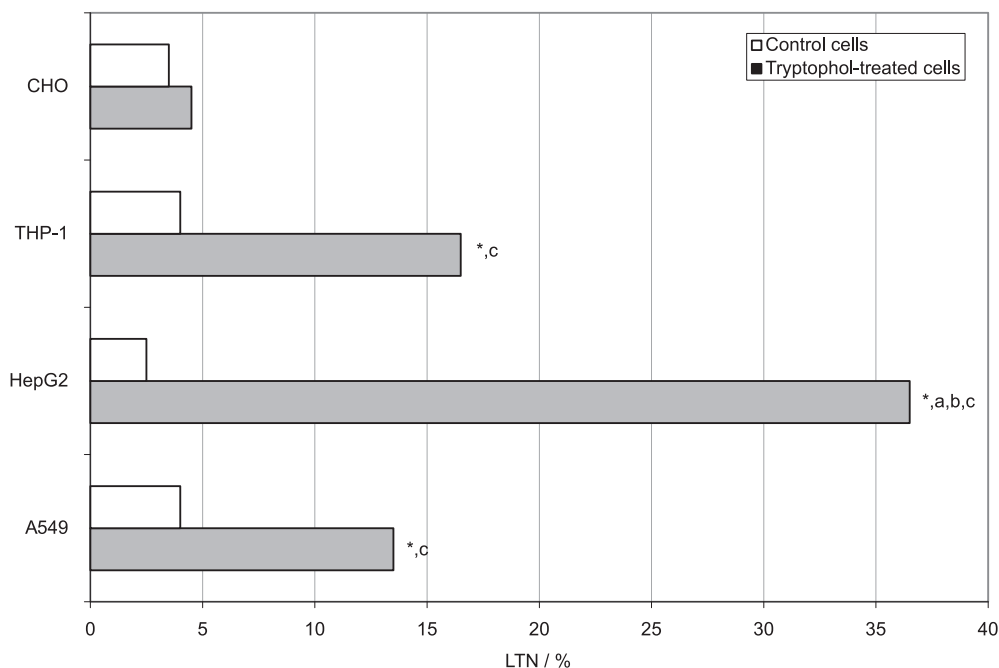


Figure 3 The percentage of long-tailed nuclei (LTN %) in A549, HepG2, THP-1, and CHO cells treated in vitro with tryptophol in the concentration of 2 mmol L⁻¹ for 24 h and in corresponding controls. Inter-group comparisons were performed using the chi-square test. Significantly increased values ($P < 0.05$) are: * vs. control; a - vs. A549 cells; b - vs. THP-1 cells; c - vs. CHO cells.

microgels that were processed following the standard protocol for the alkaline single-cell gel electrophoresis or comet assay (25). Slide preparation and other steps of the comet assay have been described in detail in our earlier paper (23). Microgels were stained with ethidium bromide (20 $\mu\text{g mL}^{-1}$). For each sample, four slides were analysed at 250x magnification under a fluorescence microscope (Leitz, Germany) coupled with a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd., United Kingdom). A total of 200 comets (4x50) per sample were measured. They were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells, and superimposed comets. Comet parameters evaluated were tail length, tail intensity (percentage of DNA in the comet tail), and tail moment.

Statistical analyses

The results are expressed as mean \pm standard error of mean, median, and range of the values measured for comet tail length, tail intensity, and tail moment.

Samples were compared based on logarithmically transformed data using the Mann-Whitney U test and one-way analysis of variance (ANOVA) followed by post-hoc Schéffe test (Statistica 9.0; StatSoft, USA).

For each control sample we also calculated the cut-off value (95th percentile) of the tail length. Comets

with tail lengths below the cut-off values were classified as “undamaged” and those with higher values as long-tailed nuclei (LTN) (26). The percentage of LTN (LTN %) was calculated for each sample. Statistical significance of differences in LTN % between tryptophol-treated and control samples was evaluated using the chi-square test (27). Statistical decisions were made at a significance level of $P < 0.05$.

RESULTS

Comet assay parameters measured in control samples indicated low level of primary DNA damage in all four cell types (Table 1, Figure 2). The cut-off values for tail length were 19.23 μm for A549 and HepG2 cells, 18.59 μm for CHO, and 16.67 μm for THP-1 cells. We found that control HepG2 cells had the lowest level of spontaneous DNA damage in this experiment with only 2.5 % of LTN (Figure 3). Differences in LTN % between the four cell types were not statistically significant.

Twenty-four-hour incubation with tryptophol resulted in increased DNA damage in all treated cells (Figure 2). Mean tail lengths measured in tryptophol-treated A549, HepG2 and THP-1 cells were significantly

higher ($P < 0.05$, Mann-Whitney U test) than in controls. Significantly higher mean tail intensities and tail moments were found in A549, CHO, and HepG2 cells (Table 1).

Considering that mean values of comet parameters might be affected by a few high scores, we thought that LTN % is a valuable additional parameter for better evaluation of DNA damage. Our results show that the most susceptible to treatment were human HepG2 cells, with 36.5 % of LTN, followed by THP-1, A549, and CHO cells. Statistical significance of these results is explained more in detail in Figure 3.

Since we evaluated DNA damage in different cell-types, which are known to differ in the amount of nuclear DNA, it makes no sense to compare cell types in terms of the tail length and tail moment. However, tail intensities measured in tryptophol-treated samples indicate that CHO cells had the lowest percentage of DNA in comet tail compared to other cell types. This difference was significant in respect to A549 and HepG2 cells ($P < 0.01$, ANOVA followed by post-hoc Schéffe test). Other differences between cell types were not statistically significant.

Microscopic analysis of 200 comets revealed two apoptotic nuclei in tryptophol-treated A549 cells and one in THP-1 cells, while none were found in HepG2 and CHO cells.

DISCUSSION

The findings of this pilot study have confirmed our working hypothesis that DNA sensitivity to tryptophol varies with cell type. They also indicate that DNA damaging effects of tryptophol are more pronounced in cell types which retain active enzymes responsible for metabolic activation of DNA reactive compounds, confirming our earlier observation (23) that the parent compound, tryptophol, has a lower genotoxic potential than its metabolite(s). In addition, our present results have shown that permanent cell lines of human origin are more susceptible to DNA damage than the animal cell line CHO. As known, inter species variability in toxic and genotoxic response is related to differences in the uptake, accumulation, metabolism, and excretion of a particular compound. Species also differ in their genetic susceptibility, in the amount of alkali-labile sites in the genome, as well as in the efficiency of DNA repair (28). All these factors should be taken into account when interpreting the results of the alkaline comet assay. This versatile method was used

here mainly because it is capable of detecting a wide variety of DNA damage in a single cell: DNA single-strand breaks, double-strand breaks, DNA-DNA/DNA-protein cross-links, base damage, alkali-labile sites, and sites subjected to DNA repair (29). The method was also recently applied to assess the genotoxicity of indoleacetic acid and 2-methylindoleacetic acid, which are structurally related with tryptophol (30).

The toxicity of tryptophol at the cell level has been poorly investigated so far. Three Japanese studies (20-22) reported that tryptophol induced apoptosis in human monoblastic leukemia cells U937. Inagaki et al. (20) observed that 12 h of exposure to $80 \mu\text{g mL}^{-1}$ of tryptophol resulted in the apoptosis of U937 cells, but not of human peripheral blood lymphocytes that were studied in parallel. In our previous study on human peripheral lymphocytes *in vitro* (23) tryptophol was applied in a dose range of (0.25 to 2.00) mmol L^{-1} (corresponding to $40.3 \mu\text{g mL}^{-1}$ to $322.4 \mu\text{g mL}^{-1}$) for 24 h, and we observed its dose-dependent cytotoxic, cytostatic and genotoxic effects. In yet another study (31), the MTT colourimetric assay on five cell lines showed that tryptophol half maximal effective concentration (EC_{50}) ranged between 2 mmol L^{-1} in THP-1 cells to 7 mmol L^{-1} in the animal CHO cell line. Based on these results, we decided that this pilot study should focus only on tryptophol concentration of 2 mmol L^{-1} , while other relevant concentrations will be tested in future studies.

Based on the types and extent of cytogenetic damage observed earlier in tryptophol-treated lymphocytes (23), we assumed that the culprit for direct DNA lesions could be an aldehyde metabolite. Such scenario is quite possible, as tryptophol is an aromatic alcohol and aldehydes are usually formed as metabolites of other alcohols (32, 33). For instance, production of acetaldehyde from ethanol by oral microbes is also observed *in vivo* and is related to the pathogenesis of oral cancer (34). Reactive aldehydes and other reactive radicals that are formed as a result of oxidative stress might cause DNA lesions, which are detectable with the alkaline comet assay. The results of our present study support this hypothesis, as all tested cells types responded positively to tryptophol treatment, especially the cells with high metabolic potential.

Since we used three human and one animal permanent cell line, a reliable interpretation of the results should include a proper parameter which is not species-dependent. As the amount of DNA in a nucleus

is species-specific, simple comparisons of comet parameters between human and animal cells are not possible. With LTN, however, we can compare DNA damage between different cell types. Our results show that HepG2 cells, which retain the most of active enzymes found in the liver, were the most susceptible to tryptophol. Previous studies on HepG2 cells indicate that these cells keep many functions of the normal human liver and are metabolically competent to activate different classes of mutagens into biologically active metabolites (35-38). The finding on high susceptibility of HepG2 cells to tryptophol deserves particular attention because of many interactions between tryptophan and alcohol in mammalian liver (39), including its metabolism into tryptophol. In addition, tryptophol is known formed in the liver after treatment with disulfiram (40).

The percentage of LTN in tryptophol-treated THP-1 and A549 cells was lower than in HepG2 cells. A549 cells possess enzymes necessary for metabolic activation (41) and have a high level of glutathione (42). We believe that this was crucial for lower LTN % in A549 than in HepG2 cells. Apparently, A549 cells better counteracted oxidative stress caused by tryptophol treatment than did HepG2. Similar findings on the sensitivity of A549 cells have been reported by Speit and Bonzheim (43), who found that the DNA damaging effect of hyperbaric oxygen, determined using the alkaline comet assay, was more pronounced in V79 than in A549 cells.

THP-1, the third cell type used in this study, are human monocytic leukaemia cells (44) related to U937 cells, which were used to study tryptophol toxicity in the past (20-22). Previous investigations indicate that the capacity to maintain cellular glutathione level was better in THP-1 than in U937 cells (45). THP-1 cells lack CYP1A1 (46) and generally have a very low content of proteins involved in metabolic activation (44). We therefore believe that they could not metabolise tryptophol the way HepG2 and A549 cells did. However, one of their important metabolic traits is a high activity of kinurenine pathway enzymes (47). As tryptophol is an end product of tryptophan catabolism, THP-1 possibly metabolised this compound in a different way than other cell types studied here. Our results, however, also suggest that these cells were able to activate some other mechanisms that led to formation of reactive species able to damage DNA. One of the candidates is myeloperoxidase. This enzyme, abundant in monocytes and other phagocytic leukocytes (48), catalyses the reaction between

hydrogen peroxide and chloride ions, which results in the formation of hypochlorous acid (HClO) (49). As a strong oxidant, HClO may interact with other small molecules by generating various reactive oxygen and nitrogen species (50), which in turn might damage DNA indirectly.

In this study we selected four types of cells that might be principal targets of low-molecular geno(toxins) secreted by *Candida albicans* during host invasion. These compounds deserve attention, as infections with the *Candida* species are involved in many of modern medical procedures. *Candida* spp. are frequently identified as agents of nosocomial pneumonias, vaginal and urinary tract infections, and sepsis. In addition, implanted devices (intravascular, central venous catheter or urinary catheters, prosthetic heart valves, cardiac pacemakers and joint replacements, etc.), are also prone to infection by *C. albicans* (51). In addition, its blastoconidia may enter the bloodstream through epithelial tissues and disseminate across organs (52). All this suggests that low-molecular-weight metabolites have to be considered as possible co-factors in *Candida* spp. invasiveness. Their role is probably as important as the production of hydrolytic enzymes during invasion and the switch from commensal to pathogenic state.

In conclusion, our results with tryptophol applied *in vitro* at 2 mmol L⁻¹ for 24 h point to its DNA damaging potential in HepG2, A549 and THP-1 cells, primarily due to the bioactivation and/or decomposition of the parent compound, which results in the formation of more genotoxic compound(s) and production of reactive species that additionally damage DNA. In contrast, notably lower levels of primary DNA damage were observed in CHO cells that lack metabolic activity (35). Taken together, our results make a solid frame for designing future studies with tryptophol, whose aim is to further clarify mechanisms involved in its toxic action. Future genotoxic studies need to focus on cell types which have not been investigated here, including vaginal epithelial cells or keratinocytes of human origin. It would also be convenient to investigate which intracellular macromolecules besides DNA are potential and/or preferential targets of tryptophol.

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Sažetak

PROCJENA GENOTOKSIČNOSTI TRIPTOFOLA NA RAZLIČITIM STANIČNIM LINIJAMA U UVJETIMA *IN VITRO*: PRELIMINARNA ISTRAŽIVANJA PRIMJENOM KOMET-TESTA U ALKALNIM UVJETIMA

Triptofol je aromatski alkohol i sekundarni metabolit oportunističkog mikroorganizma *Candida albicans*. Premda je njegova toksičnost na razini stanice vrlo slabo istražena, noviji podaci upućuju na citotoksične, citostatske i genotoksične učinke triptofola na ljudskim limfocitima te njegovu sposobnost izazivanja apoptoze u leukemijskim monocitima. Cilj ovog preliminarnog istraživanja bio je primjenom komet-testa procijeniti genotoksičnost triptofola u uvjetima *in vitro* na četiri vrste trajnih staničnih linija životinjskog i ljudskog podrijetla: stanicama jajnika, alveolarnom epitelu, stanicama jetre i monocitima. Izabrali smo one vrste stanica koje bi mogle biti potencijalne "mete" za djelovanje triptofola i srodnih mu niskomolekularnih geno(toksina) koje *Candida albicans* izlučuje tijekom infekcije domaćina. Dobiveni rezultati pokazuju da triptofol primijenjen u koncentraciji od 2 mmol L⁻¹ tijekom 24 h u uvjetima *in vitro* izaziva oštećenja DNA u HepG2, A549 i THP-1-stanicama, vjerojatno zbog bioaktivacije i/ili razgradnje roditeljske molekule koje dovode do nastanka još genotoksičnijih spojeva i stvaranja reaktivnih radikala koji dodatno oštećuju DNA. Značajno niža razina oštećenja utvrđena je u CHO-stanicama koje nemaju enzime potrebne za metaboličku aktivaciju potencijalno genotoksičnih spojeva. Buduća bi istraživanja trebala bolje razjasniti mehanizme koji su u podlozi toksičnosti triptofola i usredotočiti se na druge vrste stanica osjetljive na infekciju mikroorganizmima iz roda *Candida*, primjerice vaginalne epitelne stanice ili keratinocite ljudskog podrijetla.

KLJUČNE RIJEČI: *aromatski alkohol, Candida albicans, oštećenje DNA, sekundarni metaboliti, trajne stanične linije*

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