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**DNA REPAIR OF HYDROGEN PEROXIDE-INDUCED DAMAGE IN
HUMAN LYMPHOCYTES IN THE PRESENCE OF FOUR
ANTIMUTAGENS. A STUDY WITH ALKALINE SINGLE CELL GEL
ELECTROPHORESIS (COMET ASSAY)**

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Abstract: We assessed four antimutagenic compounds' influences on DNA repair in human lymphocytes exposed *in vitro* to hydrogen peroxide (20 μ M, 5 min, at 4°C). DNA damage and repair were estimated by means of alkaline single cell gel electrophoresis (comet assay). It was noticed that the enhancement of DNA repair was relatively strongest when fluphenazine was present in the cell culture medium. In the cases of anthocyanins and alkylresorcinols, the effects were almost 6-9 times weaker than that of FPh. The effect of todralazine on DNA repair was relatively weakest. Further study should be done on fluphenazine as a potential DNA repair-enhancing compound.

Key Words: Alkaline Comet Assay, Hydrogen Peroxide-Damaged Lymphocytes, DNA Repair, Antimutagens

INTRODUCTION

Modern analytical methods have proved that genotoxic, potentially mutagenic agents are present throughout the human environment; this omnipresence makes their complete eradication from that environment very burdensome – almost unattainable [1-3]. For these reasons, ample protection from genotoxic agents appears impossible, and regular exposure to such substances seems unavoidable. Therefore, a search for compounds which are able to enhance either DNA repair or the apoptotic elimination of genotoxically damaged cells

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from tissue/cell populations appears to be a significant branch of antimutagenic research and an important direction in the investigation of antimutagenic agents, i.e. compounds which are able to lower the frequency or rate of mutations [2-4]. We previously described the antimutagenic activity of four compounds – natural, plant-derived compounds: alkylresorcinols (from cereal grains) and anthocyanins (from *Aronia melanocarpa* fruits) and synthetic, currently used drugs: todralazine (an antihypertensive drug) and fluphenazine (a psychotropic drug). The majority of the antimutagenic effects of the tested compounds were observed in the standard *in vitro* cytogenetic tests in human lymphocyte cultures [5-10]. It was also documented that the tested antimutagens enhanced apoptosis in lymphocytes that had been genotoxically damaged with HP (0.2 mM, 20 min.) and with B[a]P (40 μ M, 90 min.) [11, 12]. The question emerges whether the tested antimutagens also influence DNA repair ability in genotoxically damaged lymphocytes *in vitro*.

Among the methods recommended for the study of both DNA damage and repair, single cell gel electrophoresis (SCGE) – also known as the comet assay – deserves special attention as a highly sensitive and rapid method to investigate the response of a single cell to DNA damage. Extensive reviews discussed in detail the technical aspects of the assay and its applications [e.g. 13-18]. In general, the assay consists of a number of consecutive steps, listed below: initially, cells are embedded in agarose, then immersed in an ice-cold fresh lysis buffer, pH 10, containing non-ionic detergents and high salts (2.5M NaCl). The lysis buffer removes the lipid membranes and most of the cellular proteins. The remaining nucleoids contain DNA in the form of long, supercoiled loops that are anchored in the residual nuclear matrix [19]. The nucleoids are then exposed to a weak electric field in the electrophoresis buffer, pH 13. After electrophoresis and staining with a fluorescent dye, the nucleoids, as analysed under a microscope, resemble comets. They contain a remnant cell nucleus (the head of the comet) and partly unwound DNA, which migrated in the electric field to the positively charged electrode (anode) of the electrophoretic unit (the tail of the comet). It is presumed that the comet tail is made up of relaxed DNA loops, and the number of loops in the tail (relative tail intensity) closely correlates with the number of DNA breaks [e.g. 15]. This assay is very sensitive in the detection of low levels of DNA damage; it allows the detection of 50-100 breaks per cell [20, 21], or 0.1 DNA strand breaks per 10^9 Da [22].

The efficiency of repair can be determined by comparing the comet tail lengths and the DNA contents in tails in the tested culture with those in the corresponding control culture, harvested at various periods of time after treatment with the genotoxic agent: the greater the decrease in the above parameters, the more efficient the repair. In the case of testing antimutagenic compounds, the reference control culture should undergo the same experimental conditions as the tested cultures, except for the absence of the tested antimutagen in the culture medium.

The aim of the present study is to evaluate the effect of the four tested antimutagens on DNA repair in lymphocyte cultures exposed *in vitro* to the genotoxic action of hydrogen peroxide.

MATERIALS AND METHODS

Chemicals

The blood cell separation solution – Histopaque-1077 – and the components of the cell culture medium – RPMI 1640, foetal calf serum (FCS) and L-glutamine – were purchased from Sigma (St. Louis, USA). Agarose type I-A and agarose type VII-low gelling temperature, dimethyl sulfoxide, Triton X-100, Trizma base and the fluorescent DNA stain 4',6-diamino-2-phenylindole (DAPI) were also purchased from Sigma (St. Louis, USA). Hydrogen peroxide (30% water solution, analytical grade), was obtained from POCH (Gliwice, Poland). The other reagents used for buffer preparation were from POCH (Gliwice, Poland).

The tested antimutagens

Alkylresorcinols (5-n-alk(en)ylresorcinols) were isolated and purified from a rye bran milling fraction at the Department of Lipids and Liposomes, University of Wrocław, following the procedure described previously [23]. The molecular weight of alkylresorcinols (AR) from such a preparation was established as being close to 376.33. The lyophilized powder was stored in a freezer. Anthocyanins (AN) were isolated from the fruits of *Aronia melanocarpa* at the Department of Fruit and Vegetable Technology, Wrocław Agricultural University, following the procedure included in Polish Patent No. PL 188707 and described in previous papers [24]. The molecular weight of the AN was taken as closely approximating 449.40. The lyophilized AN powder was stored in a freezer. Todralazine-HCl powder (TDR), research grade, molecular weight 268.7, was supplied by Polfa (Pabianice, Poland) and fluphenazine-HCl powder (FPh), research grade, molecular weight 510.4, was provided by Jelfa (Jelenia Góra, Poland). Both powders were stored at room temperature. On the day of the experiment, the tested antimutagens were dissolved in bidistilled water (AN, TDR, FPh) or in DMSO (AR), and filtered through a 0.2µm Milipore filter (Sartorius, Germany). Serial dilutions were prepared and the antimutagen solutions were added to the cell culture medium at a volume of 25 µl, at the dose needed to obtain the required final concentration. The following concentration ranges of the antimutagens were tested: FPh from 2.5µM to 7.5µM, TDR from 50µM to 200µM, AR from 12.5µM to 50µM, and AN from 25µM to 100µM.

Blood cell separation

Whole blood from fingerpricks was obtained from five healthy male volunteers, between the ages of 40 and 50, each smoking 20-30 cigarettes per day. Blood

(100µl) was drawn to heparinized micropipette tips, mixed with 1.0 ml of the complete culture medium (RPMI 1640, 10% FCS, 2mM L-glutamine) and stored for 60 min in a CO₂-incubator. Lymphocytes were separated by the single-step continuous density-gradient centrifugation technique with Histopaque-1077 [25]. The separated lymphocytes were washed in the culture medium RPMI 1640 and counted under a microscope. The cells' viability was assessed with the standard trypan blue-exclusion test [26].

Cell treatment and culture conditions

Lymphocytes were washed with Ca- and Mg-free phosphate buffered saline (PBS, pH 7.2), then suspended with chilled Ca- and Mg-free PBS containing hydrogen peroxide (HP), to a final concentration of 20µM. Cells were incubated with HP for 5min. in an ice water-bath (4°C). The incubation was terminated by dissolving the cells with excess volume of chilled PBS, spinning down and suspending with cold PBS containing Ca²⁺ and Mg²⁺ ions.

After finishing the treatment with HP, the cells were aliquoted to culture tubes containing the complete culture medium and incubated for 120min. in a CO₂-incubator at 37°C in the presence (tested culture) or in the absence (reference culture) of the tested antimutagens.

Comet assay

Alkaline single cell gel electrophoresis (comet assay) was carried out according to the procedure originally developed by Singh et al. [27, 28] with further modifications [29] and minor changes implemented by us.

After the incubation of lymphocytes for 120min. at 37°C, the cell samples were centrifuged, washed with PBS, and suspended with 100µl of PBS each. Then cell samples were mixed with an equal volume of 1% low melting point agarose (Sigma type VII) and prewarmed in a water-bath at 37°C. The suspensions were put on slides precoated with 0.5% regular agarose (Sigma type I-A), covered with coverslips and placed on ice for 5min. to allow the low melting point agarose to solidify. Then, the coverslips were removed and the slides were carefully immersed in cold (4°C) lysing solution (2.5M NaCl, 100mM Na₂EDTA, 10 mM Tris, pH 10, 1% Triton X-100 and 10% DMSO) and kept overnight in the dark at 4°C. Afterwards, the slides were rinsed (4x5 min.) with the alkaline electrophoresis buffer (300 mM NaOH, 1mM Na₂EDTA, pH 13), then placed in a horizontal gel electrophoresis unit filled with the freshly prepared alkaline electrophoresis buffer. The slides were exposed to alkali for 45min. at 4°C to permit DNA unwinding and expression of alkali-labile sites. Then, an electrophoresis was carried out (1.2 V/cm, 300 mA) for 20 min. at 4–6°C, with the slides subsequently being rinsed with neutralization buffer (0.4 M Tris, pH 7.5), 4x5 min. Finally, the slides were immersed with/in a fluorescent dye (DAPI, 1 µg/ml), covered with coverslips, and stained overnight in a

refrigerator. All the steps of the procedure from cell lysis on were conducted under dim light to prevent additional DNA damage.

Comet scoring and analysis

The slides were evaluated under a fluorescence microscope (Nikon Eclipse E-600 with a Nikon Plan Fluor 20/0.50 objective) equipped with Nikon UV 1A filter block. The images were acquired from the microscope with a Philips SC 99/PH18 camera working with WinSiS 4.18d software on Intel Celeron 400 MHz, and a 64MB RAM computer equipped with a PCI AD14 frame grabber. The WinSiS 4.18d and the PCI AD14 frame grabber were fitted to work with the SC 99/PH18 camera and supplied by Theta System Electronics GmbH (Gröbenzell, Germany). The comets were analysed with Komet 4.0 software (Kinetic Imaging Ltd., Liverpool, England). Fifty comets randomly found under a microscope were analysed in each slide. The following comet parameters were analysed: DNA contents in the comet's head and in the tail (%), and the tail moment (arbitrary units). The tail moment, according to Olive [30, 31] is the product of tail length and DNA content in the tail (Olive tail moment, OTM).

Statistical method

For the statistical examination of the results, the regression analysis of dose-effect relations was chosen [e.g. 32].

The concentrations of the tested antimutagens were recalculated and given as $\ln x$, where x = concentration of the antimutagen. The experimental results were expressed in proportion to the corresponding controls $[E/E_0]$. In the semilogarithmic scale, the dose-effect relations could be described by linear regression lines. The main purpose of the analysis was to estimate the regression line slope (a_1) and to assess if the slope was significantly different from zero. If this was the case, it could prove that the observed effects of antimutagens were significantly dose-related. The regression equations were computed by means of the Systat software (Systat, Evanston, USA).

RESULTS

Microscopic analysis proved that the comets differed considerably in their tail lengths and DNA contents in their heads and in their tails after treatment with HP (20 μ M, 5min., 4°C). Fig.1 contains representative images of comets exhibiting various degrees of DNA damage.

In Fig.1 the comets of the following cells are presented: 1A—undamaged cell (DNA in head: 95.42%, DNA in tail: 4.58%, OTM: 1.65), 1B—slightly damaged cell (DNA in head: 81.08%, DNA in tail: 18.92%, OTM: 13.33), 1C—damaged cell (DNA in head: 37.55%, DNA in tail: 62.45%, OTM: 37.34), 1D—heavily damaged cell (DNA in head: 34.11%, DNA in tail: 65.99%, OTM: 65.24), 1E—cell in apoptosis (not calculated), and 1F—necrotic cell (not calculated).

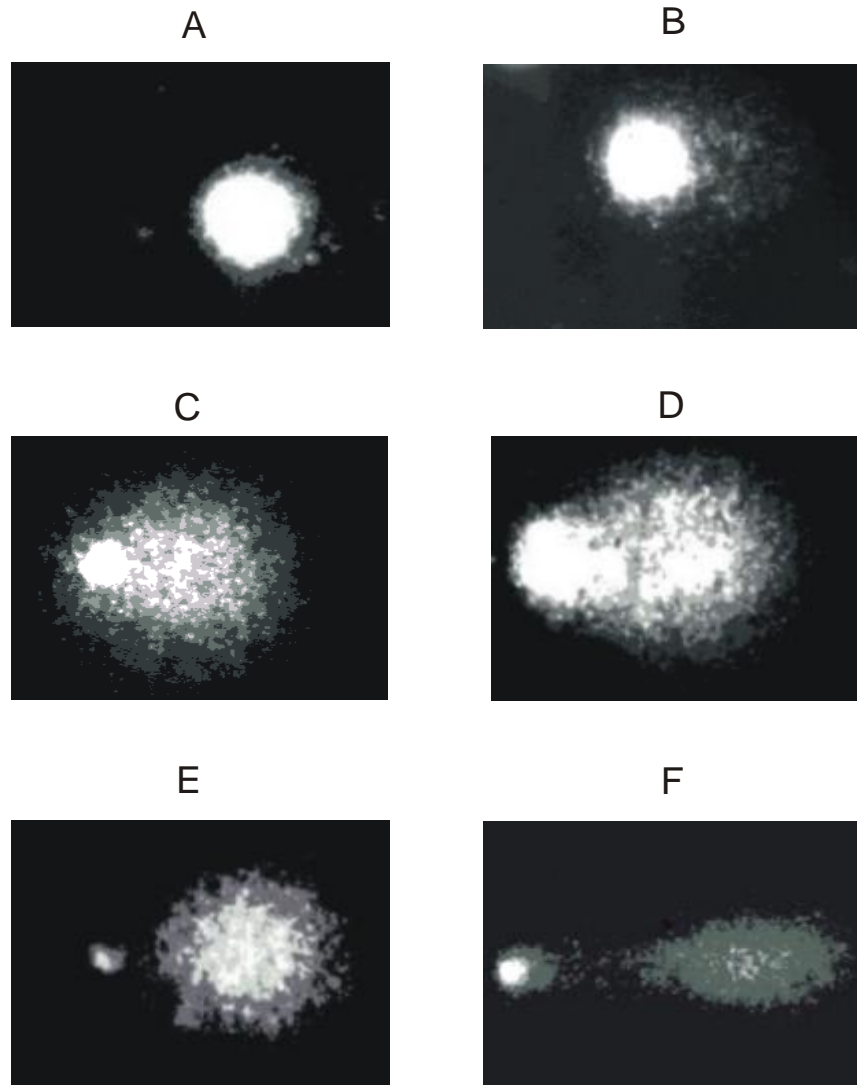


Fig.1. Digitized comet images taken from a microscopic image of the culture, in which lymphocytes were preincubated with HP (20 μ M, 5 min., 4°C), then incubated for 120 min. at 37°C in the cell culture medium.

The cells presented in Fig.1E – 1F were terminally damaged and were not analysed and counted automatically by the Komet 4.0 software. We consider the cell in Fig.1E to be apoptotic, since apoptotic cells form comets with large fan-like tails and small heads [18, 33]. In the comet provided in Fig.1E, the majority of the DNA occupied the center of the tail, since it probably contained DNA

fragments of similar high molecular weights. More recent studies demonstrated that chromatin fragmentation to large-scale 300–500 kb and 30–50 kb fragments usually precedes the degradation of DNA to small internucleosomal sections [34, 35]. It was documented that apoptosis could be a very rapid cell answer to DNA damage – the comet assay detected increasing numbers of apoptotic cells within 120min. of the irradiation of human B lymphoblasts *in vitro* [20].

The cell in Fig.1F exhibited a dim fluorescence, which reflected low DNA content both in the head and the tail. This is the expected result for necrotic (dead) cells, in which DNA was randomly digested into short fragments, easily diffusible from 0.5% agarose gel during the SCGE-procedure. We did not include the cells shown in Fig.1D – 1F in our analysis of the antimutagens' impacts on DNA repair. Comets like that in Fig. 1F constituted from 2 to 4% of the comet images randomly found under a microscopic examination of slides from various tested samples. It is well established in the literature [14, 18, 20, 33] that, based on the characteristics of the comet images, apoptotic cells can be readily distinguished from necrotic cells in the alkaline comet assay, since necrotic cells form comets with relatively larger heads and narrow tails of varying lengths.

If a single number is to be selected to describe the degree of DNA damage in an individual cell, the Olive tail moment (OTM) coefficient (the product of tail length and DNA content in the tail) is recommended, and commonly applied in such analyses as the most complex and reliable parameter measuring the degree of DNA damage [13, 30, 31].

The OTM coefficients in 50 comets randomly found under a microscope in each culture were expressed in histograms arranged in the rank of decreasing OTM coefficients (arbitrary units). The results obtained in the cultures containing the lowest concentration of each tested antimutagen and the histogram/histograms of the reference cultures (without antimutagens) are presented in Fig.2.

As can be seen in Fig.2, the culture harvested immediately after preincubation with HP revealed comets with high OTM coefficients, while the subsequent incubation of the culture for 120min. at 37°C led to a marked decrease in the comets' OTM coefficients (Fig. 2A versus Fig. 2B). It reflects the DNA repair kinetics of the HP-damaged cultures. In cultures preincubated with HP and then incubated for 120min. in the presence of the antimutagens (Fig. 2C-2D), a further considerable decrease in the OTM coefficients was noticed.

Fig.2 also shows that the OTM coefficients differed remarkably in each culture. Therefore, any grouping of the data from the histograms seemed to us an inaccurate method of comparing said histograms, as did calculating the mean values from said data. This seemed especially important in the initial study, reported here, where analysing the whole of the data on the comet lengths was preferable to the distribution of the comets to arbitrary presumed groups for data analysis. Thus, to quantify the cultures as precisely as possible, we decided to

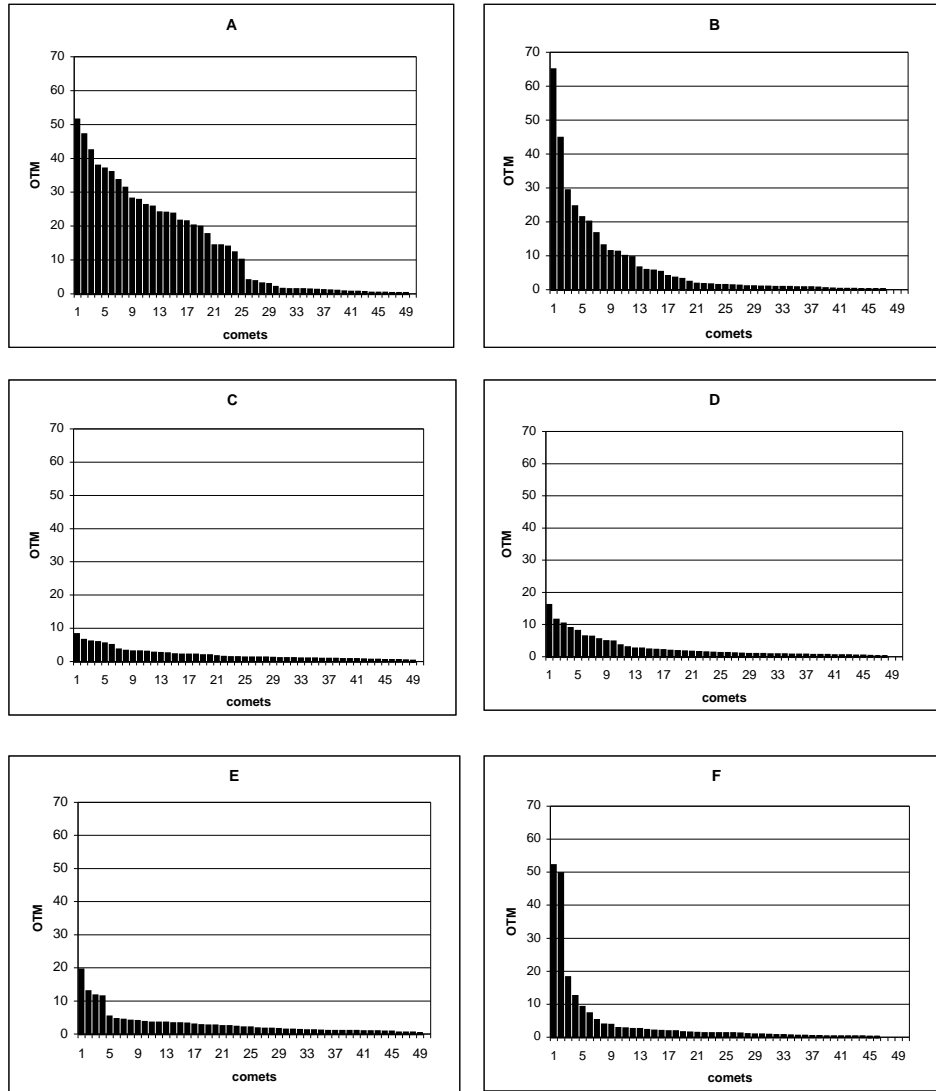


Fig.2. DNA damage by HP ($20\mu\text{M}$, 5min., 4°C) and its repair in the absence (2A, 2B) or in the presence (2C-2F) of the tested antimutagens, as revealed by the comet assay in lymphocyte cultures. Cultures 2A and 2B were preincubated with HP, then incubated with the culture medium (37°C) for 120min. – 2B, or not incubated (incubation time 0min.) – 2A. Cultures 2C-2F were preincubated with HP, then incubated with a culture medium (37°C) containing: FPh($2.5\mu\text{M}$) – 2C, TDR($50\mu\text{M}$) – 2D, AR($12.5\mu\text{M}$) – 2E, AN($25\mu\text{M}$) – 2F.

calculate the sum of OTMs as estimated for 50 comets in each culture ($\sum_{n=1}^{50} \text{OTM}$). The $\sum_{n=1}^{50} \text{OTM}$ values calculated in each culture were further processed in an analysis of the antimutagens' impact on DNA repair. The ($\sum_{n=1}^{50} \text{OTM}$) obtained with the data given in the histograms in Fig. 2 were as

follows: in the reference cultures: HP-pretreated, 0 culture time – 689.82, and HP-pretreated, 120min. of culture without antimutagens – 332.05; in the test cultures preincubated with HP and cultured for 120min. in the culture medium containing antimutagens: FPh (2.5 μM) – 95.74, TDR (50 μM) – 124.05, AR (12.5 μM) – 116.52, AN (25 μM) – 122.75.

Since the main purpose of this study is to search for the mechanisms of the antimutagenic impact of the four antimutagens, we used concentrations of the tested compounds closely corresponding to those applied in earlier studies of their antimutagenic effect [5-10]. However, to directly compare the potency of the antimutagens in their enhancement of DNA damage repair, it was necessary to express their activity in the same concentration unit. For this purpose,

the $\sum_{n=1}^{50} \text{OTM}$ values estimated for each tested concentration of the antimutagens

were referred to the corresponding culture of lymphocytes exposed to HP and cultured for 120min. without the antimutagens (E/E_0). Then the results were compared with the molecular weight of the antimutagens and presented as the specific milimolar activity of the antimutagen in lowering the OTM in lymphocyte comets. The dose-effect relations were also calculated with a regression equation for each tested antimutagen, and the results are given in Tab.1.

Tab.1. Specific milimolar activity of the tested antimutagens in the enhancement of DNA repair capacity in cells exposed to HP (20 μM , 5min., 4 $^{\circ}\text{C}$) and then cultured for 120min. in a culture medium containing the antimutagens; mean \pm SD, n=5. The dose-effect relations were calculated with regression equations.

antimutagen	specific activity [per 1 mM]	$y = a_0 + a_1 \ln x$	r	p
FPh	70.14 \pm 9.825	$y = 0.25 + 0.021 \ln x$	0.99	0.045
AR	11.88 \pm 2.022	$y = -0.16 + 0.045 \ln x$	0.99	0.032
AN	6.93 \pm 1.381	$y = -0.07 + 0.003 \ln x$	0.98	0.049
TDR	1.67 \pm 0.342	$y = -0.33 + 0.062 \ln x$	0.98	0.048

As can be seen in Tab.1, the decrease of the specific milimolar activity was relatively strongest in the case of FPh, being almost 6 times as strong as that of AR, about 10 times as strong as that of AN, and more than 40 times as strong as that of TDR. The linear regression equations show that the effects of the antimutagens were strongest in the lowest concentrations; the slopes of the regression lines ascend with increasing antimutagen concentration.

It should be stressed, however, that even in the highest tested concentrations, the $\sum_{n=1}^{50}$ OTM values were considerably lower than those for the reference culture in the case of each of the tested antimutagens. The calculated E/E_0 ratios were as follows: 0.625 (FPh 7.5 μ M), 0.8 (AR 50 μ M), 0.615 (TDR 200 μ M) and 0.904 (AN 100 μ M).

Summing up the data given in Tab.1, we conclude that the milimolar activity of FPh in lowering the sum of the OTMs in HP-pretreated lymphocyte cultures was superior among the four tested antimutagens, and the next antimutagen in the rank – AR – exerted about a 6-fold weaker effect on the enhancement of DNA repair.

DISCUSSION

It was demonstrated in biochemical studies *in vitro* that hydrogen peroxide does not react directly with DNA [36]. Inside a cell, however, it liables to the Fenton reaction in the presence of transition metal ions, which leads to the formation of highly reactive hydroxyl radicals [e.g. 37,38]. The Fe^{+2} contents in DNA-matrix attachment-sites appear to be a decisive factor of the Fenton reaction and, consequently of DNA damage in the presence of hydrogen peroxide [29, 39-41]. Once generated, hydroxyl radicals attack DNA at the sugar residue of the DNA backbone, leading to strand breaks, which are quickly repaired by the direct joining of the broken ends [e.g. 13, 15, 37]. Hydroxyl radicals also attack DNA bases, modifying both purines and pyrimidines to their hydroxyl-derivatives; for instance, to thymine glycol, 8-hydroxyguanine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine [37, 42]. The majority of these lesions are repaired by excision repair; some of them are also repaired by the system which is able to recognize regions of DNA distorted by hydroxyl radical modifications of nucleotides [43]. Another mechanism of hydrogen peroxide-originated genotoxicity appears to be the activation of nucleases by hydroxyl radicals, which effectuate in the cleavage of DNA, detected in the comet assay as single-strand breaks [37, 43]. The above mechanisms probably take place simultaneously, while a cell is exposed to hydrogen peroxide [37]. Among these chemical modifications, the generation of alkali-labile DNA adducts, the oxidation of nucleotides and the creation of abasic sites can lead to their conversion into single-strand breaks in high alkaline conditions [15, 37].

The comparison of DNA damage detected by the comet assays at pH 13 versus pH 12.1 of the electrophoresis buffer documented that in cells exposed *in vitro* to hydrogen peroxide, alkali-labile sites constituted about 20% of the overall DNA damage detected with the assay, whereas direct single-strand breaks made up more than 80% of the damage [37]. We intended to detect possibly all of the DNA damage caused by hydrogen peroxide and to assess the impact of the antimutagens on their repair. Thus, the rational choice was to carry out such an assay in highly alkaline conditions, in which both direct strand breaks and alkali-labile sites can be detected. It was established that pH values from 12.1 to 12.5 are the border value below which the alkali-labile sites are stable, and below which value they are not picked up by the comet assay [44, 45]. Therefore to reveal alkali-labile sites highly alkaline conditions of electrophoresis are recommended, i.e. at $\text{pH} \geq 13$ [e.g. 18, 44].

Single-strand breaks, contrary to double-strand breaks, are not regarded as significant lethal or mutagenic/carcinogenic DNA lesions, since they are quickly and efficiently repaired by strand-end joining [15]. Therefore, we chose a longer time of cell incubation (120min.) following exposure to hydrogen peroxide, to reveal the antimutagens' influence on more slowly-acting DNA excision repair systems. Obviously, it is necessary to evaluate the time kinetics of the observed repair-effects. However, the initial study, presented here, was aimed to establish the basic experimental conditions for future experiments, as concentration ranges of the tested antimutagens and cell culture conditions. Also in future studies a comparative analysis of the other estimable comet parameters such as tail intensities and/or tail DNA % should be included, since in recent papers it was suggested that they could provide more useful, easier to interpret results [e.g. 46].

On the basis of the preliminary experiments the direct interaction of the tested compounds with DNA could be considered non-existent. The conclusion was drawn from the experiments in which non-damaged lymphocytes were incubated for 2 h in the presence or absence of the tested compounds. The noticed shortening of the comet lengths and the decrease of the DNA content in the tails suggest that the tested compounds did not interact with DNA in a mode leading to strand breaks in the test. The shortening of the tail lengths probably reflected the repair of background DNA damage in lymphocytes obtained from heavy smokers. We established previously in the battery cytogenetic tests that lymphocytes from heavy smoker donors bore an elevated level of background genotoxic damage [7, 8, 10]. Separate studies are necessary to elucidate the impact of the tested compounds on the repair of background genotoxic damage in heavy smokers' lymphocytes.

Although the results of the alkaline comet assay presented in this paper need further experimental confirmation, at the moment we can conclude that the tested antimutagens differed markedly in their enhancement of DNA repair in HP-damaged lymphocytes. The activity of fluphenazine was considerably

stronger than that of the three other antimutagens. Therefore fluphenazine should be picked up for further studies on DNA repair-enhancing action.

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